THE RELATIVE EFFECTS OF LEUKOCYTE DEPLETION AND NITRIC OXIDE MODULATION IN AN *EX-VIVO* PORCINE NORMOTHERMIC PERFUSION MODEL OF DONATION AFTER CARDIAC DEATH IN THE KIDNEY

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Abstract

The relative effects of leukocyte depletion and nitric oxide modulation in an *ex-vivo* porcine normothermic perfusion model of donation after cardiac death in the kidney

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Introduction
The shortage of donors necessitates the use of organs from donation after cardiac death (DCD) donors. These organs demonstrate a high rate of delayed graft function (DGF) and primary non-function (PNF) when compared to those from non-DCD donors. DGF and PNF are associated with shorter graft survival times. Reperfusion injury (RI) is important in the aetiology of DGF and PNF. The roles of leukocytes and nitric oxide (NO) are pivotal in the generation of RI.
The aim of this study was to assess the effect on RI of modulating leukocyte and NO levels at the time of reperfusion.

Methods
This study utilised a porcine extra-corporeal normothermic perfusion model of RI. Kidneys were perfused with whole (WB) or leukocyte depleted blood (LDB) alone, or with an inducible nitric oxide (iNOS) inhibitor or NO donor. This model allows the collection of haemodynamic and functional data, as well as plasma and urine for biochemical analysis.

Results
Kidneys reperfused with LDB demonstrated improved blood flow and function compared to those reperfused with WB.
Initial blood flow and function in the iNOS supplemented groups was worse than in the WB/LDB perfused groups, but improved in the NO donor groups. Late blood flow plateaued in the NO donor groups but improved in the iNOS supplemented WB group. LDB and iNOS supplementation together gave poor blood flow and function throughout reperfusion.

Conclusion
Depletion of leukocytes abrogates the no-reflow phenomenon and reduces the oxidative stress caused by white cell infiltration, thereby improving blood flow and function.
The effects of nitric oxide and its inhibition on the endothelium, glomerulus and renal tubule during initial reperfusion are dependent upon the phase of reperfusion. Early benefits to blood flow by NO supplementation are offset by the generation of NO free radicals later after reperfusion.

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The time over which this research was performed and written-up has been an enlightening period of my life, with changes to my career aspirations, and personal circumstances. I thus do not simply see this work as reflecting a *de rigueur* period of research experience, but also a pivotal period of personal maturation and professional development.

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1 Introduction

1.1 End-stage Renal Disease and Transplantation

End-stage renal disease (ESRD) is a chronic disease with a growing incidence and prevalence across the world. Patients with ESRD will ultimately require some form of renal replacement therapy (RRT). In 2009, nearly 48,000 patients in the UK were receiving RRT, equating to a prevalence of 774 patients per million (ppm). The annual uptake of RRT stands at 113ppm. The population of patients receiving RRT continues to grow [1].

Of the 48,000 patients on RRT, 43% receive haemodialysis (HD), 10% peritoneal dialysis (PD) and 47% have a functioning transplant. Of the three forms of RRT transplantation is, beyond doubt, the gold-standard. The 1-year death rate in the UK for prevalent patients receiving dialysis is 13%, compared to 2.4% in the corresponding transplant population. The 5-year mortality rate for deceased-donor and live-donor recipients are 14% and 5% respectively. In patients receiving dialysis 5-year mortality rates are 13%, 22%, 33%, 52%, 71% and 82% respectively for patients aged 18-34, 35-44, 45-54, 55-64, 65-74 and >75 years. 64% of grafts will still be functioning and 68% of patients will still be alive 10-years post-transplant [1].

1.1.1 Organ procurement

Organs for kidney transplantation can be derived from two sources, live donors (LD) and deceased donors.

1.1.1.1 Live donation

The first successful LD transplant, between identical twins, was performed in 1954 [2]. Since then the donor immunological criteria have been somewhat relaxed. The increasing efficacy of immunosuppression now means that transplantation can occur across any tissue-type match and between relatives and non-relatives alike. The evolution of plasmaphoresis and newer immunoadsorption techniques allows transplantation across the ABO barrier. ABO incompatible transplantation is still in its infancy, and is not common practice.

Until the mid-1990s donor nephrectomy was peformed via a loin incision. This incision is associated with a high incidence of immediate and long-term complications, such as persistent wound discomfort and flank bulge, as well as poor cosmesis. In 1995 laparoscopic donor nephrectomy was introduced. It is now generally accepted that laparoscopic donor nephrectomy has less immediate and persistent complications compared to open nephrectomy [3]. Furthermore, laparoscopic nephrectomy does not yield organs of inferior quality to open nephrectomy. The ability to offer a procedure with good cosmesis and minimal persistent sequelae has directly increased rates of live donation [4].

In addition to scientific and technical advances, various donor strategies have evolved to optimise the LD pool. Several successful paired-donation programs have been established [5]. In a two-way exchange two incompatible recipient/donor pairs exchange donor kidneys, so that each recipient receives the kidney of the other donor. Similarly several three-way exchanges have also been performed [5]. The concept of list exchanging has also been established successfully in parts of the US. In a list

exchange a living-incompatible donor gives a kidney to a recipient on the deceased-donor waiting list. The living-incompatible recipient then becomes a priority for a deceased-donor kidney [6]. Non-directed altruistic donation, still in its infancy in the UK, has been utilized in the US for almost a decade. Most recently, and impressively, altruistically derived kidneys have been utilized to trigger a chain of paired exchanges [7]. In one such chain a single altruistic kidney, rather than benefiting a single patient on the deceased-donor waiting list, was the trigger for two paired donations, resulting in the successful transplantation of three recipients in total [7].

The successes of evolving immunosuppression and surgical techniques along with waiting list rationalization strategies have improved rates of LD transplant considerably. Often the main obstruction to live donation is donor suitability. Essentially, before live donation can be considered the following statement must be shown to be true, '*The person who gives consent to be a live organ donor should be competent, willing to donate, free from coercion, medically and psychosocially suitable, fully informed of the risks and benefits as a donor, and fully informed of the risks, benefits, and alternative treatment available to the recipient. The benefits to both donor and recipient must outweigh the risks associated with the donation and transplantation of the living donor organ.'* [8]. In order to ensure suitability the potential donor must fulfil rigorous medical and psychological criteria [9].

1.1.1.2 Deceased donation

Organs of deceased donor origin may be obtained from brain-dead donors, also known as cadaveric donors or donation after brain death (DBD) donors, or cardiac-dead

donors, so-called non-heartbeating donors or donation after cardiac death (DCD) donors.

1.1.1.2.1 Brain-dead donation

Effectively, brain death was first described by Mollaret and Goulon in 1959, and termed *coma dépassé*, meaning irreversible coma [10]. The precise definitions of brain death vary worldwide and represent a legal and ethical minefield, for example in Japan organ procurement from brain-dead donors has only been legal since 1997. The UK has adopted the definition of brain-stem death (BSD) as, *…irreversible loss of consciousness, irreversible loss of the capacity to breathe, and irreversible loss of integrated functioning* [11]. This definition allows the derivation of explicit and testable criteria for brain stem death. The diagnosis of BSD in the UK has three important components: the meeting of clearly defined preconditions; exclusion of patients not meeting preconditions; and the bedside testing of brain stem function. In some cases, such as severe facial or high spinal cord injury, there may be a need for supplementary testing. Modalities for supplementary tests include electro-encephalography, cerebral angiography and trans-cranial doppler. A patient is declared dead if on two occasions there is no respiratory effort in response to hypercarbia and a complete absence of all cranial nerve activity. Once declared dead suitable patients should always be considered for organ donation.

The main benefit of DBD organ procurement is that the initial warm ischaemic insult can be restricted to an absolute minimum.

1.1.1.2.2 Cardiac death donation

Donation after cardiac death (DCD) or non-heartbeating donation utilises organs derived from patients who die due to a cardiac arrest. This definition represents a diverse population of potential donors. The Maastricht classification of 1995 was derived to stratify donors according to the features of cardiac death [12]. The classification is summarised in Table 1.

Table 1: Summary of Maastricht classification of DCD donors.

Classification	Clinical Features of Death
I	'Dead on arrival' - found dead and brought to hospital for donation
II	'Unsuccessful resuscitation' - failed resuscitation, mostly in Emergency Department
III	'Awaiting cardiac arrest' - do not meet BSD criteria. Die after controlled withdrawal of supportive therapy (mostly intensive care)
IV	'Cardiac arrest while brain-stem dead' - cardiac death after diagnosis of BSD

This categorisation may be simplified into controlled (III) and uncontrolled (I, II, IV) cardiac death, though type IV may be controlled to some degree.

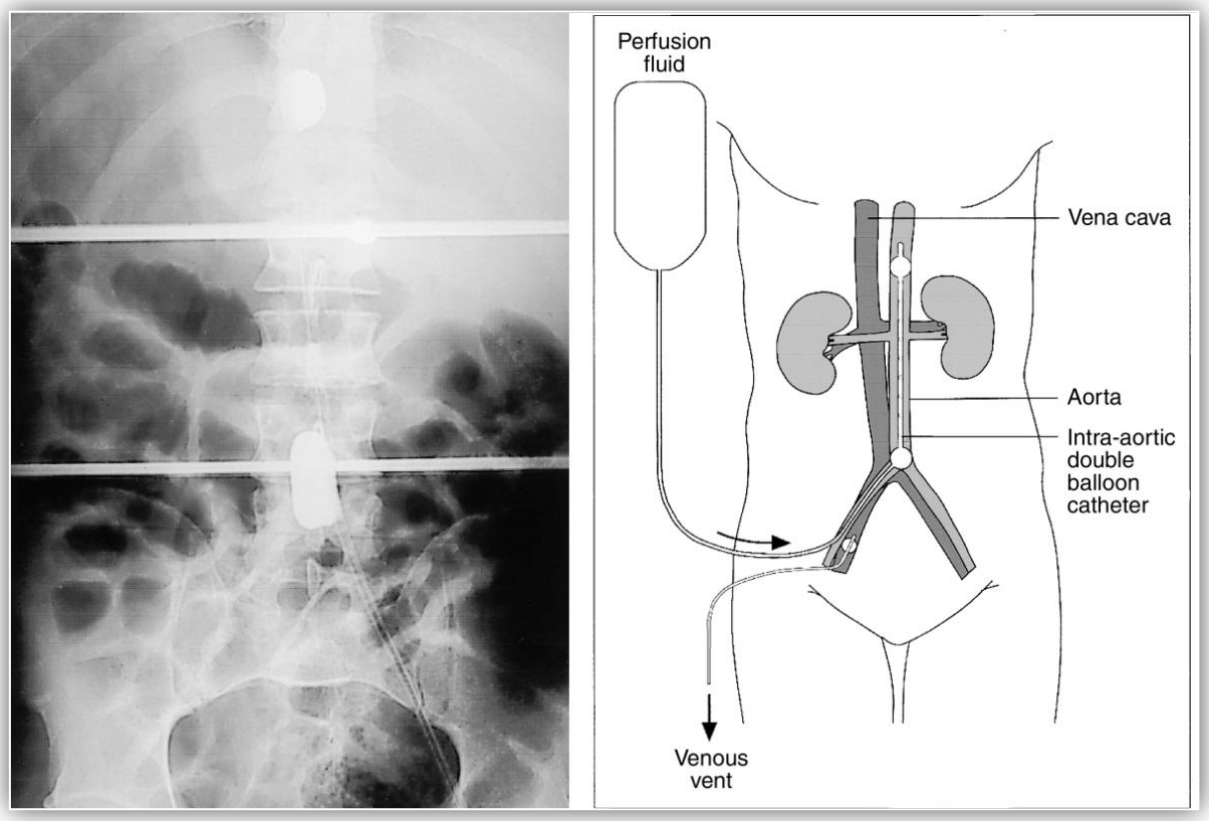
All DCD donor organs will be subjected to a period of warm ischaemia, with those in the uncontrolled category suffering the longest durations. Understandably, this has an impact on graft function, and so several donor criteria must be met before DCD can be considered. It is well-established that donor age and warm ischaemic time (WIT) are the most important selection criteria and should be considered in unison [13, 14]. Generally, a maximum age limit of 60 years and WIT of 30-45 minutes are advocated [13-15]. Potential donors older than 60 years or those with a WIT greater than 30 minutes (varies upon centre) are usually excluded from donation. Other absolute exclusions for DCD are the presence of blood-borne infection or proven *intra-venous* drug use, evidence of sepsis or severe infection, and malignancy.

If none of the exclusion criteria are met then DCD can proceed. Following pronouncement of cardiac death there is a 10 minute 'no-touch' period to ensure that brain death occurs. The duration of the 'no-touch' is hotly debated and varies worldwide. In the uncontrolled DCD setting the donor is then placed on mechanical external and artificial ventilation machine to maintain tissue oxygenation. During this period consent from relatives is gained for placement of femoral vessel catheters to allow introduction of the double-balloon triple lumen catheter (DBTLC) and commencement of *in-situ* cold preservation. The DBTLC is an ingenious device, the upper balloon is inflated in the thoracic aorta and the inflated lower balloon is lodged at the aortic bifurcation. This process allows the isolated cold preservation of the abdominal organs. A vent is placed in the femoral vein to removed perfusate. Typically 10 – 15 L of preservation fluid is instilled. A radiograph confirms adequate placement

of thoracic and abdominal balloons. Figure 1 shows a radiograph and schematic of DBTLC placement.

Unfortunately there is a high rate of organ attrition associated with DCD. Due to consent issues, DBTLC placement and device failure, and poor preservation as few as 26% of DCD kidneys are successfully transplanted [16, 17].

Figure 1. Radiograph and schematic of DBTLC placement. ***(Reproduced with kind permission of Prof. M.L. Nicholson.)***



DCD donors in the controlled group will have cardio-respiratory support withdrawn following transfer to the operating theatre. Following withdrawal of support cardiac

death is confirmed, and the 'no-touch' period observed as with uncontrolled donors, organ procurement will then proceed as for DBD donation.

As will be discussed later in this introduction warm ischaemia initiates several deleterious pathophysiological processes in the kidney. Clinically the adverse effects of prolonged WIT are observed in higher rates of delayed graft function (DGF) and primary non-function (PNF) of the graft. There is no single definition for DGF, but the diagnosis is often made when, post-transplantation, there is a requirement for dialysis or plasma creatinine levels do not fall by an arbitrary percentage [18]. Although there is conflicting data, the presence of DGF/PNF may subsequently affect allograft and patient survival and patient quality of life.

The incidence of DGF in DCD kidneys is between 48% and 84%, compared with rates of 20-60% in DBD kidneys [14]. Similarly PNF rate is higher in DCD kidneys at 8-15% versus 2-5% in DBD kidneys [14, 19]. These data demonstrated an association between WIT and the observation of DGF or PNF. There is no clear evidence that DGF in DCD kidneys increases the incidence of acute rejection or chronic allograft nephropathy *per se [20, 21*]. Allograft survival beyond the initial period post-transplant is shown to be equivalent in both DBD and DCD kidney recipients [22]. The quality of life and survival of patients receiving a DCD kidney has been shown to be higher than matched patients in the dialysis population and equivalent with DBD kidney recipients [22-24].

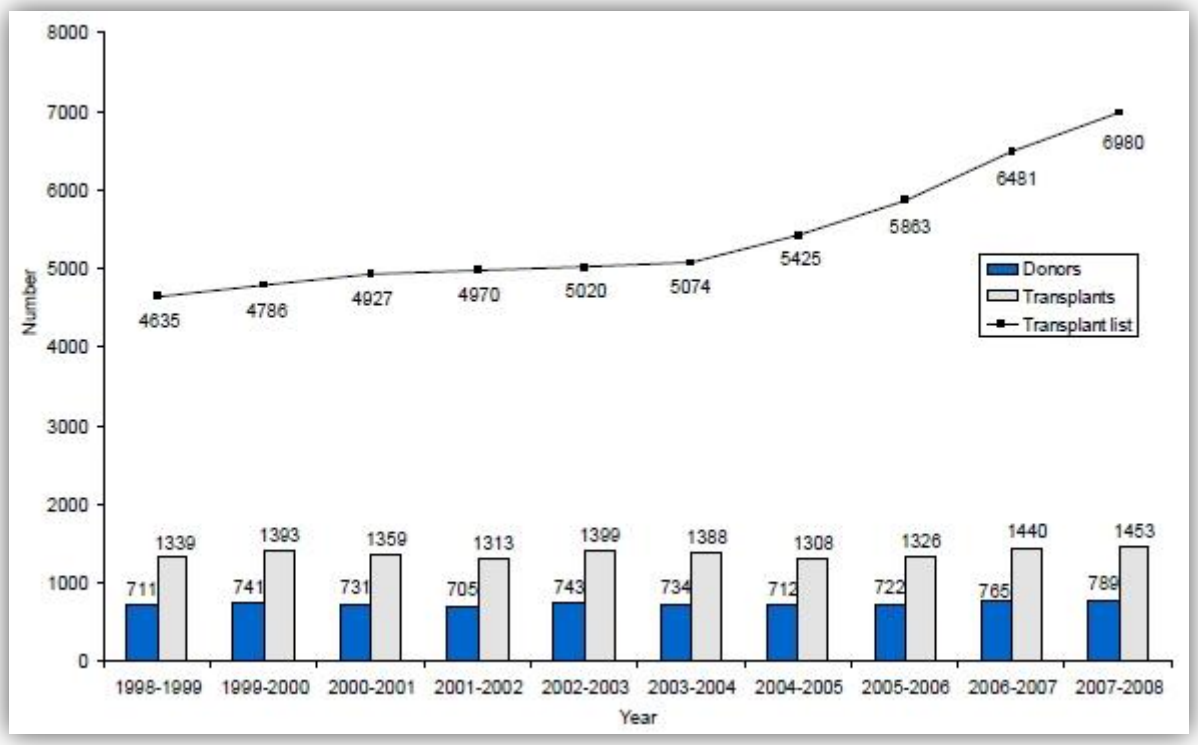
In order to minimize the rates of DGF and PNF, and the subsequent effects to patients the viability of DCD kidneys needs to be tested. Donor age and WIT are the most simple predictors of viability, however despite much research there are no sensitive methods of determining organ viability, and predicting DGF/PNF, from donors who are

<60 years old and in whom the WIT is less than 30 minutes. In addition to testing viability there is a need to improve viability in DCD kidneys to minimize the incidence of DGF and PNF. There is growing evidence that hypothermic machine perfusion confers improved viability when compared to hypothermic static preservation [25-28]. The importance of renal preservation techniques will be discussed later in this introduction.

1.1.2 Current status of transplantation

At the start of 2009 there were 7190 people on the kidney transplant waiting list, a 30% increase from the beginning of 2004 [29]. The increasing incidence and prevalence of ESRD have resulted in the transplant waiting-list growing. Unfortunately, there is a significant disparity between kidney supply and demand. The UK government triumphantly announced that the size of the organ donor register had doubled since 2001 to a current number of 16 million. This number of potential donors is undoubtedly a fine achievement, but one that has had no discernable impact on deceased donor rates. Figure 2 shows the growing disparity between organ supply and demand over the past decade.

Figure 2. Summary of UK deceased donor renal transplant waiting-list activity over the past decade.



Transplant activity, stratified for organ origin, is summarized in Table 2. The rate of DBD transplantation is, at best, static. The overall increase in transplant activity is secondary to the 79% increase in LD transplantation and a massive 127% increase in DCD transplantation over this five year period. Currently DBD, DCD and live donation account for 49%, 15% and 36% respectively of kidneys transplanted.

Table 2. Summary of 5-year UK renal transplant activity

	2004	2005	2006	2007	2008	% change 2004-2008
DBD	1295	1112	1146	1164	1118	-14%
DCD	147	200	250	276	335	+127%
Live Donation	463	543	671	690	829	+79%
Total	**1905**	**1855**	**2067**	**2130**	**2282**	**+20%**

Thus despite DCD representing the largest growth sector in kidney transplantation, these donors only provide a small percentage of the organs transplanted. Despite a tangible increase in transplantation awareness the number of DBD donors is unchanged, but the number of DCD donors has increased significantly.The reasons for this are multifactorial, but are predominantly based around improved education of medical and nursing staff regarding DCD, and changes in the pathologies of patients admitted to critical care units. So, realistically, how many organs can be gained from DCD? This question has been addressed by several groups. Predictions range from DCD equalling DBD donor numbers, to DCD accounting for four times DBD donation rates, thus eliminating the transplant waiting list and obviating the need for live donation [30-32]. Regardless of precise numbers it is clear that DCD is still a relatively untapped source of organs, and as such all techniques that increase initial donation rates and improve the viability of DCD kidneys should be intensively investigated.

1.2 The Three Phases of Renal Transplant Injury

All donor kidneys whether derived from a LD, DBD or DCD donor undergo three distinct phases of injury.

The first phase is the initial warm ischaemic period. In DBD in whom the kidneys are perfused *in situ*, this period is negligible. Live donation necessitates a warm ischaemic period of a few minutes to allow ligation of the renal vessels, kidney extraction, cannulation of the renal artery and cold flushing. In DCD the warm ischaemic period maybe up to 30 minutes, reflecting the hypo-perfusion suffered during cardiac arrest and resuscitation.

In order to minimize the deleterious effects of prolonged warm ischaemia organs are stored under hypothermic conditions. The period of cold storage may be as little as a few minutes in live donation, or greater than 24 hours when a kidney is shipped from the retrieving centre to the transplanting centre. Unfortunately, cold storage has a harmful effect on organ viability.

The final phase of renal injury is the reperfusion phase, which, hopefully, all transplanted organs will undergo. Reperfusion is perhaps the most damaging phase of organ injury.

The following part of this introduction will discuss the three phases of renal injury as separate entities. Although implicit in the text, it should be remembered that these phases are intrinsically linked and represent a synergistic, holistic mechanism of injury to the transplanted kidney.

1.2.1 Acute Ischaemic Injury

Regardless of whether a kidney is retrieved from a LD, a DBD donor or a DCD donor; it will be subject to a period of initial warm ischaemia. The duration of warm ischaemia is an important aetiological factor in the generation of DGF. This is evidenced by the higher rates of DGF observed in transplanted kidneys derived from DCD donors, in which there is a longer warm ischaemic period, when compared to LD and DBD [14, 33]. To appreciate the causation of DGF an understanding of the morphological changes and pathophysiological processes in warm ischaemic renal damage are vital.

The kidney is a heterogenous organ consisting of a number of units that work together to give function. Histology of protocol renal biopsies taken from DBD donors shortly after death demonstrates perfectly the morphological changes associated with acute ischaemia. In proximal tubules there is effacement and loss of the brush border in conjunction with tubular dilatation and distal tubular casts. Glomeruli are typically normal in appearance, and acute tubular necrosis is often limited to outer medullary tubules only [34]. More recent evidence consistently demonstrates apoptosis in distal and proximal tubules and significant deleterious changes in the peritubular vasculature [35-37].

The pathophysiological processes that occur in acute renal ischaemic injury may be divided into broad categories.

1.2.1.1 Reduction in aerobic metabolism

Unsurprisingly under prolonged ischaemic conditions oxidative metabolism is impaired. Ischaemia results in alterations to the mitochondrial electron transport chain which results in electron leakage and an increase in the univalent reduction of oxygen to superoxide [38]. Furthermore ischaemia impairs the mitochondrial antioxidant system leading to increased levels of superoxide and depleting substrates such as glutathione which protect cells from oxidative stress during reperfusion [39].

1.2.1.2 Adenosine Triphosphate depletion

Adenosine Triphosphate (ATP) is quickly degraded sequentially to Adenosine Diphosphate (ADP) and Adenosine Monophosphate (AMP) in the absence of oxygen [40]. Prolongation of ischaemia leads to the breakdown of AMP to adenine nucleotides and hypoxanthine, which contributes to the generation of reactive oxygen species (ROS) [41].

Ischaemia leads to a rise in free intracellular calcium. This is due to impaired calcium uptake by the endoplasmic reticulum in conjunction with reduced extrusion of calcium from cells [42]. Calcium accumulation has a number of deleterious effects on cell viability. Elevated intracellular calcium levels may cause degradation of DNA via endonuclease activation, as well as lysis of important structural and signaling proteins [43]. Calcium overloading in mitochondria adversely affects oxidative phosphorylation resulting in a reduction in ATP synthesis and increased superoxide formation [36]. Activation of certain phospholipases, such as phospholipase A_2 (PLA_2), are dependent upon calcium [44]. PLA_2 activation results in the lysis of membrane phospholipids and the release of pro-inflammatory free fatty acids [45].

Disruption of tubular cell structure is a well established sequel of ATP depletion. There is a redistribution of actin from the apical domain of tubular cells and microvilli to the cytoplasm, this results in the loss of membrane components into the tubular lumen that contribute to cast formation [46]. Actin breakdown leads to a loss of tethering of Na,K-ATPase to the baso-lateral cell domain. Na,K-ATPase subsequently relocates to the apical cell domain where it leads to an increase in the fractional excretion of sodium (FE_{Na}) [40]. Redistribution of basal domain β1 integrins to the apical domain results in detachment of cells form the basement membrane, and loss of tubular epithelial integrity [47].

It is worthy of note that Guanosine Triphosphate (GTP) depletion also occurs in response to renal ischaemia [48]. It has been demonstrated that GTP depletion alone can induce apoptosis in tubular cells [48]. Furthermore, supplementation of guanine nucleotides prior to the ischaemic insult has the effect of ameliorating the impact of apoptotic cell death [49].

1.2.1.3 Cell viability changes

After an ischaemic insult tubular cells may remain viable or may die. Whilst necrosis secondary to prolonged ischaemia is irreversible, shorter periods of ischaemia also enhance apoptotic processes. Apoptosis is a complex, multifactorial process, a description of which is beyond the scope of this introduction. In brief, there is growing evidence that alterations in the expression of pro-apoptotic proteins, Bax and Bid, and anti-apoptotic proteins, Bcl-2 and Bcl-x, in the Bcl-2 family, which controls mitochondrial outer membrane permeabilization, contribute to an up-regulation of apoptosis in ischaemic kidneys [50, 51]. Furthermore, p53, a pro-apoptotic

transcription factor, has been shown to be up-regulated at both the mRNA and protein translation levels [52, 53]. There is some evidence in DBD donor kidneys that there is down-regulation of the anti-apoptotic and anti-inflammatory haemoxygenase-1 (HO-1) and vascular endothelial growth factor (VEGF) in response to ischaemia [54].

1.2.1.4 Microvascular changes

Previously, much of the focus on ischaemic renal damage has been on the tubular cells. However, more recently the role of the vascular endothelium in ischaemic renal injury has become more prominent. Many of the cytoskeletal changes that affect the tubular cell, detailed above, also affect endothelial cells. Consequently, endothelial cell oedema, death and detachment may occur [55, 56].

Ischaemic injury alters the expression of a variety of adhesion molecules by endothelial cells. Intracellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin have all been shown to be up-regulated [57, 58]. The action of these molecules is to enhance leukocyte and platelet adhesion.

In addition to the above changes endothelial cells also demonstrate profound functional alterations in response to ischaemia which are only manifest upon reperfusion. The importance of the endothelium in renal injury will be discussed at greater length in the reperfusion injury section of this introduction.

1.2.1.5 Nitric Oxide and Nitric Oxide Synthase

Inducible nitric oxide synthase (iNOS) is up-regulated in the ischaemic endothelium, glomerulus and renal tubular cell [59, 60]. In response to up-regulation of iNOS there is synthesis of nitric oxide (NO). NO has a number of physiological roles that will be discussed in detail in the reperfusion section of this introduction.

Undoubtedly prolonged periods of renal ischaemia are associated with poorer function following reperfusion. However, warm ischaemic periods of up to a few minutes, as observed in LD transplantation, do not translate into worse initial graft function when compared with cadaveric organs [61]. It is thus evident that other factors play a major role in the generation of DGF.

1.2.2 Hypothermic Injury

All transplanted kidneys will endure a period of cold ischaemia. Hypothermic storage is a cornerstone of transplantation allowing the transportation of organs over great distances. In contrast to warm ischaemia, organs stored hypothermically remain viable for many hours after the cessation of normal physiological perfusion. Unfortunately, hypothermic storage is a double-edged sword with prolonged periods contributing significantly to organ damage.

Hypothermic perfusion *per se* is a risk factor for DGF with extended periods of cold ischaemia conferring greater risk of DGF [33, 62]. Moreover, the risk of DGF increases by 23% for every six hours of cold ischaemia endured [63].

The aim of hypothermic perfusion is to reduce the metabolism of the stored organ and thus prevent the aforementioned damaging processes associated with warm ischaemia [64]. Furthermore, hypothermia has direct deleterious effects on cellular metabolism which exacerbate tissue damage.

1.2.2.1 Loss of ATP precursors

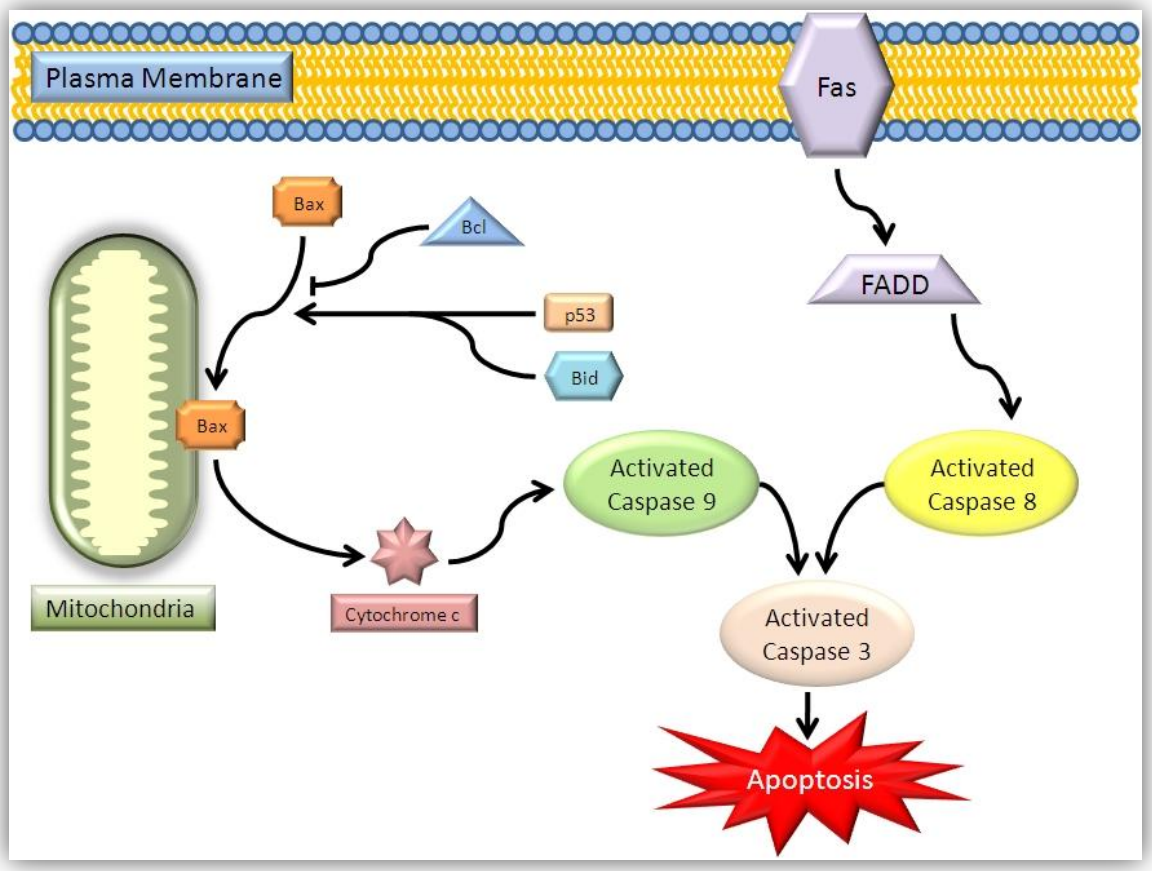
ATP synthesis within the mitochondria is only slightly limited by hypothermia. The mitochondrial transport enzyme adenine nucleotide translocase is cold-sensitive meaning that ADP is unable to enter the mitochondria for phosphorylation to ATP [65]. As a consequence cytosolic ADP is converted to ATP and AMP by adenylate kinase. AMP is subsequently broken down to adenosine which freely permeates out of the cell and is further metabolised. The net effect of this process is that the precursors of ATP synthesis are lost from the cell, inhibiting the restoration of normal metabolism upon reperfusion.

1.2.2.2 Induction of apoptosis

Cold ischaemia induces an increase in cytosolic calcium concentration in response to dysfunction of calcium pumps [66]. Under normal physiological conditions these pumps transport calcium extra-cellularly or into the endoplasmic reticulum [67]. During cold ischaemia mitochondrial uptake of calcium buffers elevated cytosolic calcium levels, thus protecting the cell [68]. If cold ischaemia is prolonged, the elevated cytoplasmic calcium concentration, in conjunction with low cytoplasmic ATP, results in activation of the calcium uniporter and subsequent mitochondrial calcium overload [69, 70]. Mitochondrial calcium overload leads to mitochondrial permeability transition pore (MPTP) formation and the loss of mitochondrial potential [71]. In turn MPTP formation leads to mitochondrial swelling, outer membrane rupture and release of pro-apoptotic factors, such as cytochrome c, apoptosis inducing factor and smac/DIABLO [72-74].

Hypothermia induces translocation of Bax, an important apoptosis regulatory protein, from the cytosol to the outer mitochondrial membrane [75]. Translocation of Bax, initiated by increased mitochondrial calcium levels, stimulates apoptosis by inducing the release of cytochrome c [75]. Bax mediated cytochrome c release is independent of MPTP formation [76]. Figure 3 shows a summary of the important steps in the induction of apoptosis.

Figure 3. Diagram showing steps in the induction of apoptosis.



1.2.3 Reperfusion Injury

The degree of injury resulting from organ reperfusion is dependent upon warm and cold ischaemia and the associated damage that prolonged durations generate. Unlike warm ischaemic and hypothermic injury the extent of reperfusion injury (RI) may not be exhibited immediately. Indeed, the effects of reperfusion may affect a renal allograft for its entire lifespan.

1.2.3.1 Reactive oxygen species (ROS)

Although accounting for less than 1% of body mass, the kidneys utilise 10% of total body oxygen consumption [77]. This metabolic demand for oxygen, to drive synthesis of ATP, is necessary for the renal tubular transport processes. The small amounts of ROS generated by this oxygen consumption are tolerated well by the normal kidney.

1.2.3.1.1 Sources of ROS

Warm ischaemia and hypothermia lead to a progressive loss of ATP and an accumulation of its breakdown products, such as hypoxanthine. During reperfusion hypoxanthine is converted to xanthine by xanthine oxidase with the consequent generation of hydrogen peroxide and superoxide. Hydrogen peroxide, which diffuses through the cell membrane, may form the oxidant Hypohalous acid via the action of myeloperoxidase. Myeloperoxidase is released by leukocytes recruited to areas of tissue injury [78]. During warm ischaemia the intracellular concentration of iron increases due to impaired binding to transferrin and ferritin [79]. This high intracellular iron concentration is a potent catalyst for the generation of the highly reactive hydroxyl radical from hydrogen peroxide [80]. Furthermore it has been shown that

iron-catalysed ROS readily produce necrosis by evading the glycine-sensitive death channel [81].

Injured endothelial/tubular cells demonstrate up-regulation of iNOS with consequent NO release. NO interacts with superoxide to form the peroxynitrite anion, as well as other reactive nitrogenous species [82, 83]. NO and peroxynitrite are particularly damaging to cellular processes. Enhanced NO release can deplete ATP, inactivate a host of enzymes required for mitochondrial electron transport and the citric acid cycle, nitrosylate tyrosine and sulphhydryl residues in proteins impairing their function and directly damage DNA [78].

In addition to the sources described above ROS may be generated by a host of other pathophysiological pathways in the post-ischaemic kidney. These pathways include; mitochondrial electron transport chains; the endoplasmic reticulum; the plasma membrane; arachidonic acid; cyclooxygenase and lipoxygenase; as well as the non-enzymatic auto-oxygenation of thiols [84].

1.2.3.1.2 Effects of ROS

The potential sources of ROS in ischaemic tissues are myriad, as are the potential targets for the deleterious effects of ROS.

ROS exert various vasoactive effects. Superoxide is known to be vasoconstrictive, largely due to NO scavenging, whereas hydrogen peroxide has both vasodilatory and vasoconstrictive effects [85]. Vasoconstrictor mediators such as endothelin, isoprostanes, and thromboxanes are induced by ROS [86-88]. Of these endothelin is the most heavily implicated in renal RI [89].

ROS impair ATP synthesis in a number of different ways. Hydrogen peroxide and other ROS lead to single strand DNA breaks, with subsequent up-regulation of poly-ADP-ribose polymerase, a DNA repair enzyme, which in turns impairs ATP synthesis [90, 91]. Glycolysis dependent ATP synthesis is inhibited by the action of hydrogen peroxide on glyceraldehyde-3-phosphate dehydrogenase [92]. Oxidants directly compromises mitochondrial ATP synthesis by inhibiting the ATP-synthetase complex [93, 94]. ROS act synergistically with elevated intracellular calcium levels to potentiate mitochondrial injury [95].

The sequelae of impaired ATP synthesis on cell structure, brought about by oxidative stress, are detailed above. It is important to appreciate that ROS can independently exert these adverse effects. The actin cytoskeleton, integrin interactions and cell permeability are all affected by oxidative stress [96-99].

ROS are injurious to cell and organelle membranes. Phospholipases, and in particular PLA_2, are activated in response to oxidative stress. The effect of this activation is to alter membrane integrity, impair membrane protein function and affect membrane to cytoskeleton interaction [100]. Sphingolipids, present on the surface of the plasma membrane, hydrolyse when exposed to oxidants yielding ceramide. Ceramide is the subject of intense research, however, its role in the induction and regulation of apoptosis is undoubted [101]. The oxidation of phospholipids yields isoprostanes which have been demonstrated to be excellent markers of oxidative stress [102].

Oxidants have an important role in the generation of the inflammatory response in RI. ROS up-regulate NF-κB and genes encoding cytokines that recruit and marginate neutrophils, monocytes and T cells [78, 103, 104].

1.2.3.2 Inflammation

Following reperfusion there are a plethora of changes to endothelial and tubular cells resulting in the release a host of pro-inflammatory cytokines, such as Tumour necrosis factor-α (TNFα); Interleukins-6 and -1β; Transforming growth factor-β (TGFβ), and chemotactic cytokines, such as Monocyte chemotactic protein-1 (MCP-1); IL-8; RANTES [105, 106]. The importance of the chemotactic cytokines and their role in the interactions between the endothelium and leukocytes will be discussed below in the leukocyte section of this introduction. Certain cytokines and their receptors are able to mediate RI independent of leukocytes.

The release of TNFα on reperfusion is in part mediated by ROS production, which activate the transcription factor NF-κB via p38 MAP kinase [107]. Inhibition of the transcriptional pathway for TNFα, or monoclonal blockade of TNFα itself can prevent apoptosis in ischaemic cells [108, 109]. Furthermore the TNF receptor family, including CD95 (Fas), TNFR-1 and CD27 have also been implicated in leukocyte independent RI [107, 110, 111]. Fas is a well established trigger for apoptotic renal tubular cell death [112]. Proximal tubular cells subjected to ATP depletion have been shown to undergo apoptosis associated with enhanced Fas expression [113]. CD27 in association with its binding partner, Siva, are induced during apoptosis in post-reperfusion tubular cells [110].

1.2.3.3 Caspases

The cysteine-aspartic acid proteases, or caspases, are a group of enzymes pivotal in the generation of apoptosis. Altered expression of caspases-1, -2 and -6 has been demonstrated in kidneys following reperfusion [114]. Furthermore, *in vitro* studies

have demonstrated increased activation of caspases-3 and -8 under hypoxic conditions [115]. Activation of caspase-3, associated with Bax translocation to the mitochondrial outer membrane and subsequent cytochrome c release, which together act to increase apoptosis, has been demonstrated during hypoxia and re-oxygenation of cultured renal cells [116, 117].

1.2.3.4 Complement

RI is a well established trigger for the activation of the complement cascade. In most tissues activation occurs via the classical pathway, however in the kidney there is strong evidence that the alternative pathway predominates [118-120]. There is recent evidence that the mannose-binding lectin pathway, a third mechanism by which complement activation occurs, may also have importance in renal RI [121]. The common step in the complement cascade occurs at the level of C3 activation with the formation of a C5-convertase that activates C5. Activation of C5 results in the creation of C5b, the first step in the formation of the membrane attack complex (MAC), and C5a. Previously, the MAC had been considered the predominant mechanism by which complement exerted its effects in RI. The MAC inserts into the membrane of target cells inducing direct cell injury and necrosis [122]. Sub-lethal levels of MAC activate neutrophils and the endothelium by up-regulating adhesion molecules and the release of cytokines, ROS and hydrolytic enzymes [123, 124]. More recently the role of C5a in RI has gained importance. C5a is a potent chemo-attractant that recruits leukocytes, stimulates ROS production, modulates cytokine release and up-regulates adhesion molecule expression [125-128]. C5a expression is observed to be markedly up-regulated in tubules following RI [129].

1.2.3.5 Leukocyte actions and interactions

Leukocytes interact with the injured tubule and endothelium in a complex manner. Local tissue injury initiates a cascade of events that results in the triggering, tethering, strong adhesion and trans-endothelial migration of leukocytes. The molecular processes underlying these observations will be expounded below.

1.2.3.5.1 Local injury

Ischaemic, hypothermic and reperfusion injury induce endothelial and tubular injury. In response to this injury there is up-regulation of a number of cytokines, such as TNFα, interferon-γ (INFγ), IL-1, IL-2 and IL-8 [130]. This in turn leads to up-regulation of adhesion molecules on the endothelium, tubule and leukocytes. In post-ischaemic tissues, complement activation and increased levels of lipid mediators, such as leukotrienes and platelet activating factor (PAF), are chemotactic for neutrophils [131].

In response to cytokine release there is enhanced expression of certain endothelial and leukocyte adhesion molecules. ICAM-1, selectin and integrin expression have been shown to be increased [132-134].

1.2.3.5.2 Tethering, margination and transmigration

Initial capture of leukocytes and slow margination is mediated by selectin interactions. There are three molecules within the selectin family: E-, L- and P-selectin. E-selectin is expressed by activated endothelial cells. L-selectin is expressed on all leukocytes. P-selectin is expressed on platelets and activated endothelial cells [135]. Originally L-selectin and endothelial derived P-selectin were considered important in leukocyte tethering [136]. However, recent studies have demonstrated that platelet derived P-

selectin is more important in the context of leukocyte adhesion [137]. Selectin binding is weak and readily reversible.

In order for transmigration to occur the leukocyte must become flattened and firmly adherent to the endothelial surface. This process is mediated by ICAM-1 and the integrins [134]. ICAM-1 is expressed by activated endothelial cells and has been shown to the main counter-receptor for the leukocyte derived β2-integrins, lymphocyte function antigen-1 (LFA-1) and Macrophage-1 antigen (Mac-1) [58].

Trans-endothelial migration (TEM) is the final step for leukocyte infiltration into an injury tissue. Transmigration is extremely complex and still poorly understood. The main mechanism by which TEM occurs is by diapedesis through lateral adherens junctions, although there are proposed mechanisms for trans-cellular migration of leukocytes [138, 139].

1.2.3.5.3 Leukocyte subsets

The role of different leukocyte subtypes in RI is still debated. Neutrophils are the first cells to infiltrate, but experimental blockade/depletion of neutrophils give variable protection from injury [140]. Furthermore, in humans neutrophil infiltration is not a prominent feature in post-ischaemic injury [141]. Macrophage infiltration occurs later than neutrophil infiltration. Some protection against RI has been observed experimentally by selective macrophage depletion [142]. Recently the importance of the T lymphocyte in RI has been realised. T cells recruit later than macrophages and are observed to localize to areas of greatest injury [143]. There is a wealth of experimental data demonstrating protection from RI in T cell depleted and knock-out

models [144, 145]. The identification of seemingly protective (Th2) and deleterious (Th1) phenotypes further complicates the role of T lymphocytes in RI [146].

1.2.3.5.4 Leukocyte effects

Once recruited to the site of injury leukocytes exert a host of effects. Platelet and leukocyte adhesion to the injured endothelium causes capillary plugging and the no-reflow phenomenon, which leads to further ischaemic damage. Adherent and infiltrating leukocytes release a number of pro-inflammatory cytokines, including TNFα, TGFβ, MCP-1,IL-1, IL-6, IL-8, that serve to worsen tissue injury [141, 147]. ROS are generated by white cells further augmenting cell death. There is also a release of proteolytic and matrix degrading enzymes that enhance damage and further stimulate leukocyte infiltration. The potential deleterious impact of infiltrating leukocytes with regard to allograft immunogenicity must also be appreciated.

1.2.3.6 Nitric oxide and nitric oxide Synthase

NO is a diatomic free radical. NO is synthesized from the terminal guanidino group of L-arginine during its oxidation to L-citrulline by a group of enzymes called nitric oxide synthases (NOS).

1.2.3.6.1 Nitric oxide synthase

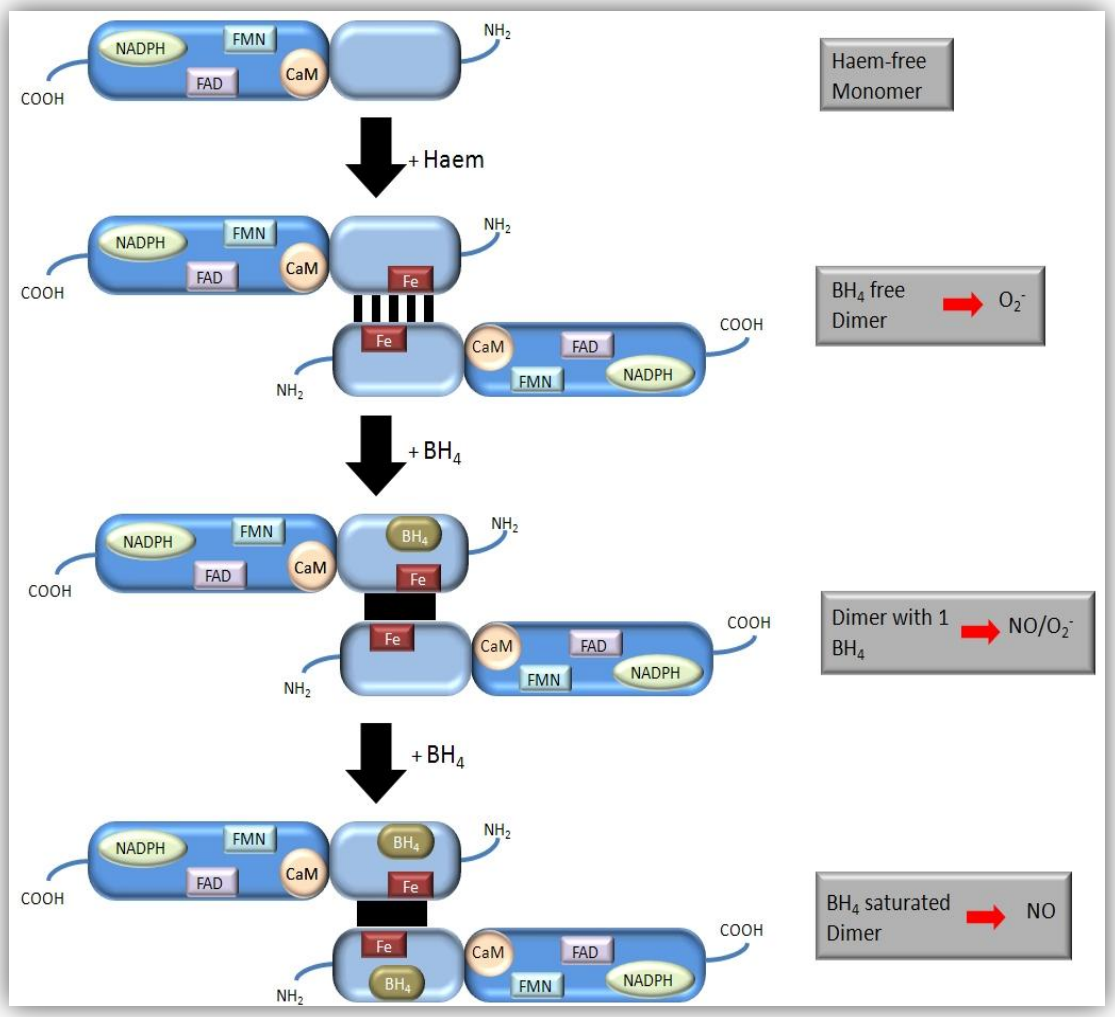
There are three distinct isoforms of NOS. Endothelial NOS (eNOS) and neuronal NOS are constitutively expressed, whereas the third isoforms, inducible NOS (iNOS), is only expressed in response to inflammatory/cytokine stimulus.

Structurally NOS is composed of two identical monomers. Each monomer is divided into two major domains, the C-terminal reductase domain and the N-terminal oxygenase domain, linked by a calmodulin binding domain [148]. The C-terminal

contains binding sites for nicotinamide-adenine-dinucleotide phosphate (NADPH) and the flavins, flavine adenine dinucleotide and flavine mononucleotide. The N-terminal binds the substrate L-arginine in addition to haem and tetrahydrobiopterin (BH_4). The interposed calmodulin binding domain plays a pivotal role in the structure and function of NOS [149]. NO can only be generated by dimeric NOS. Haem is essential for the dimerisation of all NOS subtypes, whilst BH_4 is required for iNOS dimerisation and stabilisation of eNOS dimers [150-152]. L-arginine is necessary for the dimerisation of iNOS [150].

Catalytically, L-arginine is initially hydroxylated, then oxidized by electrons derived from NADPH, via the flavins, to yield NO and L-citrulline. NOS, however, is not only capable of generating NO, but also superoxide. In the presence of low L-arginine and/or low BH_4 concentrations both monomeric and dimeric forms of NOS generate superoxide[153, 154]. Importantly, the dimeric forms of NOS seem to share a single BH_4 molecule, meaning that dimeric NOS can produce both NO and superoxide simultaneously [149]. High BH_4 levels have been shown to inhibit superoxide formation via a scavenging mechanism [155]. Figure 4 shows the catalytic function of NOS dependent upon cofactor presence.

Figure 4. Diagram showing the catalytic functions of NOS depending upon presence of cofactors.



1.2.3.6.2 Nitric oxide physiology and pathophysiology

The physiological effects of NO are divided into direct and indirect categories. The direct effects are those mediated by the NO *per se*, whereas the indirect effects are mediated by NO free radicals such as peroxynitrite.

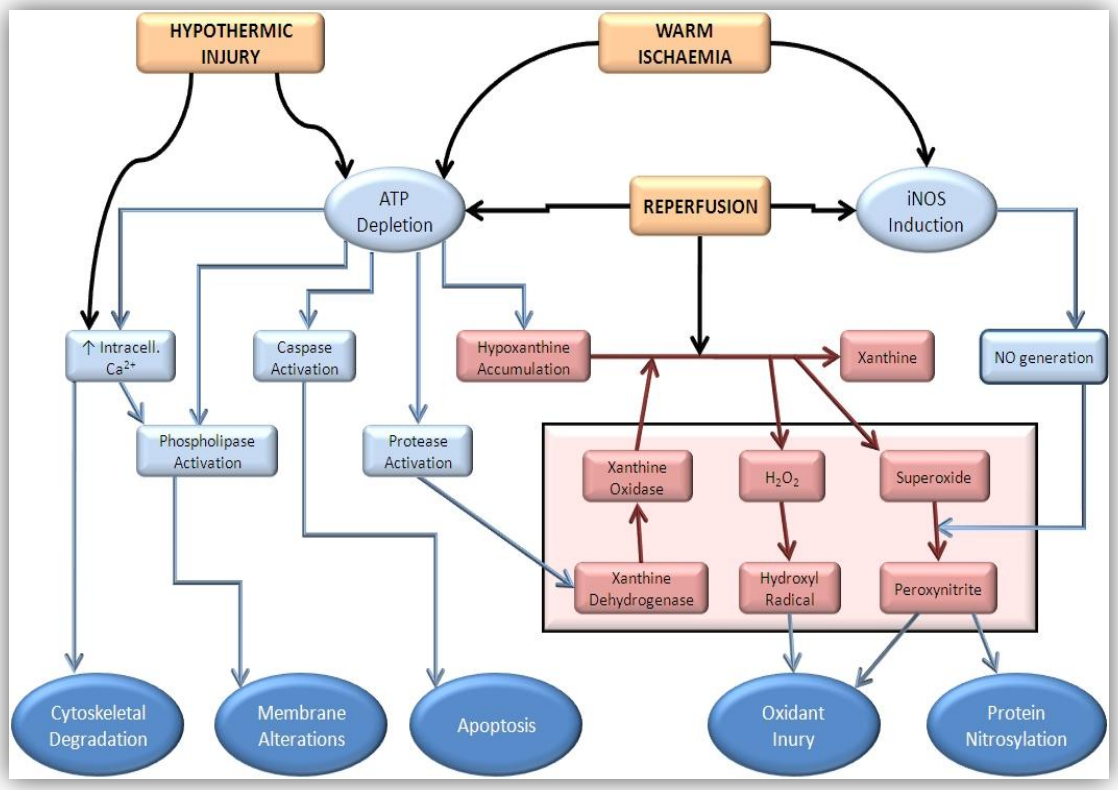
NO acts directly in a tissue protective manner to regulate vascular tone, inhibit platelet aggregation and leukocyte adhesion, scavenge ROS and maintain vascular homeostasis

by preserving normal vascular permeability, inhibiting smooth muscle proliferation and stimulating endothelial regeneration.

In response to ischaemia, and subsequent cytokine release, there is up-regulation of iNOS. At the time of reperfusion there is a surge in superoxide production from eNOS and iNOS, due to a progressive loss of L-arginine and BH_4 during ischaemia and cold storage [156, 157]. The NOS derived superoxide, in addition to that from other cellular sources, reacts with NO to produce peroxynitrite. Peroxynitrite causes tissue damage by inducing apoptosis, DNA fragmentation, lipid peroxidation, protein lysis and nitration and enzyme inactivation.

Figure 5 shows a schematic summarising the event in ischaemic, hypothermic and reperfusion injury.

Figure 5. Schematic showing the main processes in ischaemic, hypothermic and reperfusion injury.



1.2.3.6.3 Role of NO donors and NOS inhibitors

The use of NO donors and NOS inhibitors experimentally aids the clarification of the physiological pathways involved in the pathogenesis of RI. Furthermore, these experiments also offer possible strategies for the clinical amelioration of RI.

The administration of L-arginine has been shown in several RI models to abolish microvascular constriction and reduce neutrophil infiltration [158]. Sodium nitroprusside (SNP) is a well established NO donor and has been shown to improve RI associated vasospasm [158].

The non-specific NOS inhibitors, N-methyl-L-arginine acetate (L-NMMA) and N^{w}-nitro-L-arginine methyl ester (L-NAME), have been used to assess the impact of NOS inhibition on RI. These results demonstrated that inhibition of NOS was generally associated with less tissue injury, although the effect may be dependent upon the time of inhibitor administration [159, 160]. N-(3-(Aminomethyl)benzyl)acetamidine, or 1400W for short, is a novel, highly sensitive and specific inhibitor of iNOS with very limited crossover inhibition of eNOS [161]. Experimental models using 1400W have shown that it is able to ameliorate RI [162-164]. Conversely, some studies have demonstrated that selective iNOS blockade enhanced RI [164].

Undoubtedly the investigation of RI using NO donors and NOS inhibitors is fraught with potential confounding factors. There are a multitude of different models for RI, in a multitude of different species and different tissues. Ischaemic, hypothermic and reperfusion protocols in the published studies are highly disparate. The multifunctional role of NOS itself further skews results when it is appreciated that even in baseline conditions it produces potentially helpful NO and harmful superoxide.

When these factors are applied to an organ as complex as the kidney it is easy to conclude that the understanding of the roles of NO and NOS are far from completely understood.

1.3 Renal Preservation

Up until quite recently the preservation step in transplantation was considered a static phase; nothing is actively done to the organ and little harm is caused to the organ due to the slowed physiology associated with hypothermia. However, with growing evidence that prolonged cold ischaemic times (CIT) adversely affect organ quality and that intervention during hypothermic storage maybe able to improve organ viability, the importance of organ preservation techniques once again comes to the fore.

1.3.1 The origins of renal preservation

Originally, donor kidneys did not undergo any form of preservation. In 1954 Hume *et al* reviewed nine cases of renal transplantation in which the mean WIT was 136 minutes [165]. In this series five of the transplants never gained function, and four*...secreted urine from 37 to 180 days*. Although the potential protective role of hypothermia on renal physiology had been suggested in the 1940s, it was only in the 1960s that hypothermic techniques were utilised and recommended for renal preservation [166]. In 1964, Ackermann and Barnard demonstrated the benefits of hypothermia and continuous autologous blood perfusion for renal preservation in canines [166]. These same experiments showed that renal function, following

hypothermic flush-out and surface cooling with a saline-based solution, was massively inferior to continuous organ perfusion. Five years later, in an attempt to simplify organ preservation, Collins *et al* revisited static hypothermic preservation but trialled different perfusates [167]. These results demonstrated that surface cooling and hypothermic flush-out with, in essence an intracellular fluid, improved the quality of stored organs to a level approaching that of continuously perfused organs. These experiments showed for the first time that long-term preservation, 30 hours in canine kidneys, did not necessitate complex apparatus and fluid compositions, and thus heralded the concept of organ transportation. Collins demonstrated this potential by shipping canine kidneys from Los Angeles to London, Sydney and Tel Aviv for successful transplantation. Concurrently, Belzer refined hypothermic perfusion utilising plasma only [168]. In 1972, Claes and Blohme further refined the technique by demonstrating equivalent results between plasma and albumin preservation [169]. By the 1980s hypothermic machine perfusion (HMP), with plasma/albumin and static preservation with crystalloid perfusates, were established as the methods of choice for renal preservation. Although there had been little change to the methodologies of perfusion or static preservation, the 1980s heralded a greater understanding of the deleterious processes occurring in hypothermic and ischaemic kidneys. Further elucidation of the deleterious processes affecting donor kidneys, coupled with the need for utilizing increasing numbers of marginal organs, such as those from DCD donors, have driven the refinement of current preservation techniques.

1.3.2 Current status of renal preservation

Since the 1980s University of Wisconsin solution has been accepted as the benchmark preservation fluid, although several newer fluids have been developed, albeit with preservation of specific organs in mind.

There is still some debate between the relative merits of static cold storage (SCS) and HMP. The current evidence suggests that HMP, although more technically demanding and costly is superior to SCS in a number of respects. Lower rates of DGF and better function over longer preservation times are reported [170-172]. It has been suggested that these findings are related to replacement of metabolic substrates, such as adenine nucleotides, clearance of catabolic products, prevention of tissue oedema and improvement of cortical microcirculation, which have all been observed during machine preservation [26-28]. These findings are of particular significance in DCD donor kidneys [22, 173, 174]. Furthermore, HMP techniques confer the potential for testing organ viability that SCS cannot. Perfusate flow characteristics during HMP maybe predictive for initial graft function and kidney viability in general [175-177]. HMP also allows the administration of drugs to the kidney.

The current status of organ supply versus demand necessitates the use of sub-optimal donor organs, such as those from DCD donors, organs with long cold storage times and organs from donors with adverse co-morbidities. The goal of any renal preservation technique should be to allow easy viability testing, organ resuscitation to optimize the function of the organ prior to transplantation, and should not damage the organ any further. HMP goes some way to providing solutions to these Grails of preservation. Unfortunately, HMP still requires deleterious cooling of the organ being preserved,

thus damaging the graft further and consequently limiting the resuscitative potential of the technique.

1.3.3 The future of organ preservation – normothermic preservation

Perfusion techniques allow the assessment of potential functionality markers such as perfusion pressure and renal blood flow and metabolic markers in the perfusate such as oxygen consumption and acid-base balance. It would seem logical that, by avoiding hypothermic damage and reperfusion injury, replication of physiological perfusion could provide an ideal storage method. For these reasons, there has been a resurgence of interest in normothermic preservation (NP). This enthusiasm is primarily for the potential role of NP in DCD kidney preservation.

Historically, Kootstra first described the NP of isolated kidneys with a view to transplantation in 1980. This groups' later work demonstrated that hypothermically stored kidneys could be resuscitated by normothermic *ex-vivo* perfusion [178]. After 3 days of SCS and a period of normothermic *ex-vivo* perfusion kidneys could be stored for a further 3 days yet remain viable following transplantation. These results were not replicated by 6 days SCS alone.

Due to a presumed lack of substrates, hormones and an accumulation of waste products, the maximal duration of NP of kidneys was restricted to a few hours. Under these conditions, there is evidence of increasing vascular resistance and oedema coupled with an evident inability to supply the appropriate nutrients and metabolic requirement of the kidney [179]. In order to counteract the pathophysiological

limitations associated with normothermic blood perfusion Braisle *et al* developed an acellular perfusion fluid [180]. This solution was designed to provide all the metabolic needs of the organ, including oxygen requirement, yet maintain the normal functions of the vascular endothelium that may be compromised with other methods of storage. From 1997 onwards, this group developed and improved upon this solution demonstrating its ability to improve initial function in organs with prolonged WIT when compared to HMP and potentially to ameliorate RI [180, 181]. Having shown the potential for this technique in the resuscitation of DCD donor analogues, the group moved to demonstrate the techniques potential as an absolute alternative to SCS/HMP. In 2003, Brasile showed that after 24 and 48 hours of warm perfusion, initial function of transplanted kidneys was better than in kidneys cold stored or cold perfused for the same duration [182]. The potential of this technique to provide accurate viability testing, by assessing oxygen and glucose consumption as well as renal blood flow parameters, was also suggested [183]. In 2005, the group moved beyond resuscitation of organs and considered the concept of *ex-vivo* organ repair. Canine kidneys were subjected to two hours warm ischaemia and perfused, as previously described, but in one group the perfusate was supplemented with fibroblast growth factors. This experiment showed improved initial creatinine clearance and improved cellular repair mechanisms in the group administered with growth factor [184]. In addition, four human organs, not suitable for transplantation, were perfused and gene transfection with the Ad5.CMV5GFP reporter gene attempted. Histological analysis showed successful transfection of the endothelium and expression of green fluorescent protein (GFP) at the end of the 24-hour perfusion period.

The decade of work performed by this group has shown the potential power of normothermic perfusion as a direct alternative to SCS/HMP for kidneys from all donors. The minimization of reperfusion injury, organ resuscitation and, latterly, organ repair has highlighted the importance of NP in DCD donor organs. The main drawback of this technique is the perfusate itself which, by nature of its complexity, may be prohibitively costly when compared with traditional storage techniques.

Perhaps the most cost effective fluid for normothermic perfusion is donor blood. There are well-established problems with long-term NP with blood due to haemolysis and potential damage to the endothelium. However, in the context of short-term perfusion of up to a few hours, successful resuscitation, viability testing and repair can be initiated. Renal normothermic blood perfusion research has, in recent times, been spearheaded by the Leicester group in the UK. The Leicester model utilizes organs and blood derived from slaughterhouse pigs. Blood is oxygenated via a membrane oxygenator and supplemented with lipids and glucose to satisfy the metabolic need of the kidney. In 2002, Metcalfe showed that porcine kidneys exposed to minimal warm ischaemia had better initial function after 16 hours normothermic blood-perfusion than when compared to HMP kidneys [185]. Having demonstrated that blood perfusion was not only equivalent but also better than hypothermic perfusion, the model moved towards the optimization of organ function following a period of hypothermic storage and assessment of organ viability [186-188]. Kidneys perfused with leukocyte-depleted blood and whole blood were compared after receiving a short warm ischaemic insult and 2 hours SCS. In this model of DBD kidney preservation

improved renal blood flow and initial function were demonstrated in the leukocyte-depleted group [189].

The future reliance upon DCD kidneys and the importance of optimizing and assessing function in these organs led the group to focus upon development of a porcine DCD model [190]. Hosgood *et al* demonstrated the importance of the cold storage period in the generation of RI in DCD kidneys [191]. Subsequently, amelioration of RI and restoration of initial function to that seen with short SCS/WIT kidneys was achieved with various therapeutic interventions. Bagul *et al* showed an increase in oxygen utilization by DCD kidneys when perfusate was supplemented with erythropoeitin and also demonstrated an improvement in initial function with the carbon monoxide donor CORM-3 [192-194].

2 Hypothesis and Aims

2.1 Hypothesis

The theory underpinning this study is that circulating leukocytes and NO levels at reperfusion are pivotal in dictating initial function of transplanted DCD kidneys. Therefore, the overarching hypotheses of this study are:

- If initial graft function is related to the presence of circulating leukocytes at reperfusion, then depletion of leukocytes will effect initial graft function.
- If initial graft function is related to NO levels at reperfusion, then modulation of NO levels or NOS activity will effect initial graft function.

2.2 Aims

The aims of this study are as follows:

- Demonstrate that reperfusion with leukocyte depleted blood improves initial graft function in this model of DCD transplantation.
- Demonstrate that reperfusion with blood supplemented with a specific iNOS inhibitor/SNP improves initial graft function.
- Demonstrate a synergistic effect of leukocyte depletion and iNOS inhibition/NO supplementation on graft function.

Graft function will be quantified with a number of indices described in the Materials and Methods chapter.

3 Materials and Methods

3.1 Tissue procurement and preparation

3.1.1 Kidney and blood retrieval

Large white slaughterhouse pigs of 60-70kg in weight were stunned by electrocution. Following stunning pigs were culled by exsanguination resulting from division of the internal jugular/innominate vein. Approximately one litre of fresh blood was collected into a sterile receptacle, to which 25,000 units of Heparin (Multiparin; CP Pharmaceuticals, Wrexham, UK) was added. The blood was then transferred into CPDA-1 blood bags (Baxter Healthcare, Thetford UK) and stored at 4^{o}C until use.

Following blood collection, a midline laparotomy incision was made into the pig cadaver and intra-peritoneal viscera removed *en-bloc*. The pig cadaver was then laid onto an operating table. Kidneys were then cleanly dissected free from the cadaver. Following a defined *in situ* period of warm ischaemia kidneys were transferred from the cadaver to a separate sterile area. The kidneys were placed into a cold bath of crushed frozen saline (Normal Saline; Baxter healthcare, Norfolk, UK) and hyperosmolar citrate solution (Soltran; Baxter healthcare, Norfolk, UK). The renal artery was cannulated with a Tibbs vascular cannula of an appropriate size. The kidneys were then flushed with 250ml of hyperosmolar citrate solution (Soltran; Baxter healthcare, Norfolk, UK) at a hydrostatic pressure of 100cmH_2O and a temperature of 4^{o}C. Following the cold flush the organs were weighed and wedge biopsies taken. Biopsies were fixed in formalin. Kidneys were then placed on ice in

hyperosmolar citrate solution (Soltran; Baxter healthcare, Norfolk, UK) and transferred to the laboratory in a insulated transplant box.

Organs were subjected to a WIT of 25 minutes from the time of exsanguination until commencement of the cold flush. Kidneys were cold stored for a period of 2 hr or 18 hours.

3.1.2 Kidney preparation

Following the cold ischaemic period the organs were removed from ice and transferred to the back bench in the laboratory for preparation prior to reperfusion. The kidneys were placed into a cold bath of crushed frozen saline (Normal Saline; Baxter Healthcare, Thetford, UK) and hyperosmolar citrate solution (Soltran; Baxter Healthcare, Thetford, UK). The renal artery and vein were cannulated with custom tubing obtained from the normothermic perfusion circuit (Medtronic, Tolochenaz, Switzerland). The ureter was cannulated with a Nelaton 14Fr catheter (Pennine Healthcare, Derby, UK). Canullae were secured with 2/0 polyglactin ligatures. The kidneys were then flushed with 100ml of Ringer's lactate solution (Baxter Healthcare, Thetford, UK), at a temperature of 4^{o}C. The kidneys were reweighed and a further wedge biopsy taken. Biopsies were divided and fixed in formalin.Kidneys were then transferred to the normothermic perfusion system (NPS) for reperfusion.

3.1.3 Blood preparation

White cell filtration was performed prior to addition to the NPS. Retrieved whole blood (WB) was passed through a LeukoGuard® RS white cell filter (Pall Medical, Portsmouth, UK). Following filtration the leukocyte depleted blood (LDB) was added to the reservoir of the NPS.

Non-white cell filtered, whole blood was added directly to the reservoir of the NPS.

3.2 The normothermic perfusion system

3.2.1 Pre-perfusion preparation

The NPS was composed of a number of commercially available clinical-grade cardiopulmonary bypass components. The individual components consist of a centrifugal blood pump and remote control console (550 Bio-pump® and 550 Bio-console®; Medtronic, Tolochenaz, Switzerland), a heat exchanger (GD120; Grant, Cambridge, UK), a 5-litre venous reservoir (Medtronic) and a Minimax Plus® membrane oxygenator (Medtronic, Tolochenaz, Switzerland). 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) were connected to the system to allow the accurate administration of additional fluids and the measurement of urine output. The connective tubing required was purchased in the form of a

custom kit (Medtronic, Tolochenaz, Switzerland). Figure 6 shows a schematic of the NPS circuit.

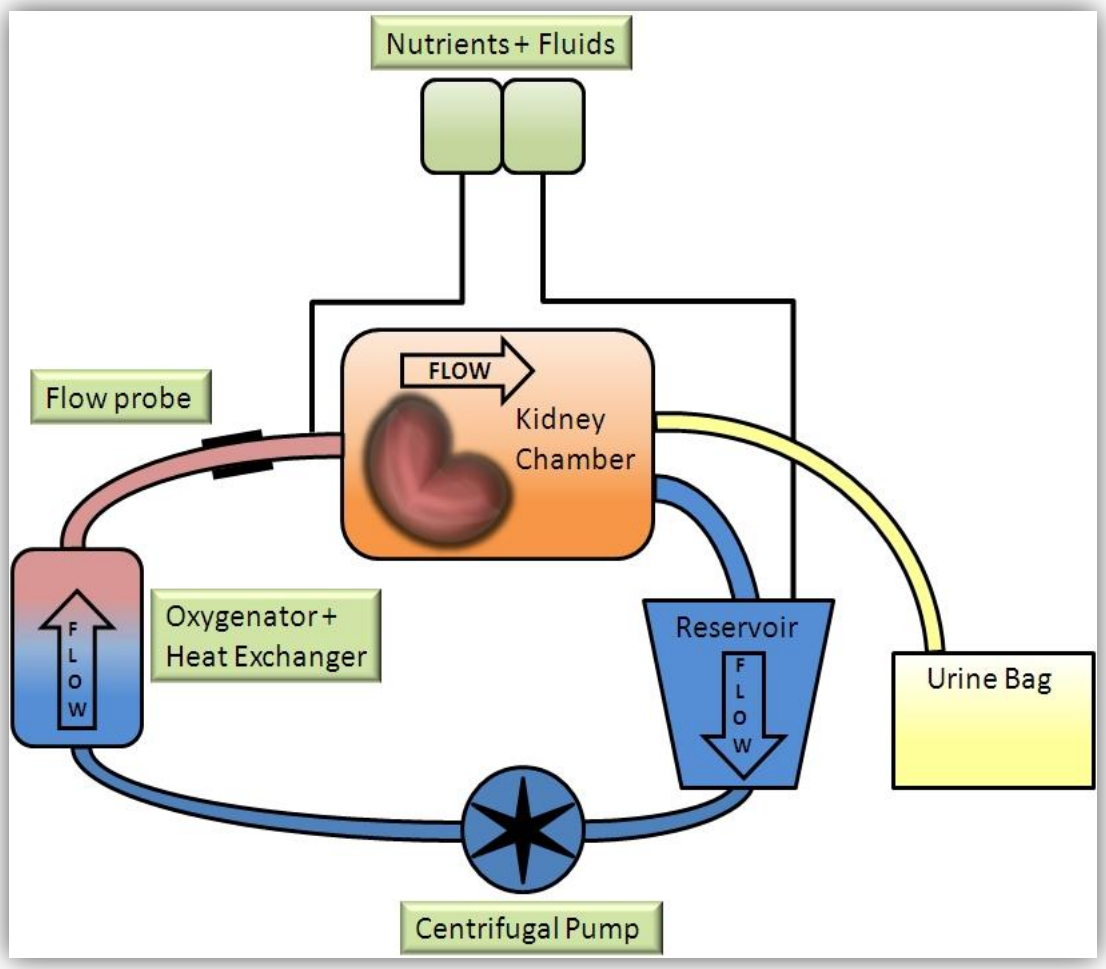
Prior to placing the kidney in the NPS circuit the system was primed with perfusate. The main constituents of the perfusate were 500ml of blood and 500ml of Ringer's Lactate solution (Baxter Healthcare, Thetford, UK). Additives to this priming solution are summarized in Table 3. This mixture was placed in the reservoir and run through the circuit until a temperature of 37^{o}C is achieved. A creatinine bolus (Sigma-Aldrich, Steinheim, Germany) was added to the perfusate to achieve an initial circulating concentration of 1000µmol/l.

Prior to reperfusion of the kidney a full blood count and blood urea, serum creatinine and electrolyte levels were taken. An arterial/venous blood gas was also taken at this time.

Table 3. Summary of additives to pump priming solution.

Additive	**Volume**	**Rationale**
Cefuroxime (Stragen, Reigate, UK)	750mg	Antibiosis
Mannitol (Sigma-Aldrich, Steinheim, Germany)	5g	Diuretic/ vasodilator
Sodium Bicarbonate 8.4% (Fresenius, Runcorn, Cheshire)	12ml	Buffer
Nutriflex lipid peri® (B. Braun, Sheffield, UK)	20ml/hr	Nutrition
Glucose 5% (Baxter Healthcare, Thetford, UK)	7ml/hr	Nutrition
Supplement to Nutriflex Solution (1250ml)		
Actrapid® (Novo Nordisk, Crawley, UK)	100u	Glycogenesis
Sodium Bicarbonate 8.4% (Fresenius, Runcorn, Cheshire)	25ml	Buffer

Figure 6. Schematic of the normothermic perfusion system.



3.2.2 Preparation of experimental agents

Experimental agents were added as either a bolus dose prior to perfusion of the kidney or as a continual infusion throughout the reperfusion period.

3.2.2.1 N-(3-[Aminomethyl]benzyl)acetamidine (1400W)

The iNOS inhibitor 1400W was obtained from Sigma-Aldrich (Steinheim, Germany). The 1400W powder was reconstituted with 100% ethanol in accordance with manufacturer's recommendations to a concentration of 200µg/ml. In accordance with

the literature 1400W was added to the perfusate at a dose of 10mg/kg and 1mg/kg of kidney weight [161, 163].

3.2.2.2 Sodium Nitroprusside (SNP)

The NO donor SNP was obtained fromSigma-Aldrich (Steinheim, Germany). SNP powder was reconstituted at a concentration of 100µg/ml of 5% glucose solution (Baxter Healthcare, Thetford, UK). The SNP was added to the NPS as a continuous infusion at a rate of 15ml/hr for the full duration of the reperfusion period.

3.2.3 Reperfusion

The cannulated artery, vein and ureter were attached to the arterial limb, venous limb and urine bag respectively of the NPS. Mean arterial blood pressure (MAP) was increased to 85mmHg, on a 550 Bio-Console® (Medtronic, Tolochenaz, Switzerland), a TX-50 Bio-Probe® (Medtronic, Tolochenaz, Switzerland), was placed in the arterial limb of the NPS circuit to allow direct measurement of renal blood flow. Temperature was recorded continuously and maintained at 37-39^{o}C by altering the temperature of the heat-exchanger water-bath. All kidneys were reperfused for three hours. Measurement of MAP, renal blood flow, temperature and urine output were recorded at 0, 5, 10, 15, 60, 120 and 180 minutes post-reperfusion. During the reperfusion period urine and plasma samples and renal biopsies were taken. Figure 7 shows a photograph of the NPS. Table 4 summarises the timing schedule for all samples taken.

Figure 7. Photograph of the normothermic perfusion system.

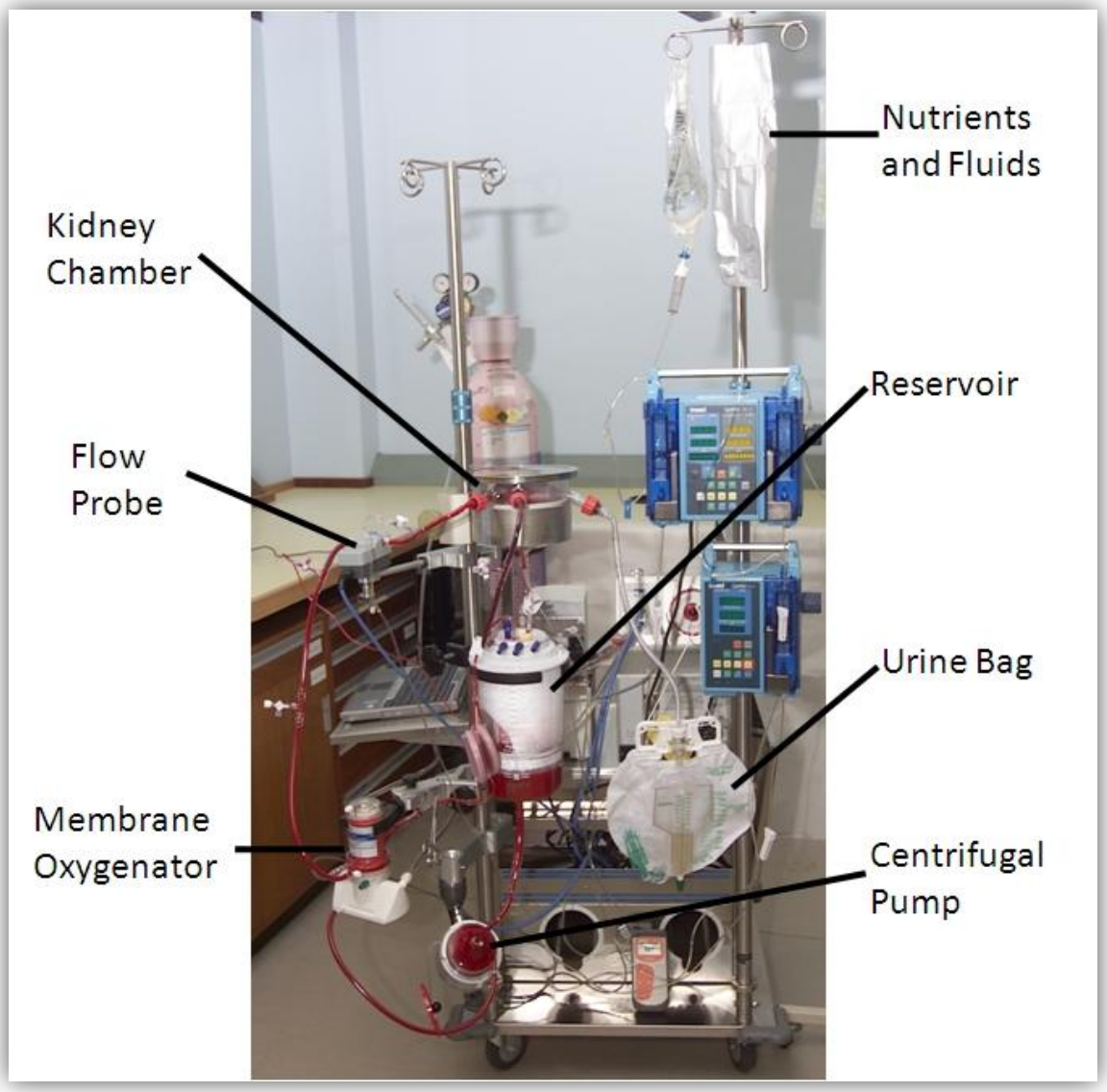


Table 4. Summary of samples taken at each time point. FBC; full blood count, UE; plasma urea, creatinine and electrolytes, A/VBG; arterial/venous blood gas, UC; urine chemistry, SCS; static cold storage. SFP/U; snap frozen plasma/urine, RB; renal biopsy

Time Point (minutes)	Samples taken
Pre-SCS	RB
Post-SCS	RB
0 (primed circuit)	FBC, UE, A/VBG, SFP
60	UE, UC, A/VBG, RB, SFP/U
120	UE, UC
180	FBC, UE, UC, A/VBG, RB, SFP/U

3.2.4 Post-reperfusion

Following three hours of reperfusion the kidneys were taken off the NPS. Further post-reperfusion biopsies were taken and the kidneys were re-weighed.

Kidneys and blood products were discarded via a locally agreed method for clinical waste products.

3.3 Experimental Design

The number of kidneys used in each group was six (n=6).

3.3.1 Control groups

Two control groups were used throughout this study. Group one consisted of kidneys subjected to 25 min WIT and 2hr SCS time, reperfused with WB and no experimental agents. Group two consisted of kidneys subjected to 25 min WIT and 18hr SCS time, reperfused with WB and no experimental agents.

3.3.2 Experimental groups

All experimental groups received 25 min WIT and 18hr SCS. Experimental groups are summarized in Table 5. Experimental order is shown in figure 8.

Table 5. Summary of groups. LDB; leukocyte depleted blood, WB; whole blood, SNP; sodium nitroprusside.

Group	Perfusate	Experimental agent	Group name
1	WB	-	2hr Control
2	WB	-	18hr Control
3	LDB	-	LDB
4	WB	1400W	WB+1400W
5	LDB	1400W	LDB+1400W
6	WB	SNP	WB+SNP
7	LDB	SNP	LDB+SNP

3.3.3 Sample analysis

3.3.3.1 Full blood count analysis

Full blood counts were performed in the haematology department of a local hospital using an ADVIA®-2120 Haematology System (Siemens, Surrey, UK).

3.3.3.2 Urea, creatinine and electrolyte analysis

Urea, creatinine and electrolyte quantification was performed in the biochemistry department of a local hospital using an ADVIA®-1800 Chemistry System (Siemens, Surrey, UK).

3.3.3.3 Blood gas analysis

Blood gas analysis was performed immediately using a using a Rapidlab™-248 (Bayer Healthcare, MA, USA) blood gas analyser.

3.3.3.4 Histology

As mentioned above wedge biopsies were taken at various time points. Biopsies were fixed in 10% formal saline, dehydrated and embedded in paraffin wax. Sections of 4µm were cut and stained with haematoxylin and eosin for evaluation under light microscopy. Sections were scored over five fields, assessing changes in four morphological variables**;** tubular dilation, tubular debris, vacuolation and interstitial infiltration. Three trained assessors blinded to the experimental group scored samples from 0 to 3 according to the level of damage; 0 representing normal, 1 – mild , 2 – moderate and 3 severe morphological changes.

3.3.3.5 Urinary nitric oxide quantification

Urine samples, taken at 60 and 180 minutes post-reperfusion were immediately snap frozen in liquid nitrogen and stored at -80^{o}C. Urine levels of nitric oxide were quantified using a total nitric oxide assay kit (Assay Designs, MI, USA) according to the manufacturers instructions. This assay relies on the conversion of nitric oxide to nitrate and the subsequent conversion of nitrate to nitrite by the enzyme nitrate reductase. Nitrite is detected colourimetrically at 540nm as an azodye product of the Griess reaction.

3.3.3.5.1 Assay reagents and preparation

The reaction buffer provided in the kit is a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) based buffer. The final concentration of reaction buffer was achieved by diluting 10ml of supplied concentrate with 90ml of deionized water (dH_2O).

Nicotinamide adenine dinucleotide (NADH) reagent was reconstituted by the addition of 1ml of dH_2O. The reconstituted reagent was stored at -20^oC until use. The final NADH dilution was composed of 0.9ml of reagent diluted in 1.8ml of dH_2O, vortexed and stored on ice.

Nitrate reductase enzyme was reconstituted with 1ml of the provided nitrate reductase storage buffer. This mixture was vortexed and left at room temperature for 15 minutes. This cycle was repeated and the mixture stored on ice until the final dilution.

The volume of final enzyme dilution required was dependent on the number well used in the assay. The final dilution was composed of a mix of the reconstituted enzyme solution and reaction buffer. The final volume was derived using the following equations:

A. $Volume\ of\ Nitrate\ enzyme\ solution\ (\mu l) =$

$[Number\ of\ experimental\ wells + 14\ (standards\ and\ blanks)] \times 10\mu l$

B. $Volume\ of\ Reaction\ buffer\ \ (\mu l) = A \times 1.5\mu l$

Final enzyme dilution, composed of the volume of nitrate enzyme solution derived in A, diluted in the volume of reaction buffer derived in B. This solution was stored on ice and had to be used within 15 minutes of preparation.

Standards were prepared from the provided nitrate standard 1000µmol/l solution. Six eppendorf tubes were labeled 1 to 6. 900µl of reaction buffer was pippetted (Gilson, Middleton, WI, USA) into tube 1 and 500µL of reaction buffer into tubes 2-6. 100µL of the

1,000µmol/l standard was pipette into tube 1 and vortexed thoroughly. 500µl of tube 1 was added to tube 2 and vortexed thoroughly. This process was continued for tubes 3-6. The concentration of nitrate standard in tubes 1-6 was therefore 100, 50, 25, 12.5, 6.25 and 3.125µmol/l respectively.

Frozen urine samples were thawed at room temperature. Samples were centrifuged (Hereaus Sepatech, Thermo Scientific, USA) at 10,000 rpm for ten minutes. Supernatant was aspirated and stored on ice.

3.3.3.5.2 Assay procedure

1. 200µL of reaction buffer added into duplicate blank wells.
2. 50 µL of standards 1-6 added into duplicate wells.
3. 50 µL of reaction buffer added into duplicate wells (zero standard).
4. 50 µL of samples added into duplicate wells.
5. 25 µL of final NADH dilution added into all zero standard, standard and sample wells.
6. 25 µL of the nitrate reductase final enzyme dilution added into all zero standard, standard and sample wells.
7. All reagents mixed well by gentle agitation of the 96-well plate. Plate sealer was applied and the plate incubated for 30 minutes at 37°C.
8. 50µL of the provided Griess reagent I added into each well, except the blank wells.
9. 50µL of the provided Griess reagent II added into each well, except the blank wells.
10. The 96-well plate was agitated once more, to ensure adequate mixing of reagents.

11. The plate was then incubated at room temperature for 10 minutes.
12. Using a spectrophotometer (Multiskan EX, Labsystems) the optical density of each well was read at a wavelength of 540nm after blanking was performed against the blank wells.

3.3.3.6 Urine 8-Isoprostane quantification

Urinary measurement of 8-Isoprostane was chosen as a marker of oxidative stress. Urine samples, taken at 60 and 180 minutes post-reperfusion were immediately snap frozen in liquid nitrogen and stored at -80^{o}C. Levels of urine 8-Isoprostane were determined using an enzyme-linked immunosorbent assay (ELISA) technique (Cayman Chemical Co, MI, USA) according to manufacturers instructions.

3.3.3.6.1 Assay reagents and preparation

The provided vial of enzyme immunoassay (EIA) buffer concentrate was diluted in 90ml of dH_2O.

1ml of wash buffer concentrate was diluted in 400ml of dH_2O and 200µl of Tween-20 added.

Frozen urine samples were thawed at room temperature. Samples were centrifuged (Hereaus Sepatech, Thermo Scientific, USA) at 10,000 rpm for ten minutes. Supernatant was aspirated and stored on ice.

100µl of the provided 8-isoprostane standard was pipetted into an Eppendorf tube and diluted with 900µl of dH_2O. Eight Eppendorf tubes were labeled 1-8. 900µl of EIA buffer was added to tube 1 and 750µl of EIA buffer to tubes 2-8. 100µl of standard solution was added to tube 1 and vortexed. 500µl of tube 1 was added to tube 2 and

vortexed thoroughly. This process was continued for tubes 3-8. Serial dilutions yielded 8-isoprostane standards with final dilutions of 500, 200, 80, 32, 12.8, 5.1, 2.0, 0.8pg/ml respectively in tubes 1-8.

8-isoprostane AChE tracer was reconstituted with 6ml of EIA buffer.

8-isoprostane EIA antiserum was reconstituted with 6ml of EIA buffer.

The vial of Ellman's reagent concentrate was reconstituted with 20ml of dH_2O.Ellman's reagent was reconstituted immediately prior to use.

3.3.3.6.2 Assay procedure

1. 100µl of EIA buffer was added to non-specific binding (NSB) wells and 50µl to maximum binding (MB) wells.
2. 50µl of 8-isoprostane standard dilutions 1-8 were added to wells in duplicate.
3. 50µl of sample were added to each well in duplicate.
4. 50µl of 8-isoprostane AChE tracer was added to each well except the total activity (TA) well and the blank wells.
5. 50µl of 8-isoprostane EIA antiserum was added to each well except the TA, NSB and blank wells.
6. The 96-well plate was covered with plate sealer and incubated at 4^oC for 18 hours.
7. Wells were emptied and rinsed five times with wash buffer.
8. 200µl of Ellman's reagent was added to each well.
9. 5µl of 8-isoprostane AChE tracer was added to the TA well.
10. The 96-well plate was sealed and placed on an orbital shaker with an opaque cover. The plate was left to develop for 120 minutes.

11. The plate sealer was removed carefully. Using a spectrophotometer (Multiskan EX, Labsystems) the optical density of each well was read at a wavelength of 420nm. Blanking was performed against the blank wells.
12. Data was evaluated against the standard curves.

3.3.4 Statistical analysis

Values are presented as mean ± sd. Continuous variables were plotted against time and the area under the curve (AUC) for individual perfusion experiments calculated using Excel® (Microsoft, Reading, UK) and Graphpad Prism (GraphPad Software, San Diego California USA) software.

Mean values, and mean AUC values were compared using the Kruskal-Wallis ANOVA test (K-W) with Dunn's post-test (D P-T) and the Mann-Whitney test (GraphPad InStat, GraphPad Software, San Diego California USA). $P<0.05$ was considered as statically significant. AUC was calculated using the trapezoidal method.

Correlation between AUC creatinine level, AUC fractional excretion of Sodium, urine total nitric oxide level and urine 8-isoprostane levels were assessed with Spearman's rank correlation (non-parametric correlation). $P< 0.05$ was considered significant.

3.3.5 Formulae for calculation of functional data

3.3.5.1 Corrected creatinine clearance

$$uCrCl = \frac{U_{Cr} \times V}{P_{Cr}}$$

Equation 1. Uncorrected creatinine clearance calculation. U_{Cr}; Urinary Creatinine (µmol/l), V; Urine flow rate (ml/min), P_{Cr}; Plasma creatinine (µmol/l), uCrCl; Uncorrected creatinine clearance (ml/min)

Creatinine clearance is then corrected for kidney weight by the following equation:

$$cCrCl = \frac{uCrCl \times 100}{W}$$

Equation 2. Corrected creatinine clearance calculation. W; Pre-perfusion kidney weight (g), cCrCl; Corrected creatinine clearance (ml/min/100g)

3.3.5.2 Intra-renal resistance

$$IRR = \frac{AP}{RBF}$$

Equation 3. Intra-renal resistance calculation. IRR; Intra-renal resistance (mmHg/ml/min), AP; Arterial pressure (mmHg), RBF; Renal blood flow (ml/min)

3.3.5.3 Fractional excretion of sodium

$$FE_{Na} = \frac{U_{Na} \times P_{Cr}}{P_{Na} \times U_{Cr}} \times 100$$

Equation 4. Fractional excretion of sodium calculation. FE_{Na}; Fractional excretion of sodium, U_{Na}; Urinary sodium (µmol/l), UO; Urine output, P_{Na}; Plasma sodium (µmol/l), P_{Cr}; Plasma Creatinine (µmol/l), U_{Cr}; Urinary creatinine (µmol/l).

3.3.5.4 Oxygen consumption

$$VO_2 = \frac{\left(AP_{aO_2} - VP_{aO_2}\right) \times RBF}{W}$$

Equation 5. Oxygen consumption calculation. VO_2; Oxygen consumption (kPa.ml/min/100g), AP_{aO_2}; Arterial partial pressure of oxygen (kPa), VP_{aO_2}; Venous partial pressure of oxygen (kPa).

4 Results

4.1 Haematology

4.1.1 Leukocyte counts

There was a significant difference in leukocyte counts between WB and LDB perfused groups both pre and post-reperfusion. There were significantly decreased leukocyte counts in post-reperfusion samples when compared to pre-reperfusion samples in the 2hr control, 18hr control and WB+1400W groups. The leukocyte count in the LDB group was significantly higher post-reperfusion. These data are summarized in table 6.

Table 6. Table showing leukocyte counts (x10^9/l) in each group pre- and post-reperfusion. P values shown are between WB and LDB perfused groups and pre- and post-reperfusion in each experiment.

	Mean Leukocyte Count (SD)			
	Group	**Pre-reperfusion**	**Post-reperfusion**	**p-value (pre:post)**
1.	2hr Control	5.3 (1.3)	3.4 (0.3)	**0.01**
2.	18hr Control	5.7 (1.6)	3.7 (1.2)	**0.01**
3.	LDB	0.1 (0)	0.6 (0.2)	**0.01**
	P value	**0.003**	**0.001**	
4.	WB + 1400W	5.8 (0.5)	4.2 (0.8)	**0.001**
5.	LDB + 1400W	0.1 (0)	0.4 (0.1)	**NS**
	P value	**0.01**	**0.001**	
6.	WB + SNP	5.6 (1.3)	4 (0.7)	**NS**
7.	LDB + SNP	0.4 (0.4)	0.6 (0.4)	**NS**
	P value	**0.002**	**0.005**	

4.1.2 Platelet counts

There was a significant difference in platelet counts between WB and LDB perfused groups both pre and post-reperfusion. There was no significant change in platelet count pre and post-reperfusion. Results are shown in table 7.

Table 7. Table showing platelet counts ($x10^9$/l) in each group pre- and post-reperfusion. P values shown are between WB and LDB perfused groups.

		Mean Platelet Count (SD)	
	Group	**Pre-reperfusion**	**Post-reperfusion**
1.	2hr Control	92 (22)	97 (12)
2.	18hr Control	78 (53)	76 (47)
3.	LDB	6 (8)	17 (22)
	p-value	**0.002**	**0.008**
4.	WB + 1400W	87 (7)	86 (12)
5.	LDB + 1400W	11 (3)	12 (4)
	p-value	**0.01**	**0.001**
6.	WB + SNP	75 (42)	73 (33)
7.	LDB + SNP	6 (1)	6 (2)
	p-value	**0.002**	**0.002**

4.1.3 Haematocrit level

There was no difference in haematocrit level between groups or between pre and post-reperfusion samples. These data are summarized in table 8.

Table 8. Table showing haematocrit (l/l) in each group pre- and post-reperfusion. P values shown are between WB and LDB perfused groups.

		Mean Haematocrit (SD)	
	Group	**Pre-reperfusion**	**Post-reperfusion**
1.	2hr Control	0.22 (0.01)	0.23 (0.02)
2.	18hr Control	0.17 (0.03)	0.15 (0.03)
3.	LDB	0.19 (0.01)	0.18 (0.02)
	p-value	**NS**	**NS**
4.	WB + 1400W	0.19 (0.01)	0.18 (0.02)
5.	LDB + 1400W	0.16 (0.01)	0.16 (0.08)
	p-value	**NS**	**NS**
6.	WB + SNP	0.18 (0.01)	0.16 (0.03)
7.	LDB + SNP	0.16 (0.03)	0.15 (0.03)
	p-value	**NS**	**NS**

4.2 Kidney weight

There was some variation in post-static cold storage kidney weight between the experimental groups. Statistical analysis demonstrated a significant difference in post-SCS weight between the 2hr Control and 18hr Control, 2hr Control and LDB+1400W and LDB+1400W and WB+SNP groups.

All kidneys gained weight following the reperfusion period. The percentage weight gain was highly variable between groups. Weight gain was statistically significant in WB+1400W, LDB+1400W and LDB+SNP groups. These data are shown in table 9.

Table 9. Table showing kidney weight (g), post-SCS and post-reperfusion. K-W; Kruskal-Wallis.

	Mean Kidney Weight (SD)				
	Group	**Post-SCS**	**Post-reperfusion**	**% weight gain**	**p-value**
1.	2hr control	255 (24)	270 (24)	6 (4)	**NS**
2.	18hr control	194 (23)	240 (47)	23 (13)	**NS**
3.	LDB	207 (37)	222 (41)	7 (9)	**NS**
4.	WB + 1400W	210 (27)	287 (32)	37 (8)	**0.002**
5.	LDB + 1400W	157 (5)	204 (22)	29 (11)	**0.029**
6.	WB + SNP	243 (25)	263 (51)	8 (11)	**NS**
7.	LDB + SNP	215 (5)	228 (7)	6 (5)	**0.016**
	K-W p- value	**0.001**	**0.01**		
	Dunn's post test (p<0.05)	**1 vs 2, 1 vs 5, 5 vs 6**	**4 vs 5**		

4.3 Histology

Histological analysis demonstrated a statistical difference ($p<0.05$) between pre and post-reperfusion tubular dilatation and tubular cell vacuolation in the 2hr control, 18hr control and LDB groups. Tubular dilatation alone was significant in the WB+1400W group. There were no statistically significant intra-group differences. These results are summarized in table 10.

Table 10. Table showing histological differences between pre-reperfusion and post-reperfusion renal biopsies. Values are means (SD) of a semi-quantitative scoring system (0=normal, 1=mild, 2=moderate, 3=severe morphological changes).

	Histological Variable									
		Tubular dilatation		Tubular Debris		Tubular Cell Vacuolation		Interstitial Infiltration		
	Group	Pre	Post	Pre	Post	Pre	Post	Pre	Post	
1.	2hr control	0.3 (0.5)	1.1 (1)*	1.2 (0.6)	1.1 (1)	0.3 (0.6)	2.3 (0.8)*	0.8 (0.5)	0.7 (0.8)	
2.	18hr control	0.9 (0.7)	1.6 (0.6)*	1.4 (0.5)	1.1 (0.7)	0.5 (0.8)	1.1 (1.1)*	1.3 (0.5)	1.3 (0.5)	
3.	LDB	0.7 (0.5)	1.8 (0.5)*	1.4 (0.5)	1.4 (0.6)	0.2 (0.4)	1.7 (1.2)*	0.9 (0.4)	1 (0.6)	
4.	WB + 1400W	2.0 (0.5)	1.3 (0.5)*	1.5 (0.6)	1.9 (0.5)*	0.5 (0.7)	0.4 (0.5)	1.0 (0.6)	1.0 (0.7)	
5.	LDB + 1400W	1.6 (1.0)	1.1 (0.5)	1.6 (0.5)	1.3 (0.5)	0.4 (0.9)	0.2 (0.4)	0.6 (0.4)	0.7 (0.6)	
6.	WB + SNP	1.4 (0.8)	1.1 (0.9)	1.8 (0.6)	1.6 (0.6)	0.4 (0.5)	0.3 (0.6)	0.6 (0.5)	0.8 (0.8)	
7.	LDB + SNP	2.0 (0.6)	1.4 (0.5)*	1.3 (0.7)	1.2 (0.6)	0.5 (0.6)	0.1 (0.4)	1.1 (0.3)	0.9 (0.7)	

4.4 Functional data

For ease of comparison the functional data will be presented in four parts. A summary of the groups compared in each part is shown in table 11.

Table 11. Table showing the composition of the four study parts.

Part	Groups within each part (group number)			
1	2hr control (1)	18hr control (2)	LDB (3)	
2	18hr control (2)	LDB (3)	WB + 1400W (4)	LDB + 1400W (5)
3	18 hr control (2)	LDB (3)	WB + SNP (6)	LDB + SNP (7)
4	WB + 1400W (4)	LDB + 1400W (5)	WB + SNP (6)	LDB + SNP (7)

4.4.1 Part One

4.4.1.1 Haemodynamics

In all groups initial RBF was low. After 10 minutes of reperfusion RBF improved in all groups. After 180 minutes of reperfusion RBF in the LDB group was significantly higher than in both the 2hr control and 18hr control groups. RBF in the 2hr control group was better at all time points than in the 18hr control group, but this did not reach statistical significance over the entire 180 minutes reperfusion period. For the 2hr control, 18hr control and LDB groups respectively, AUC RBF was 11015±1450, 6361±2623, 14492±3255. RBF characteristics are shown in figure 9. Mann-Whitney test between groups: 2hr control vs 18hr control p=0.005, 2hr control vs LDB p=NS, 18hr control vs LDB p=0.005.

IRR demonstrated similar characteristics. The corresponding intra-renal resistance is shown in figure 9. IRR was lower in 2hr control and LDB groups when compared to the 18hr control group, however for the entire 180 minutes reperfusion period statistical significance was only reached between the 2hr control and LDB groups. AUC IRR was 104±9, 288±196, 113±51 for 2hr control, 18hr control and LDB groups respectively. Mann-Whitney test between groups: 2hr control vs 18hr control p=0.001, 2hr control vs LDB p=NS, 18hr control vs LDB p=0.005.

Figure 8. Graph showing mean renal blood flow in 2hr control, 18hr control and LDB groups over 180 minutes of reperfusion. Values shown are mean ±SD.

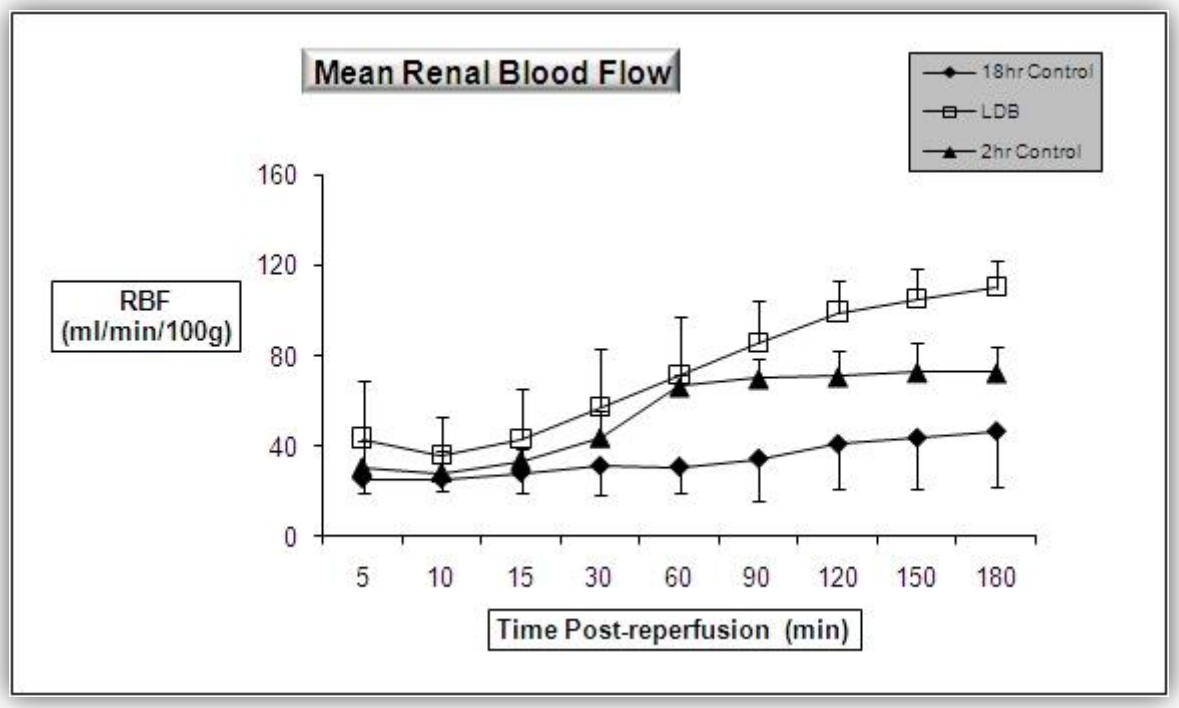
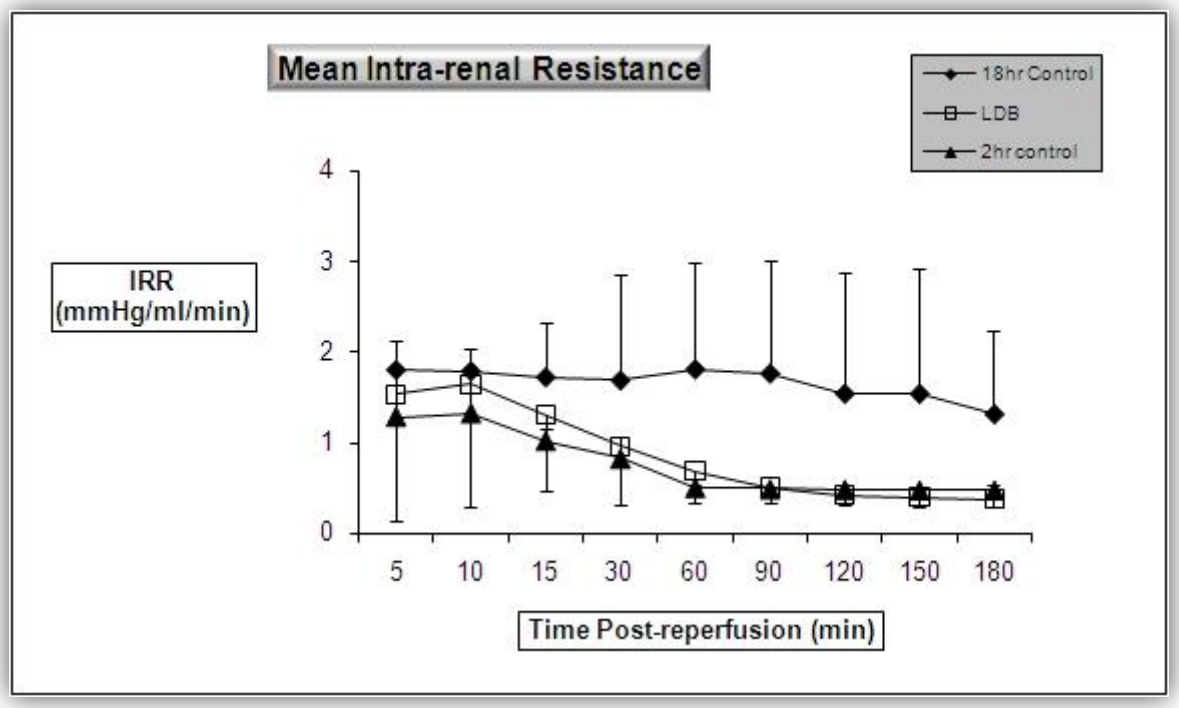


Figure 9. Graph showing intra-renal resistance in 2hr control, 18hr control and LDB groups over 180 minutes of reperfusion. Values shown are mean ±SD.



4.4.1.2 Creatinine clearance

Corrected creatinine clearance was significantly different at all three time points post-reperfusion. These results are summarised in table 12. Mann-Whitney test between groups: 60min; 2hr control vs 18hr control p=0.005, 2hr control vs LDB p=0.026, 18hr control vs LDB p=0.025. 120min; 2hr control vs 18hr control p=0.008, 2hr control vs LDB p=0.004, 18hr control vs LDB p=0.016. 180min; 2hr control vs 18hr control p=0.001, 2hr control vs LDB p=0.002, 18hr control vs LDB p=0.015.

Table 12. Table showing mean corrected creatinine clearance (ml/min/100g) at 60, 120 and 180 minutes post-reperfusion in 2hr control, 18hr control and LDB groups. Values shown are mean ±SD.

		Mean Corrected Creatinine Clearance (SD)		
	Group	**60min**	**120min**	**180min**
1.	2hr control	6.7 (5.2)	11.7 (6.1)	9.4 (4.9)
2.	18hr control	0.5 (0.2)	0.4 (0.2)	0.5 (0.4)
3.	LDB	2.1 (2.0)	2.3 (1.1)	1.9 (1.4)

4.4.1.3 Urine output

Total urine output was significantly greater in both the LDB and the 2hr control group when compared to the 18hr control group. Results are summarised in table 13. Mann-Whitney test between groups: 60min; 2hr control vs 18hr control p=0.003, 2hr control vs LDB p=NS, 18hr control vs LDB p=0.021. 120min; 2hr control vs 18hr control p=0.003, 2hr control vs LDB p=NS, 18hr control vs LDB p=0.005. 180min; 2hr control vs 18hr control p=0.001, 2hr control vs LDB p=NS, 18hr control vs LDB p=0.015. Total 2hr control vs 18hr control p=0.003, 2hr control vs LDB p=NS, 18hr control vs LDB p=0.005.

Table 13. Table showing hourly and total urine output (ml) in 2hr control, 18hr control and LDB groups. Values shown are mean ±SD.

		Mean Hourly Urine Output (SD)			
	Group	**1hr**	**2hr**	**3hr**	**Total**
1.	2hr control	267 (85)	220 (44)	245 (111)	732 (129)
2.	18hr control	64 (30)	47 (34)	46 (34)	156 (83)
3.	LDB	197 (168)	183 (87)	169 (116)	550 (338)

4.4.1.4 Fractional excretion of sodium

The fractional excretion of sodium was lower in the LDB and 2hr control groups when compared to the 18hr control groups. However, statistical significance was only reached between the 2hr control and 18hr control groups. Results are summarised in table 14. Mann-Whitney test between groups: 60min; 2hr control vs 18hr control p=0.004, 2hr control vs LDB p=0.009, 18hr control vs LDB p=NS. 120min; 2hr control vs 18hc p=0.004, 2hr control vs LDB p=0.004, 18hr control vs LDB p=NS. 180min; 2hr control vs 18hr control p=0.001, 2hr control vs LDB p=0.002, 18hr control vs LDB p=NS.

Table 14. Table showing mean fractional excretion of sodium (%) at 60, 120 and 180 minutes post-reperfusion in 2hr control, 18hr control and LDB groups. Values shown are mean ±SD.

		Mean Fractional Excretion of Sodium (SD)		
	Group	**60min**	**120min**	**180min**
1.	2hr control	29 (22)	9 (5)	11 (4)
2.	18hr control	121 (62)	82 (44)	84 (53)
3.	LDB	81 (8)	70 (12)	71 (21)

4.4.1.5 Hydrogen ion concentration

There was no difference in pH between groups pre-reperfusion. pH was lower in the 18hr control group compared to the other groups, but only reached statistical significance when compared to the 2hr control group. These data are summarised in table 15. Mann-Whitney test between groups: Pre-reperfusion; 2hr control vs 18hr control p=NS, 2hr control vs LDB p=NS, 18hr control vs LDB p=NS. 60min; 2hr control vs 18hr control p=0.012, 2hr control vs LDB p=0.030, 18hr control vs LDB p=NS. 180min; 2hr control vs 18hr control p=0.003, 2hr control vs LDB p=0.006, 18hr control vs LDB p=NS.

Table 15. Table showing mean pH values pre-reperfusion, 60min and 180min post-reperfusion in 2hr control, 18hr control and LDB groups. Values shown are mean ±SD.

		Mean pH (SD)		
	Group	**Pre**	**1hr**	**3hr**
1.	2hr control	7.43 (0.04)	7.39 (0.03)	7.49 (0.03)
2.	18hr control	7.43 (0.03)	7.32 (0.04)	7.30 (0.08)
3.	LDB	7.45 (0.02)	7.34 (0.03)	7.38 (0.07)

4.4.1.6 Oxygen consumption

Oxygen consumption was greater in the 2hr control and LDB groups compared to the 18hr control group. This reached statistical significance in both groups at 60 minutes and the LDB group at 180 minutes post-reperfusion. Results are summarised in table 16. Mann-Whitney test between groups: 60min; 2hr control vs 18hr control p=0.001, 2hr control vs LDB p=NS, 18hr control vs LDB p=0.022. 180min; 2hr control vs 18hr control p=0.073, 2hr control vs LDB p=0.017, 18hr control vs LDB p=0.001.

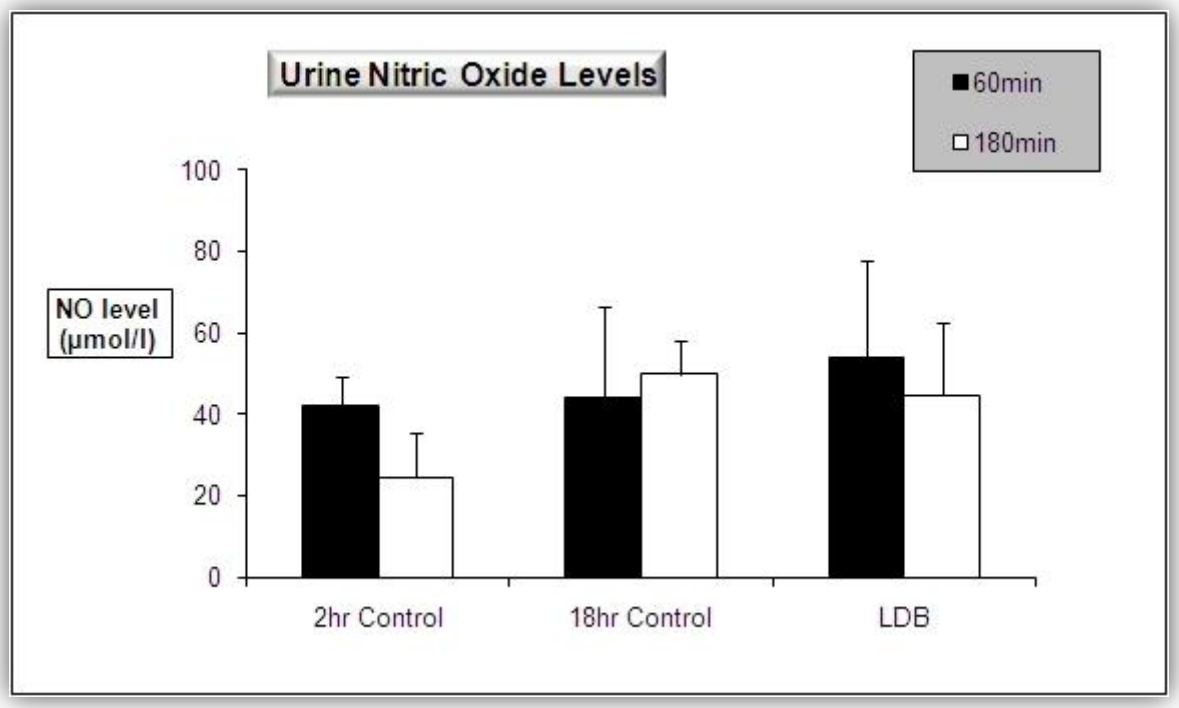
Table 16. Table showing mean oxygen consumption (kPa.ml/min/100g) at 60 and 180 minutes post-reperfusion in 2hr control, 18hr control and LDB groups. Values shown are mean ±SD.

		Mean Oxygen Consumption (SD)	
	Group	**1hr**	**3hr**
1.	2hr control	39.5 (6.2)	43.1 (10.8)
2.	18hr control	19.3 (6.8)	28 (13.9)
3.	LDB	44.3 (16.8)	64.7 (5.5)

4.4.1.7 Urinary nitric oxide quantification

There were no significant differences in the total urinary NO levels 60 minutes post-reperfusion. At 180 minutes post-reperfusion urinary NO levels were significantly lower in the 2hr control group compared to the 18hr control (p=0.013) and the LDB group (p=0.026). These data are shown in figure 10.

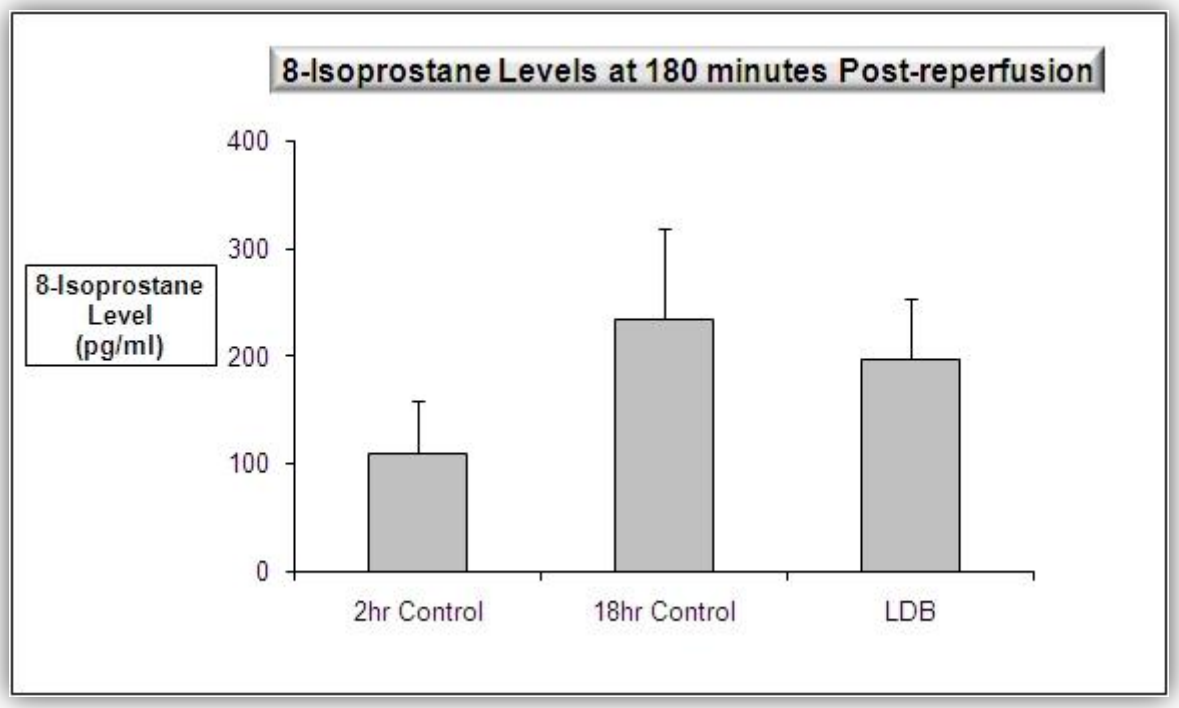
Figure 10. Histogram showing urinary nitric oxide levels at 60 and 180 minutes post-reperfusion in 2hr control, 18hr control and LDB groups. Values shown are mean ±SD.



4.4.1.8 Urinary 8-isoprostane quantification

Urinary levels of 8-isoprostane were measured at 180minutes post-reperfusion. 8-Isoprostane levels in the 2hr control group were statistically lower than those in the 18hr control group (p=0.017), but not the LDB group (p=0.067). These results are shown in figure 11.

Figure 11. Histogram showing urinary 8-Isoprostane levels at 180 minutes post-reperfusion in 2hr control, 18hr control and LDB groups. Values shown are mean ±SD.



4.4.2 Part two

4.4.2.1 Dose response

The effect of two doses, 10mg/kg kidney weight and 1mg/kg kidney weight of 1400W was assessed. No statistical differences in RBF, IRR, UO, cCrCl, or FE_{Na} was observed. These data are summarized in figures 12-16.

For all subsequent experiments a dose of 1mg/kg of 1400W was used.

Figure 12. Histogram showing AUC renal blood flow after 180 minutes of reperfusion with 1mg/kg or 10mg/kg of 1400W in whole blood perfusate.

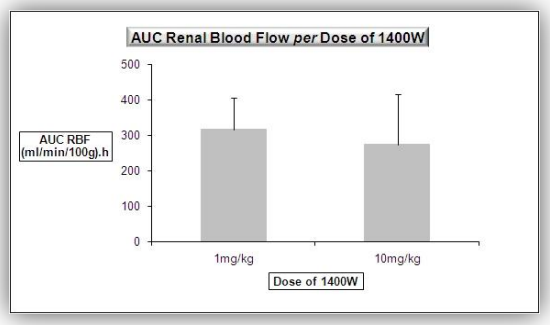


Figure 13. Histogram showing AUC intra-renal resistance after 180 minutes of reperfusion with 1mg/kg or 10mg/kg of 1400W in whole blood perfusate.

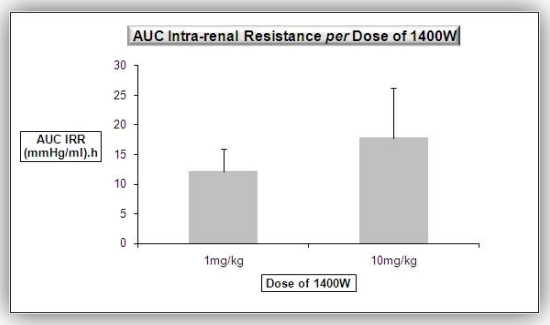


Figure 14. Histogram showing total urine output after 180 minutes of reperfusion with 1mg/kg or 10mg/kg of 1400W in whole blood perfusate.

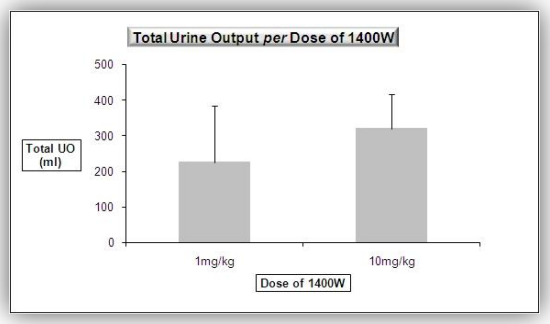


Figure 15. Histogram showing AUC creatinine clearance after 180 minutes of reperfusion with 1mg/kg or 10mg/kg of 1400W in whole blood perfusate.

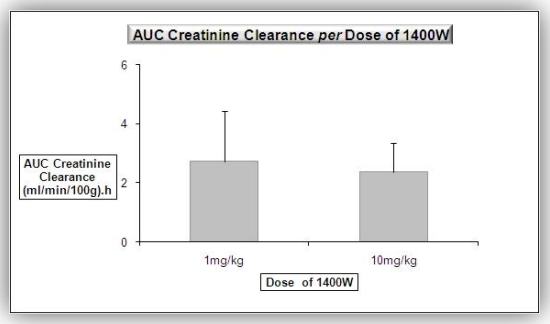
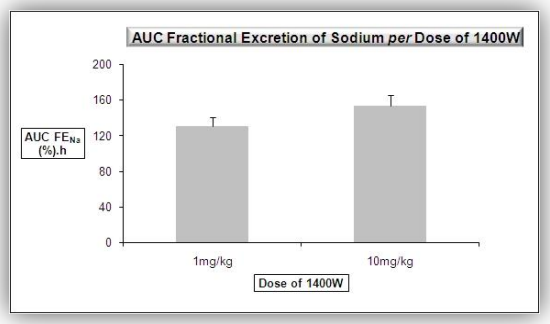


Figure 16. Histogram showing AUC fractional excretion of sodium after 180 minutes of reperfusion with 1mg/kg or 10mg/kg of 1400W in whole blood perfusate.



4.4.2.2 Haemodynamics

Blood flow was initially poor in all 4 groups (18hr control, LDB, WB+1400W and LDB+1400W). After 10 minutes of reperfusion blood flow in the 18hr control and LDB groups improved, however flow in the WB+1400W and LDB+1400W groups continued to decline. When analyzed separately AUC RBF for 5-30 minutes of reperfusion was 705±190, 995±458, 333±112, 180±144 for the 18hr control, LDB, WB+1400W and LDB+1400W groups respectively. Mann-Whitney test between groups: 18hr control vs LDB p=NS, 18hr control vs WB+1400W p=0.01, 18hr control vs LDB+1400W p=0.017, LDB vs WB+1400W p=0.032, 18hr control vs LDB+1400W p=0.036, WB+1400W vs LDB+1400W p=NS.

After 30 minutes of reperfusion the renal blood flow in the WB+1400W group had begun to improve, this continued until the end of the 180 minute reperfusion period at which point it was equal to the RBF in the 18hr control group. The renal blood flow in the LDB+1400W group continued to deteriorate from 30 minutes until the end of reperfusion. AUC for 30-180 minutes of reperfusion was 4727±2327, 11417±1969, 5982±3563, 581±750 in the 18hr control, LDB, WB+1400W and LDB+1400W groups respectively. Mann-Whitney test between groups: 18hr control vs LDB p=0.001, 18hr control vs WB+1400W p=NS, 18hr control vs LDB+1400W p=0.018, LDB vs WB+1400W p=0.015, LDB vs LDB+1400W p=0.014, WB+1400W vs LDB+1400W p=0.014. These results are summarized in figure 17.

The intra-renal resistance demonstrated similar characteristics as the RBF. At 10 minutes post-reperfusion the IRR in the WB+1400W and LDB+1400W groups was higher than in the 18hr control and LDB groups, this divergence continued up to 30

minutes or reperfusion. At 30 minutes post-reperfusion the WB+1400W group demonstrated a rapid fall in IRR to the level of the 18hr control and LDB groups. The IRR in the LDB+1400W group remained high throughout the remainder of the reperfusion period. AUC IRR for 5-30 minutes of reperfusion was 43±16, 32±23, 91±51, 162±33 for the 18hr control, LDB, WB+1400W and LDB+1400W groups respectively. Mann-Whitney test between groups: 18hr control vs LDB p=NS, 18hr control vs WB+1400W p=0.014, 18hr control vs LDB+1400W p=0.006, LDB vs WB+1400W p=0.015, LDB vs LDB+1400W p=0.01, WB+1400W vs LDB+1400W p=0.067. For the period 60-180 minutes post-reperfusion AUC IRR was 192±149, 56± 17, 145±92, 952±203 respectively in the 18hr control, LDB, WB+1400W and LDB+1400W groups. Mann-Whitney test between groups: 18hr control vs LDB p=0.008, 18hr control vs WB+1400W p=NS, 18hr control vs LDB+1400W p=0.006, LDB vs WB+1400W p=0.020, LDB vs LDB+1400W p=0.014, WB+1400W vs LDB+1400W p=0.01. These data are presented in figure 18.

Figure 17. Graph showing mean renal blood flow in the 18hr control, LDB, WB+1400W and LDB+1400W groups over 180 minutes of reperfusion. Values shown are mean ±SD.

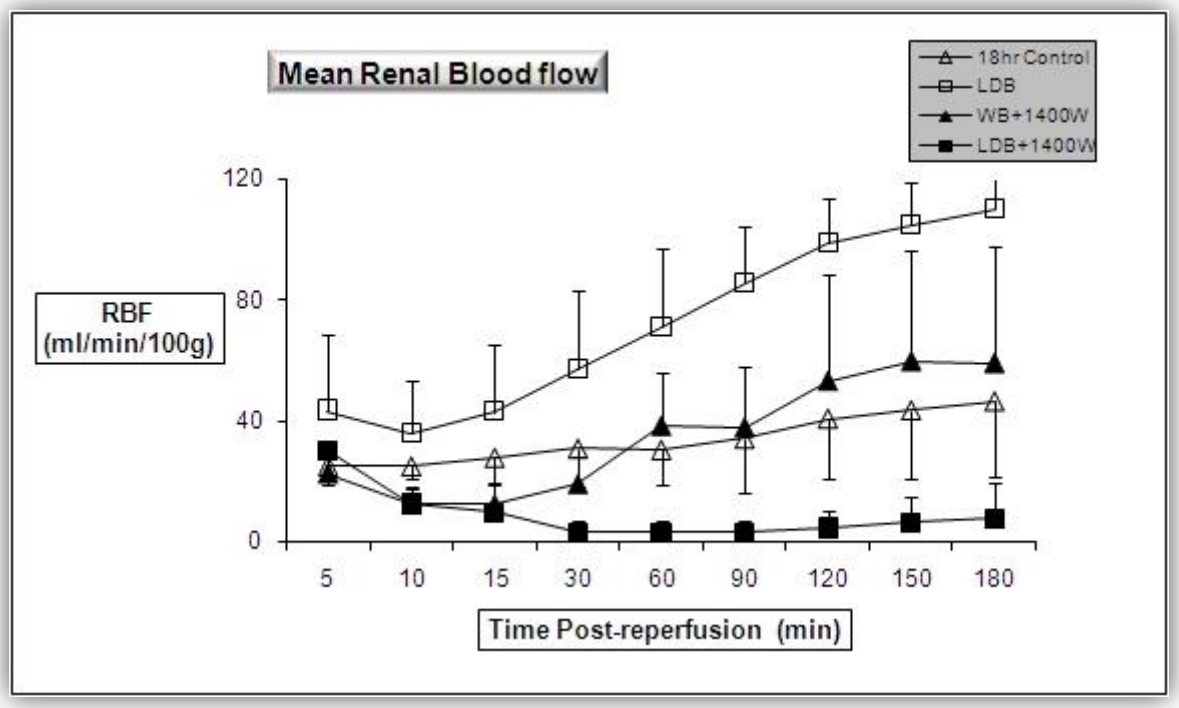
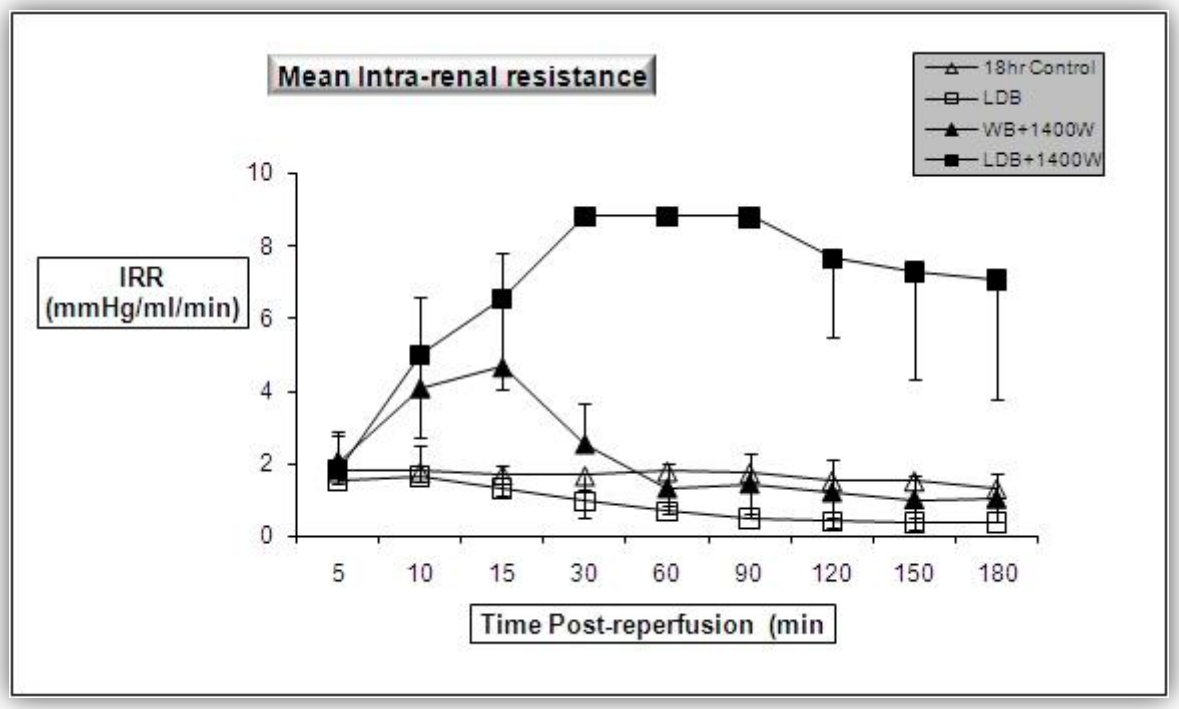


Figure 18. Graph showing intra-renal resistance in the 18hr control, LDB, WB+1400W and LDB+1400W groups over 180 minutes of reperfusion. Values shown are mean ±SD.



4.4.2.3 Creatinine clearance

The cCrCl data was statistically different at all time points. Generally cCrCl was highest in the LDB followed by the WB+1400W, 18hr control and LDB+1400W groups respectively. These results are summarized in table 17. Mann-Whitney test between groups: 60min; 18hr control vs LDB p=0.025, 18hr control vs WB+1400W p=0.030, 18hr control vs LDB+1400W p=NS, LDB vs WB+1400W p=NS, LDB vs LDB+1400W p=0.014, WB+1400W vs LDB+1400W p=0.014. 120min; 18hr control vs LDB p=0.016, 18hr control vs WB+1400W p=0.014, 18hr control vs LDB+1400W p=0.063, LDB vs WB+1400W p=NS, LDB vs LDB+1400W p=0.031, WB+1400W vs LDB+1400W p=0.058. 180min; 18hr control vs LDB p=0.015, 18hr control WB+1400W p=0.032, 18hr control vs LDB+1400W p=0.028, LDB vs WB+1400W p=NS, LDB vs LDB+1400W p=0.013, WB+1400W vs LDB+1400W p=0.023.

Table 17. Table showing mean corrected creatinine clearance (ml/min/100g) at 60, 120 and 180 minutes post-reperfusion in the 18hr control, LDB, WB+1400W and LDB+1400W groups. Values shown are mean ±SD.

		Mean Corrected Creatinine Clearance (SD)		
	Group	**60min**	**120min**	**180min**
2.	18hr control	0.5 (0.2)	0.4 (0.2)	0.5 (0.4)
3.	LDB	2.1 (2)	2.3 (1.1)	1.9 (1.4)
4.	WB + 1400W	1.2 (0.6)	1.3 (0.7)	1 (0.8)
5.	LDB + 1400W	0.2 (0.2)	0 (0)	0.1 (0.1)

4.4.2.4 Urine output

Urine output was highest in the LDB group, followed by the WB+1400W, 18hr control and LDB+1400W groups respectively. Statistically the difference in total urine output was significant between in the WB+1400W vs LDB+1400W, LDB vs LDB+1400W and 18hr control vs WB+1400W groups. These data are summarized in table 18. Mann-Whitney test between groups: 60min; 18hr control vs LDB p=0.021, 18hr control vs WB+1400W p=NS, 18hr control vs LDB+1400W p=0.070, LDB vs WB+1400W p=NS, LDB vs LDB+1400W p=0.010, WB+1400W vs LDB+1400W p=0.010. 120min; 18hr control vs LDB p=0.005, 18hr control vs WB+1400W p=0.022, 18hr control vs LDB+1400W p=0.031, LDB vs WB+1400W p=NS, LDB vs LDB+1400W p=0.031, WB+1400W vs LDB+1400W p=0.031. 180min; 18hr control vs LDB p=0.015, 18hr control vs WB+1400W p=NS, 18hr control vs LDB+1400W p=0.028, LDB vs WB+1400W p=NS, LDB vs LDB+1400W p=0.013, WB+1400W vs LDB+1400W p=0.017. Total; 18hr control vs LDB p=0.005, 18hr control vs WB+1400W p=0.008, 18hr control vs LDB+1400W p=NS, LDB vs WB+1400W p=NS, LDB vs LDB+1400W p=0.010, WB+1400W vs LDB+1400W p=0.010.

Table 18. Table showing hourly and total urine output (ml) in the 18hr control, LDB, WB+1400W and LDB+1400W groups. Values shown are mean ±SD.

		Mean Hourly Urine Output (SD)			
	Group	**1hr**	**2hr**	**3hr**	**Total**
2.	18hr control	64 (30)	47 (34)	46 (34)	156 (83)
3.	LDB	197 (168)	183 (87)	169 (116)	550 (338)
4.	WB + 1400W	125 (66)	113 (62)	82 (54)	320 (96)
5.	LDB + 1400W	29 (9)	0 (0)	6 (13)	35 (20)

4.4.2.5 Fractional excretion of sodium

There was no overall trend or any statistical significance in the fractional excretion of sodium between any of the groups at any time-point. These data are summarised in Table 19. The low urine urine output in the LDB+1400W group removed the possibility of statistical analysis. Mann-Whitney test between groups: 60min; 18hr control vs LDB p=0.024, 18hr control vs WB+1400W p=NS, 18hr control vs LDB+1400W p=NS, LDB vs WB+1400W p=NS, LDB vs LDB+1400W p=NS, WB+1400W vs LDB+1400W p=NS. 120min; 18hr control vs LDB p=NS, 18hr control vs WB+1400W p=NS, 18hr control vs LDB+1400W n/a, LDB vs WB+1400W p=NS, LDB vs LDB+1400W n/a, WB+1400W vs LDB+1400W n/a. 180min; 18hr control vs LDB p=NS, 18hr control vs WB+1400W p=NS, 18hr control vs LDB+1400W n/a, LDB vs WB+1400W p=NS, LDB vs LDB+1400W n/a, WB+1400W vs LDB+1400W n/a.

Table 19. Table showing mean fractional excretion of sodium (%) at 60, 120 and 180 minutes post-reperfusion in the 18hr control, LDB, WB+1400W and LDB+1400W groups. Values shown are mean ±SD.

		Mean Fractional Excretion of Sodium (SD)		
	Group	**60min**	**120min**	**180min**
2.	18hr control	121 (61)	82 (44)	84 (53)
3.	LDB	81 (8)	70 (12)	71 (21)
4.	WB + 1400W	78 (17)	62 (19)	61 (14)
5.	LDB + 1400W	79 (38)	n/a	93 (0)

4.4.2.6 Hydrogen ion concentration

There was no difference in pH pre-perfusion between any of the groups. There was a general trend of increasing acidosis in the 18hr control, WB+1400W and LDB+1400W groups throughout the reperfusion period. At the end of reperfusion the pH in the WB+1400W group was statistically lower than that in the LDB group, otherwise there were no other significant intra-group differences. These data are shown in Table 20.

Mann-Whitney test between groups: Pre; 18hr control vs LDB p=NS, 18hr control vs WB+1400W p=NS, 18hr control vs LDB+1400W p=NS, LDB vs WB+1400W p=NS, LDB vs LDB+1400W p=NS, WB+1400W vs LDB+1400W p=0.042. 60min; 18hr control vs LDB p=NS, 18hr control vs WB+1400W p=NS, 18hr control vs LDB+1400W p=NS, LDB vs WB+1400W p=0.024, LDB vs LDB+1400W p=NS, WB+1400W vs LDB+1400W p=NS. 180min; 18hr control vs LDB p=NS, 18hr control vs WB+1400W p=NS, 18hr control vs LDB+1400W p=NS, LDB vs WB+1400W p=0.004, LDB vs LDB+1400W p=NS, WB+1400W vs LDB+1400W p=NS.

Table 20. Table showing mean pH values pre-reperfusion, 60min and 180min post-reperfusion in the 18hr control, LDB, WB+1400W and LDB+1400W groups. Values shown are mean ±SD.

		Mean pH (SD)		
	Group	**Pre**	**1hr**	**3hr**
2.	18hr control	7.43 (0.03)	7.32 (0.04)	7.3 (0.08)
3.	LDB	7.45 (0.02)	7.34 (0.03)	7.38 (0.07)
4.	WB + 1400W	7.47 (0.04)	7.28 (0.05)	7.24 (0.04)
5.	LDB + 1400W	7.41 (0.04)	7.3 (0.01)	7.28 (0.02)

4.4.2.7 Oxygen consumption

There was increased oxygen consumption throughout the reperfusion in all groups. Results are shown in table 21. Mann-Whitney test between groups: 60min; 18hr control vs LDB p=NS, 18hr control vs WB+1400W p=0.022, 18hr control vs LDB+1400W p=0.010, LDB vs WB+1400W p=0.041, LDB vs LDB+1400W p=0.013, WB+1400W vs LDB+1400W p=0.013. 180min; 18hr control vs LDB p=NS, 18hr control vs WB+1400W p=0.001, 18hr control vs LDB+1400W p=0.028, LDB vs WB+1400W p=0.041, LDB vs LDB+1400W p=0.023, WB+1400W vs LDB+1400W p=0.013.

Table 21. Table showing mean oxygen consumption (kPa.ml/min/100g) at 60 and 180 minutes post-reperfusion in the 18hr control, LDB, WB+1400W and LDB+1400W groups. Values shown are mean ±SD.

		Mean Oxygen Consumption (SD)	
	Group	**1hr**	**3hr**
2.	18hr control	19.3 (6.8)	28 (13.9)
3.	LDB	44.3 (16.8)	64.7 (5.5)
4.	WB + 1400W	24.1 (11)	36.4 (22.8)
5.	LDB + 1400W	1.1 (2.2)	3.9 (7.9)

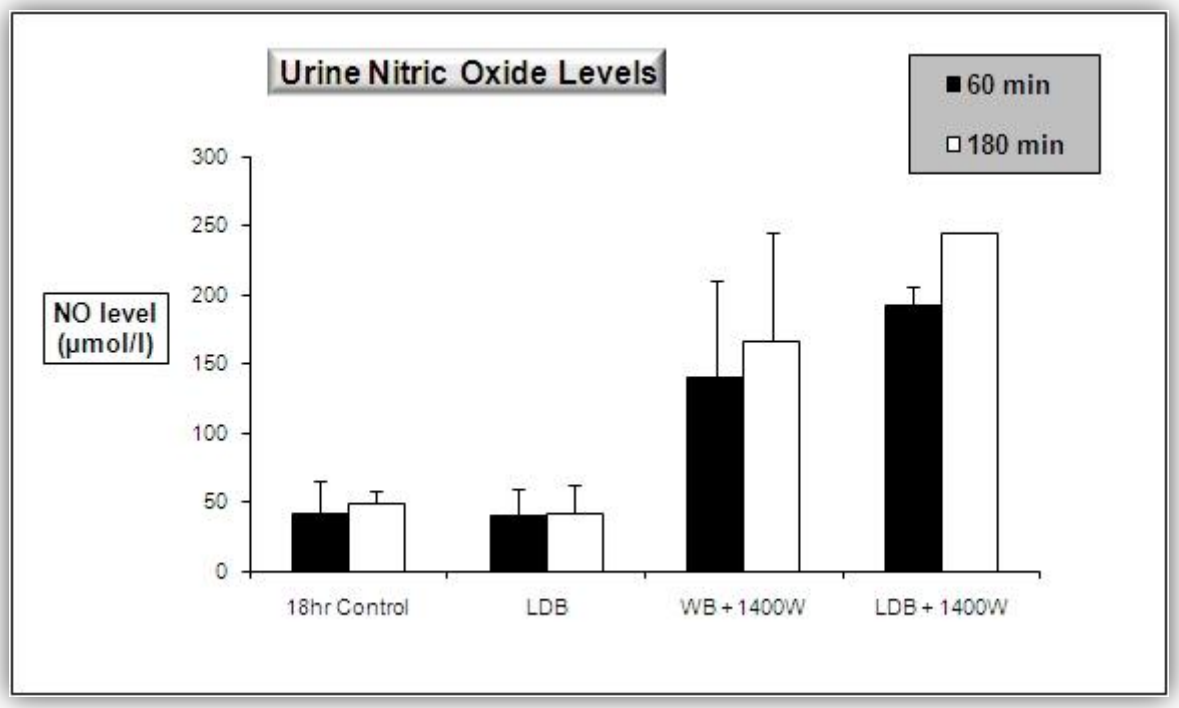
4.4.2.8 Urinary nitric oxide quantification

Urinary nitric oxide (NO) levels were significantly higher in the WB+1400W and LDB+1400W groups at both 60 and 180 minutes post-reperfusion when compared with the 18hr control and LDB groups. The differences in urinary NO levels were statistically significant at 60 minutes post-reperfusion between the 18hr control vs WB+1400W, 18hr control vs LDB+1400W, LDB vs WB+1400W, and LDB vs LDB+1400W groups, and after 180 minutes between 18hr control vs WB+1400W and LDB vs WB+1400W groups. The NO levels in the LDB+1400W group were not compared statistically because of sample number limitations. These data are summarised in figure 19 and table 22.

Table 22. Table showing individual p values between in the 18hr control, LDB, WB+1400W and LDB+1400W groups when urinary nitric oxide levels were compared at 60 and 180 minutes post-reperfusion.

		P value between groups					
	Group	18hr Control		LDB		WB + 1400W	
		60 mins	180 mins	60 mins	180 mins	60 mins	180 mins
2.	18hr Control	-	-	-	-	-	-
3.	LDB	**NS**	**NS**	-	-	-	-
4.	WB + 1400W	**0.002**	**0.004**	**0.002**	**0.009**	-	-
5.	LDB + 1400W	**0.01**	**n/a**	**0.01**	**n/a**	**NS**	**n/a**

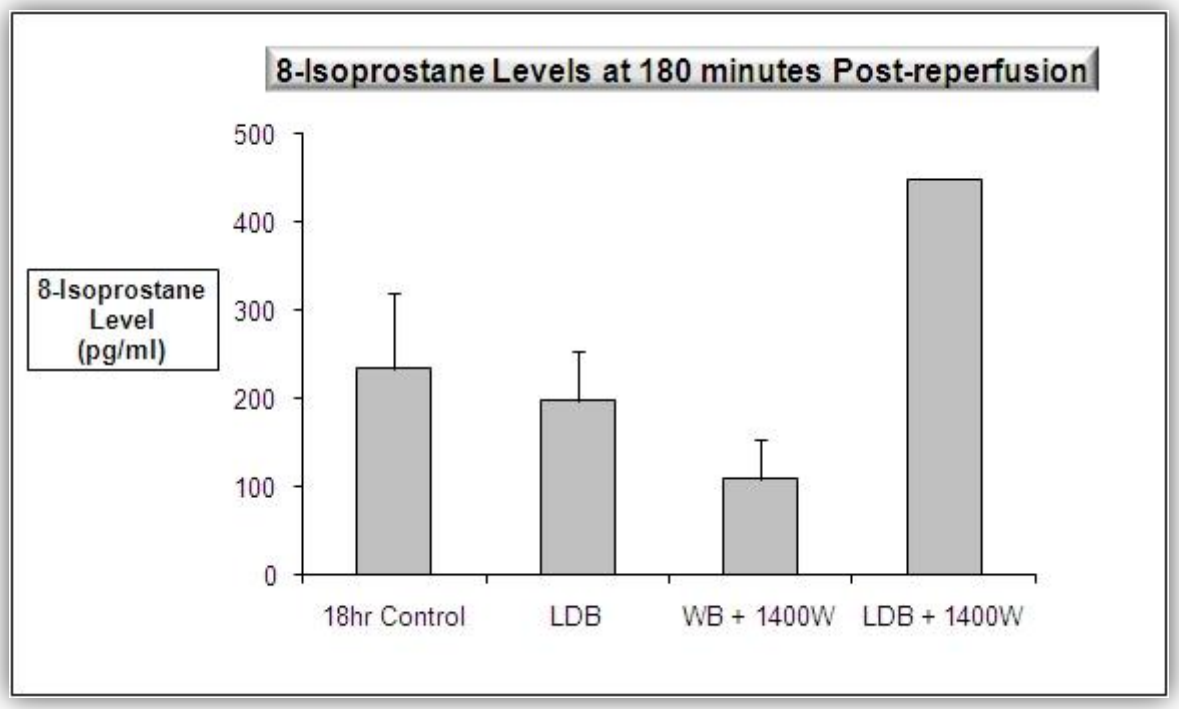
Figure 19. Histogram showing urinary nitric oxide levels at 60 and 180 minutes post-reperfusion in the 18hr control, LDB, WB+1400W and LDB+1400W groups. Values shown are mean ±SD.



4.4.2.9 Urinary 8-isoprostane quantification

Urinary levels of 8-isoprostane were measured at 180 minutes post-reperfusion. There were statistically lower levels of 8-Isoprostane expression in the WB+1400W group when compared with the 18hr control and LDB groups (p=0.03 and 0.038, respectively). The level of expression observed in the LDB+1400W group was particularly high, but because of limited sample numbers were not used for statistical comparison with the other groups. These results are shown in figure 20.

Figure 20. Histogram showing urinary 8-Isoprostane levels at 180 minutes post-reperfusion in the 18hr control, LDB, WB+1400W and LDB+1400W groups. Values shown are mean ±SD.



4.4.3 Part three

4.4.3.1 Haemodynamics

In all groups initial renal blood flow (RBF) was low. After 10 minutes of reperfusion RBF improved in all groups for the remainder of the reperfusion period. RBF in the WB+SNP and LDB+SNP groups improved throughout the first 30 minutes of reperfusion to a greater degree than in the 18hr control and LDB groups, but for the remainder of reperfusion improvements in RBF in these two groups was minimal. AUC RBF for the 5-30 and 30-180 minutes of reperfusion were analysed separately. After 60 minutes of reperfusion RBF in the WB+SNP and LDB+SNP groups was higher than that in the 18hr control group, but lower than the LDB group, this trend continued until the end of the reperfusion period. Blood flow in the 18hr control group was statistically lower than in both the LDB and LDB+SNP groups. AUC RBF 5-30 minutes of reperfusion were 705±190, 995±458, 1072±383 and 1529±820 in the 18hr control, LDB, WB+SNP and LDB+SNP groups respectively. Mann-Whitney test between groups: 18hr control vs LDB p=0.020, 18hr control vs WB+SNP p=0.051, 18hr control vs LDB+SNP p=0.005, LDB vs WB+SNP p=NS, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS. AUC 30-180 minutes of reperfusion were 4727±2327, 11417±1969, 8085±970 and 9819±2031 in the 18hr control, LDB, WB+SNP and LDB+SNP groups respectively. Mann-Whitney test between groups: 18hr control vs LDB p=0.001, 18hr control vs WB+SNP p=0.035, 18hr control vs LDB+SNP p=0.005, LDB vs WB+SNP p=0.026, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS. During the initial 30 minutes of perfusion RBF in the LDB and LDB+SNP groups was significantly higher than in the 18hr control group. The RBF in the WB+SNP group, though higher, did not reach statistical significance when compared with the 18hr control group. During the remainder of reperfusion the RBF in the LDB

group was significantly higher than that in the 18hr control and WB+SNP groups, whilst the RBF in the LDB+SNP group was statistically higher than in the 18hr control group 2 only. These results are shown in figure 21.

Intra-renal resistance demonstrated reciprocal characteristics. During the initial 30 minutes of reperfusion IRR was higher in all groups than during the remainder of reperfusion. The IRR in the SNP supplemented groups fell markedly at 30 minutes and remained at a low level for the remainder of reperfusion. IRR was analysed separately during the period 5-30 and 30-180 minutes of reperfusion. AUC IRR for 5-30 minutes post-reperfusion was 43±16, 32±23, 25±13 and 22±13 in the 18hr control, LDB, WB+SNP and LDB+SNP groups respectively. Mann-Whitney test between groups: 18hr control vs LDB p=0.051, 18hr control vs WB+SNP p=0.035, 18hr control vs LDB+SNP p=0.008, LDB vs WB+SNP p=NS, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS. AUC IRR for 30-180 minutes post-reperfusion was 192±149, 56± 17, 66±6 and 58±13 in the 18hr control, LDB, WB+SNP and LDB+SNP groups respectively. Mann-Whitney test between groups: 18hr control vs LDB p=0.008, 18hr control vs WB+SNP p=0.035, 18hr control vs LDB+SNP p=0.005, LDB vs WB+SNP p=NS, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS. For 5-30 minutes of reperfusion the IRR was higher in the 18hr control group compared to all other groups, though this only reached significance versus the WB+SNP and LDB+SNP groups. During this period there was no statistical difference in IRR between the LDB, WB+SNP and LDB+SNP groups. During 30-180 minutes of reperfusion IRR was significantly higher than all the other groups, once again there was no statistical difference in IRR between the LDB, WB+SNP and LDB+SNP groups. These results are shown in figure 22.

Figure 21. Graph showing mean renal blood flow in the 18hr control, LDB, WB+SNP, LDB+SNP groups over 180 minutes of reperfusion. Values shown are mean ±SD.

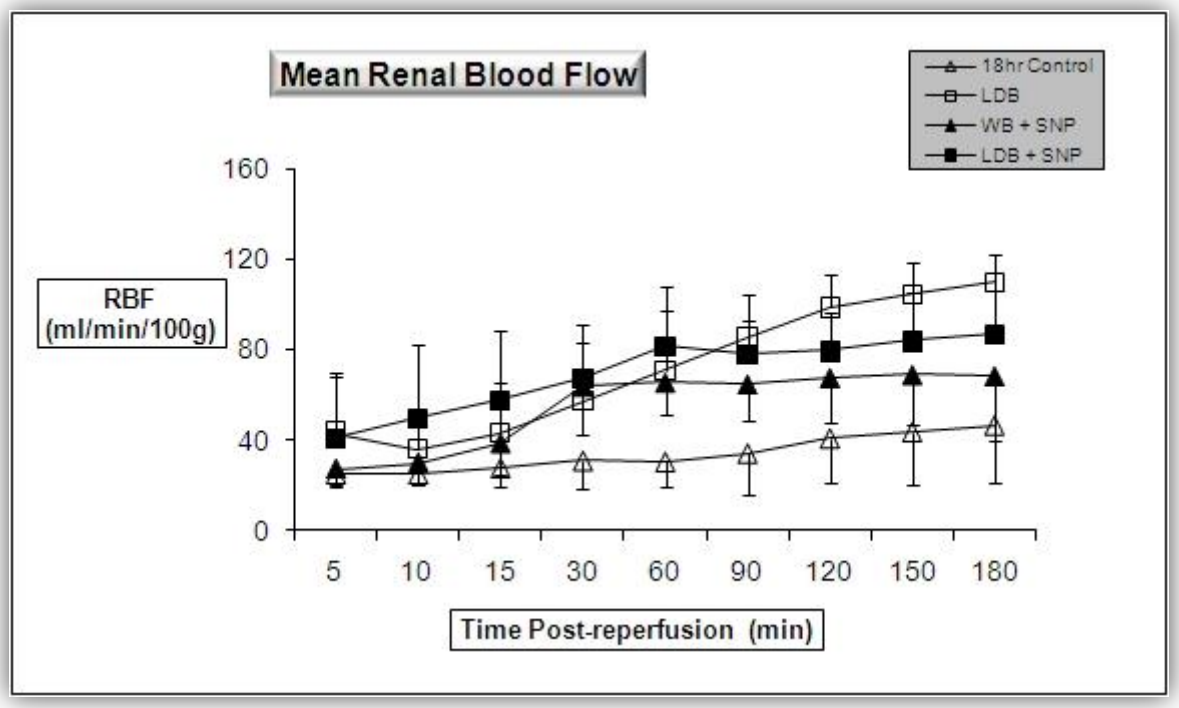
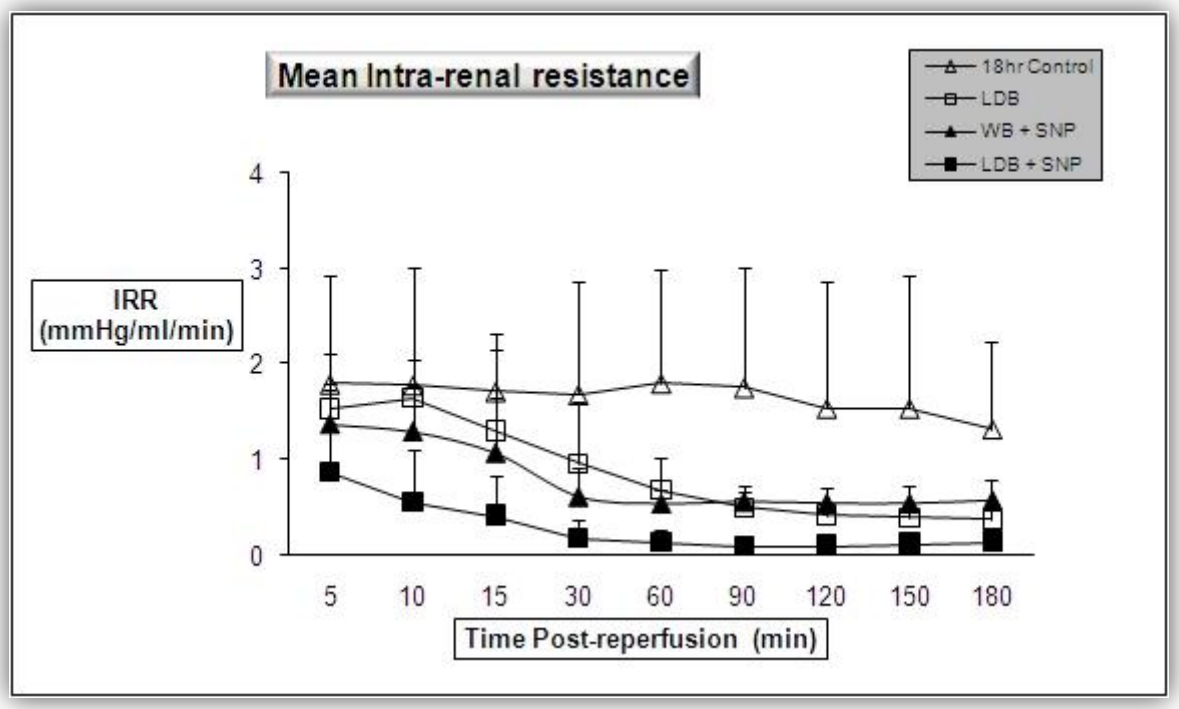


Figure 22. Graph showing mean intra-renal resistance in the 18hr control, LDB, WB+SNP and LDB+SNP groups over 180 minutes of reperfusion. Values shown are mean ±SD.



4.4.3.2 Creatinine clearance

At 60 minutes post-reperfusion cCrCl was lowest in the 18hr control group and highest in the LDB + SNP group. Although not statistically significant throughout the first 60 minute period of reperfusion the WB+SNP and LDB+SNP groups demonstrated similar CrCl to the LDB group. However, by 120 minutes of reperfusion CrCl in the WB+SNP and LDB+SNP groups had begun to deteriorate in comparison to the LDB group. At 180 minutes post-reperfusion this deterioration had continued and the WB+SNP group demonstrated worse CrCl than the 18hr control group. CrCl was highest in the groups perfused with LDB. These data are shown in table 26. Mann-Whitney test between groups: 60min; 18hr control vs LDB p=0.025, 18hr control vs WB+SNP p=0.013, 18hr control vs LDB+SNP p=0.005, LDB vs WB+SNP p=NS, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS. 120min; 18hr control vs LDB p=0.016, 18hr control vs WB+SNP p=NS, 18hr control vs LDB+SNP p=0.008, LDB vs WB+SNP p=0.030, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=0.037. 180min; 18hr control vs LDB p=0.015, 18hr control vs WB+SNP p=NS, 18hr control vs LDB+SNP p=0.032, LDB vs WB+SNP p=0.004, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=0.026.

Table 23. Table showing mean corrected creatinine clearance (ml/min/100g) at 60, 120 and 180 minutes post-reperfusion in the 18hr control, LDB, WB+SNP and LDB+SNP groups. Values shown are mean ±SD.

		Mean Corrected Creatinine Clearance (SD)		
	Group	**60min**	**120min**	**180min**
2.	18hr control	0.5 (0.2)	0.4 (0.2)	0.5 (0.4)
3.	LDB	2.1 (2)	2.3 (1.1)	1.9 (1.4)
6.	WB + SNP	2.2 (1.3)	0.7 (0.6)	0.4 (0.4)
7.	LDB + SNP	3.6 (2.2)	1.7 (0.6)	1.5 (0.9)

4.4.3.3 Urine output

Urine output for the initial 60 minutes of reperfusion was highest in the SNP supplemented groups. However throughout the remainder of the reperfusion period the urine output in these groups deteriorated. By 180 minutes of reperfusion the urine output in the WB+SNP group was significantly lower than that in the LDB group. These results are shown in table 24. Mann-Whitney test between groups: 60min; 18hr control vs LDB p=0.021, 18hr control vs WB+SNP p=0.006, 18hr control vs LDB+SNP p=0.003, LDB vs WB+SNP p=NS, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS. 120min; 18hr control vs LDB p=0.005, 18hr control vs WB+SNP p=NS, 18hr control vs LDB+SNP p=0.054, LDB vs WB+SNP p=0.013, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS. 180min; 18hr control vs LDB p=0.015, 18hr control vs WB+SNP p=NS, 18hr control vs LDB+SNP p=NS, LDB vs WB+SNP p=0.002, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=0.041. Total; 18hr control vs LDB p=0.005, 18hr control vs WB+SNP p=NS, 18hr control vs LDB+SNP p=0.005, LDB vs WB+SNP p=NS, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS.

Table 24. Table showing hourly and total urine output (ml) in the 18hr control, LDB, WB+SNP and LDB+SNP groups. Values shown are mean ±SD.

		Mean Hourly Urine Output (SD)			
	Group	**1hr**	**2hr**	**3hr**	**Total**
2.	18hr control	64 (30)	47 (34)	46 (34)	156 (83)
3.	LDB	197 (168)	183 (87)	169 (116)	550 (338)
6.	WB + SNP	198 (88)	45 (42)	23 (21)	266 (126)
7.	LDB + SNP	252 (124)	101 (42)	97 (64)	450 (169)

4.4.3.4 Fractional excretion of sodium

There was no overall trend or any statistical significance in the fractional excretion of sodium between any of the groups at any time point. These data are summarized in table 25. Mann-Whitney test between groups: 60min; 18hr control vs LDB p=NS, 18hr control vs WB+SNP p=NS, 18hr control vs LDB+SNP p=0.009, LDB vs WB+SNP p=NS, LDB vs LDB+SNP p=0.041, WB+SNP vs LDB+SNP p=NS. 120min; 18hr control vs LDB p=NS, 18hr control vs WB+SNP p=NS, 18hr control vs LDB+SNP p=NS, LDB vs WB+SNP p=NS, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS. 180min; 18hr control vs LDB p=NS, 18hr control vs WB+SNP p=NS, 18hr control vs LDB+SNP p=NS, LDB vs WB+SNP p=NS, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS.

Table 25. Table showing mean fractional excretion of sodium (%) at 60, 120 and 180 minutes post-reperfusion in the 18hr control, LDB, WB+SNP and LDB+SNP groups. Values shown are mean ±SD.

		Mean Fractional Excretion of Sodium (SD)		
	Group	**60min**	**120min**	**180min**
2.	18hr control	121 (61)	82 (44)	84 (53)
3.	LDB	81 (8)	70 (12)	71 (21)
6.	WB + SNP	66 (18)	51 (20)	48 (25)
7.	LDB + SNP	57 (14)	42 (13)	38 (13)

4.4.3.5 Hydrogen ion concentration

There was no difference in pre-perfusion pH between any of the groups. There was a general trend of increasing acidosis in the 18hr control and WB+SNP groups throughout the reperfusion period. The LDB+SNP group demonstrated a slight recovery in pH between 120 and 180 minutes post-reperfusion. Significance was only reached between the LDB vs WB+SNP groups at 120 minutes post-reperfusion. These data are shown in table 26. Mann-Whitney test between groups: Pre; 18hr control vs LDB p=NS, 18hr control vs WB+SNP p=NS, 18hr control vs LDB+SNP p=NS, LDB vs WB+SNP p=NS, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=0.030. 60min; 18hr control vs LDB p=NS, 18hr control vs WB+SNP p=0.071, 18hr control vs LDB+SNP p=NS, LDB vs WB+SNP p=0.016, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS. 180min; 18hr control vs LDB p=NS, 18hr control vs WB+SNP p=NS, 18hr control vs LDB+SNP p=NS, LDB vs WB+SNP p=0.030, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS.

Table 26. Table showing mean pH values pre-reperfusion, 60min and 180min post-reperfusion in the 18hr control, LDB, WB+SNP and LDB+SNP groups. Values shown are mean ±SD.

		Mean pH (SD)		
	Group	**Pre**	**1hr**	**3hr**
2.	18hr control	7.43 (0.03)	7.32 (0.04)	7.3 (0.08)
3.	LDB	7.45 (0.02)	7.34 (0.03)	7.38 (0.07)
6.	WB + SNP	7.5 (0.07)	7.26 (0.05)	7.28 (0.04)
7.	LDB + SNP	7.43 (0.05)	7.31 (0.04)	7.32 (0.09)

4.4.3.6 Oxygen consumption

There was increased oxygen consumption throughout the reperfusion in all groups. Results are shown in table 27. Mann-Whitney test between groups: 60min; 18hr control vs LDB p=0.022, 18hr control vs WB+SNP p=0.001, 18hr control vs LDB+SNP p=0.001, LDB vs WB+SNP p=NS, LDB vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS. 180min; 18hr control vs LDB p=0.001, 18hr control vs WB+SNP p=NS, 18hr control vs LDB+SNP p=0.014, LDB vs WB+SNP p=0.037, LDB vs LDB+SNP p=0.041, WB+SNP vs LDB+SNP p=NS.

Table 27. Table showing mean oxygen consumption (kPa.ml/min/100g) at 60 and 180 minutes post-reperfusion in the 18hr control, LDB, WB+SNP and LDB+SNP groups. Values shown are mean ±SD.

		Mean Oxygen Consumption (SD)	
	Group	**1hr**	**3hr**
2.	18hr control	19.3 (6.8)	28 (13.9)
3.	LDB	44.3 (16.8)	64.7 (5.5)
6.	WB + SNP	44.1 (11.7)	40.9 (16.9)
7.	LDB + SNP	50 (14)	48.4 (12.6)

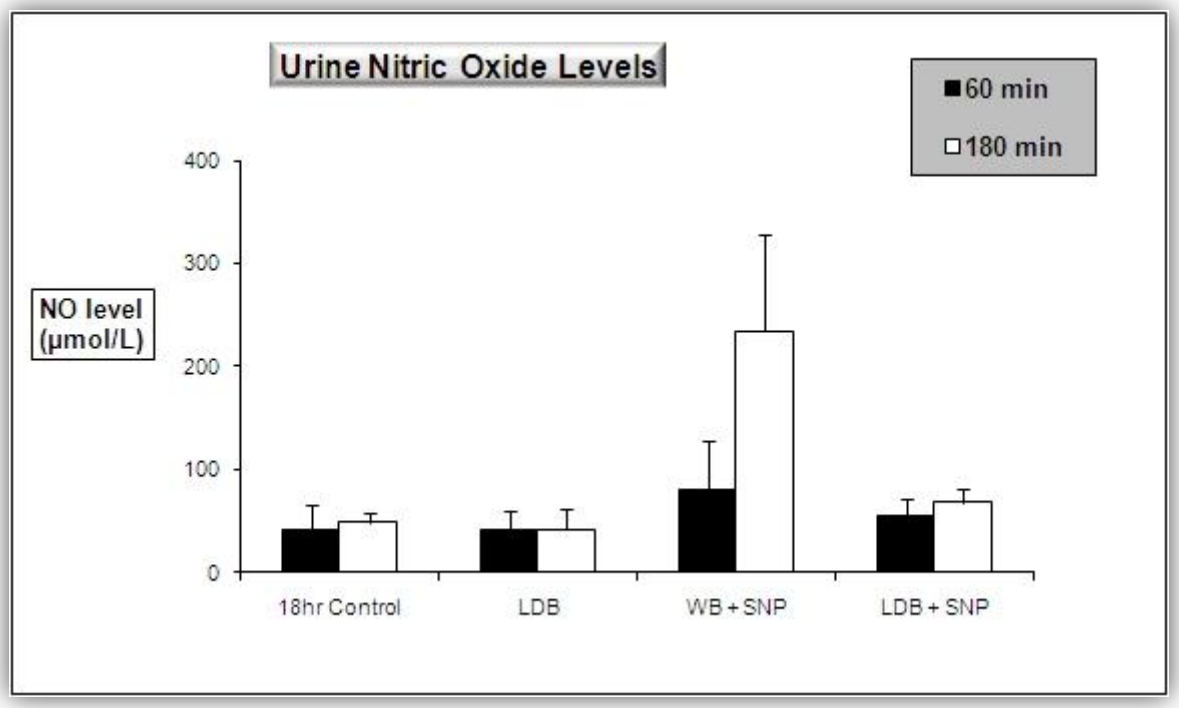
4.4.3.7 Urinary nitric oxide quantification

Urinary nitric oxide quantification at 60 minutes reperfusion showed no difference between the 18hr control, LDB and LDB+SNP groups, but a slightly elevated level in the WB+SNP group when compared with the 18hr control group. Following 180 minutes of reperfusion the level of NO was marginally, but significantly, elevated in the LDB+SNP group when compared with the 18hr control and LDB groups. The level of NO in the WB+SNP group following 180 minutes of reperfusion was massively elevated when compared with the 18hr control, LDB, and LDB+SNP groups. These results and statistics are summarised in table 28 and figure 23.

Table 28. Table showing individual p values between groups when urinary nitric oxide levels were compared at 60 and 180 minutes post-reperfusion in the 18hr control, LDB, WB+SNP and LDB+SNP groups.

		P value between groups					
	Group	18hr Control		LDB		WB + SNP	
		60 mins	180 mins	60 mins	180 mins	60 mins	180 mins
2.	18hr Control	-	-	-	-	-	-
3.	LDB	**NS**	**NS**	-	-	-	-
6.	WB + SNP	**0.045**	**0.014**	**NS**	**0.014**	-	-
7.	LDB + SNP	**NS**	**0.023**	**NS**	**0.023**	**NS**	**0.02**

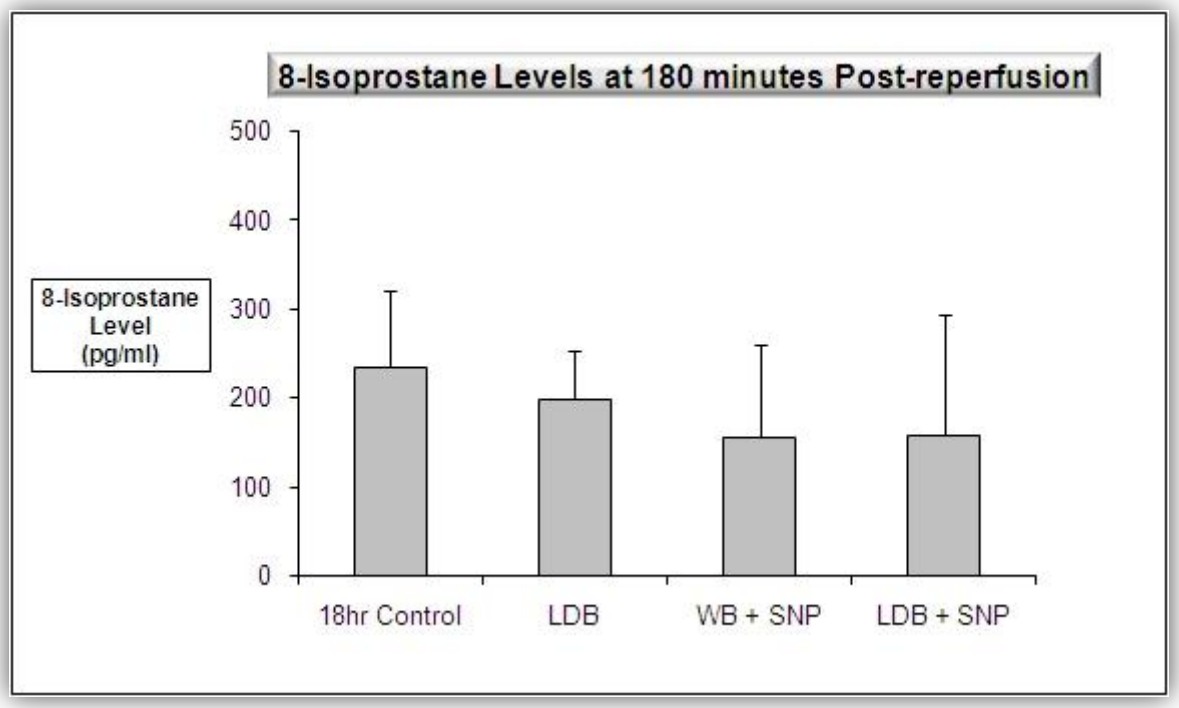
Figure 23. Histogram showing urinary nitric oxide levels at 60 and 180 minutes post-reperfusion in the 18hr control, LDB, WB+SNP and LDB+SNP groups. Values shown are mean ±SD..



4.4.3.8 Urinary 8-isoprostane quantification

Urinary levels of 8-isoprostane were measured at 180minutes post-reperfusion. Although the levels of 8-Isoprostane were lower in the SNP supplemented groups, this did not reach statistical significance. These data are shown in figure 24.

Figure 24. Histogram showing urinary 8-Isoprostane levels at 180 minutes post-reperfusion in the 18hr control, LDB, WB+SNP and LDB+SNP groups. Values shown are mean ±SD.



4.4.4 Part four

4.4.4.1 Haemodynamics

Renal blood flow was significantly higher in the SNP supplemented groups when compared to the 1400W supplemented groups during the initial 30 minutes of reperfusion. AUC RBF was 333±112, 180±144, 1072±383 and 1529±820 in the WB+1400W, LDB+1400W, WB+SNP and LDB+SNP groups respectively. Mann-Whitney test between groups: WB+1400W vs LDB+1400W p=NS, WB+1400W vs WB+SNP p=0.004, WB+1400W vs LDB+SNP p=0.004, LDB+1400W vs WB+SNP p=0.024, LDB+1400W vs LDB+SNP p=0.024, WB+SNP vs LDB+SNP p=NS. During the reperfusion period from 30 – 180 minutes the blood flow in the SNP supplemented groups plateaued, the blood flow in the WB+1400W group continued to increase to the level of the flow in the SNP groups. The blood flow in the LDB+1400W group remained poor. AUC RBF was 5982±3563, 581±751, 8085±970 and 9819±2031 in the WB+1400W, LDB+1400W, WB+SNP and LDB+SNP groups respectively. Mann-Whitney test between groups: WB+1400W vs LDB+1400W p=0.01, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=NS, LDB+1400W vs WB+SNP p=0.014, LDB+1400W vs LDB+SNP p=0.014, WB+SNP vs LDB+SNP p=NS. These data are summarized in figure 25.

The intra-renal resistance reciprocated the renal blood flow findings. During the initial 30 minutes of reperfusion intra-renal resistance in the 1400W supplemented groups was high and in the SNP groups it was low. AUC IRR was 92±51, 161±33, 25±13 and 22±13 in the WB+1400W, LDB+1400W, WB+SNP and LDB+SNP groups respectively. Mann-Whitney test between groups: WB+1400W vs LDB+1400W p=NS, WB+1400W vs

WB+SNP p=0.004, WB+1400W vs LDB+SNP p=0.002, LDB+1400W vs WB+SNP p=0.01, LDB+1400W vs LDB+SNP p=0.01, WB+SNP vs LDB+SNP p=NS. During 30 - 180 minutes of reperfusion resistance in the WB+SNP and LDB+SNP groups remained low. Intra-renal resistance in the LDB+1400W group remained high whilst that in the WB+1400W group fell to the levels observed in the WB+SNP and LDB+SNP groups. AUR IRR between 30-180 minutes post-reperfusion was 145±92, 952±203, 66±6, 58±13 for the WB+1400W, LDB+1400W, WB+SNP and LDB+SNP groups respectively. Mann-Whitney test between groups: WB+1400W vs LDB+1400W p=0.01, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=0.009, LDB+1400W vs WB+SNP p=0.01, LDB+1400W vs LDB+SNP p=0.01, WB+SNP vs LDB+SNP p=NS. These data are shown in figure 26.

Figure 25. Graph showing mean renal blood flow in the WB+1400W, LDB+1400W, WB+SNP and LDB+SNP groups over 180 minutes of reperfusion. Values shown are mean ±SD.

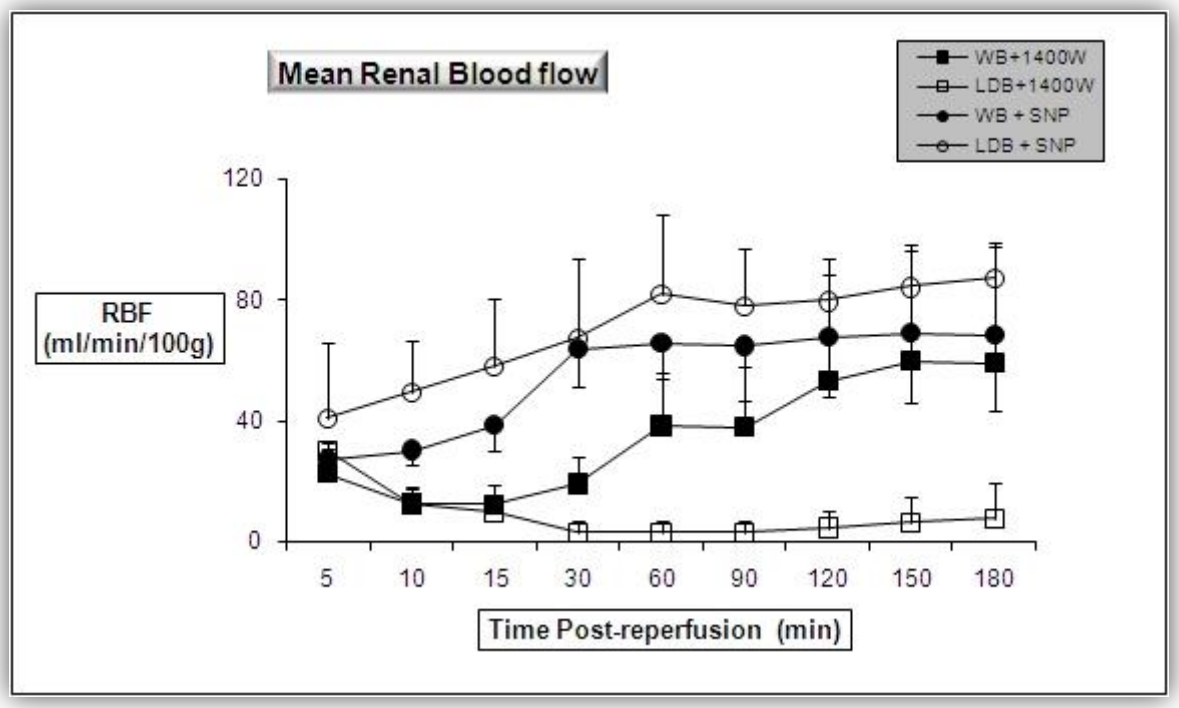
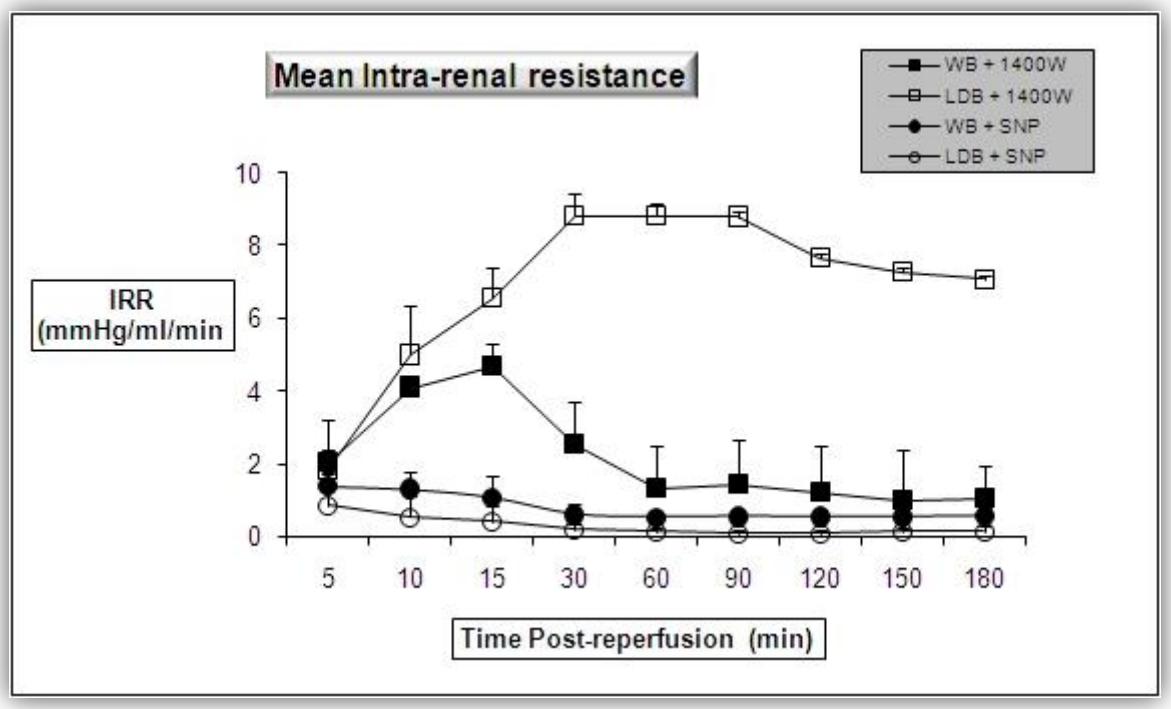


Figure 26. Graph showing mean intra-renal resistance in the WB+1400W, LDB+1400W, WB+SNP and LDB+SNP groups over 180 minutes of reperfusion. Values shown are mean ±SD.



4.4.4.2 Creatinine clearance

Creatinine clearance was poor in all groups. CrCl in the SNP supplemented groups was significantly better than that in the 1400W supplemented groups after 60 minutes of reperfusion. However, by 120 minutes of reperfusion the CrCl in both SNP groups had deteriorated significantly, although the clearance in the LDB+SNP group remained higher than that in the WB+SNP group. By 180 minutes post-reperfusion the CrCl in the SNP groups continued to deteriorate, such that it approached the levels observed in the 1400W groups. These results are shown in Table 29. Mann-Whitney test between groups: 60 min; WB+1400W vs LDB+1400W p=0.01, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=0.009, LDB+1400W vs WB+SNP p=0.014, LDB+1400W vs LDB+SNP p=0.014, WB+SNP vs LDB+SNP p=NS. 120min; WB+1400W vs LDB+1400W n/a, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=NS, LDB+1400W vs WB+SNP n/a, LDB+1400W vs LDB+SNP n/a, WB+SNP vs LDB+SNP p=0.04. 180min; WB+1400W vs LDB+1400W p=0.019, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=NS, LDB+1400W vs WB+SNP p=NS, LDB+1400W vs LDB+SNP p=0.013, WB+SNP vs LDB+SNP p=0.026.

Table 29. Table showing mean corrected creatinine clearance (ml/min/100g) at 60, 120 and 180 minutes post-reperfusion in the WB+1400W, LDB+1400W, WB+SNP and LDB+SNP groups. Values shown are mean ±SD.

		Mean Corrected Creatinine Clearance (SD)		
	Group	**60min**	**120min**	**180min**
4.	WB + 1400W	1.2 (0.6)	1.3 (0.7)	1 (0.8)
5.	LDB + 1400W	0.2 (0.2)	0 (0)	0.1 (0.1)
6.	WB + SNP	2.2 (1.3)	0.7 (0.6)	0.4 (0.4)
7.	LDB + SNP	3.6 (2.2)	1.7 (0.6)	1.5 (0.9)

4.4.4.3 Urine output

Urine output for all groups was highest in the first 60 minutes of reperfusion. Urine output decreased throughout the period of reperfusion until 180 minutes. These data are summarized in table 30. Mann-Whitney test between groups: 60min; WB+1400W vs LDB+1400W p=0.01, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=NS, LDB+1400W vs WB+SNP p=0.01, LDB+1400W vs LDB+SNP p=0.01, WB+SNP vs LDB+SNP p=NS. 120min; WB+1400W vs LDB+1400W n/a, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=NS, LDB+1400W vs WB+SNP n/a, LDB+1400W vs LDB+SNP n/a, WB+SNP vs LDB+SNP p=NS. 180min; WB+1400W vs LDB+1400W p=0.019, WB+1400W vs WB+SNP p=0.026, WB+1400W vs LDB+SNP p=NS, LDB+1400W vs WB+SNP p=NS, LDB+1400W vs LDB+SNP p=0.023, WB+SNP vs LDB+SNP p=0.041. Total; WB+1400W vs LDB+1400W p=0.01, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=NS, LDB+1400W vs WB+SNP p=0.01, LDB+1400W vs LDB+SNP p=0.01, WB+SNP vs LDB+SNP p=NS.

Table 30. Table showing hourly and total urine output (ml) in the WB+1400W, LDB+1400W, WB+SNP and LDB+SNP groups. Values shown are mean ±SD.

		Mean Hourly Urine Output (SD)			
	Group	**1hr**	**2hr**	**3hr**	**Total**
4.	WB + 1400W	125 (66)	113 (62)	82 (54)	320 (96)
5.	LDB + 1400W	29 (9)	0 (0)	6 (13)	35 (20)
6.	WB + SNP	198 (88)	45 (42)	23 (21)	266 (126)
7.	LDB + SNP	252 (124)	101 (42)	97 (64)	450 (169)

4.4.4.4 Fractional excretion of sodium

The FE_{Na} improved in all groups, except the LDB+1400W group, during the reperfusion period. Statistical analysis of the LDB+1400W group was precluded by the paucity of urine produced. Respectively, the FE_{Na} was lowest in the LDB+SNP group followed by WB+SNP group and the WB+1400W group. Throughout the reperfusion period the FE_{Na} in the WB+1400W group was statistically higher than in the LDB+SNP group. These data are shown in table 31. Mann-Whitney test between groups: 60 min; WB+1400W vs LDB+1400W n/a, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=0.015, LDB+1400W vs WB+SNP n/a, LDB+1400W vs LDB+SNP n/a, WB+SNP vs LDB+SNP p=NS. 120min; WB+1400W vs LDB+1400W n/a, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=0.009, LDB+1400W vs WB+SNP n/a, LDB+1400W vs LDB+SNP n/a, WB+SNP vs LDB+SNP p=NS. 180min; WB+1400W vs LDB+1400W n/a, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=0.015, LDB+1400W vs WB+SNP n/a, LDB+1400W vs LDB+SNP n/a, WB+SNP vs LDB+SNP p=NS.

Table 31. Table showing mean fractional excretion of sodium (%) at 60, 120 and 180 minutes post-reperfusion in the WB+1400W, LDB+1400W, WB+SNP and LDB+SNP groups. Values shown are mean ±SD.

		Mean Fractional Excretion of Sodium (SD)		
	Group	**60min**	**120min**	**180min**
4.	WB + 1400W	78 (17)	62 (19)	61 (14)
5.	LDB + 1400W	79 (38)	n/a	93 (0)
6.	WB + SNP	66 (18)	51 (20)	48 (25)
7.	LDB + SNP	57 (14)	42 (13)	38 (13)

4.4.4.5 Hydrogen ion concentration

All groups became increasing acidotic during the reperfusion period. There was no statistically difference in pH between the groups. Mann-Whitney test between groups: Pre; WB+1400W vs LDB+1400W p=0.042, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=NS, LDB+1400W vs WB+SNP p=NS, LDB+1400W vs LDB+SNP p=NS, 6&7 p=0.030. 60min; WB+1400W vs LDB+1400W p=NS, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=NS, LDB+1400W vs WB+SNP p=NS, LDB+1400W vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS. 180min; WB+1400W vs LDB+1400W p=NS, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=NS, LDB+1400W vs WB+SNP p=NS, LDB+1400W vs LDB+SNP p=NS, WB+SNP vs LDB+SNP p=NS.

Table 32. Table showing mean pH values pre-reperfusion, 60min and 180min post-reperfusion in the WB+1400W, LDB+1400W, WB+SNP and LDB+SNP groups. Values shown are mean ±SD.

		Mean pH (SD)		
	Group	**Pre**	**1hr**	**3hr**
4.	WB + 1400W	7.47 (0.04)	7.28 (0.05)	7.24 (0.04)
5.	LDB + 1400W	7.41 (0.04)	7.3 (0.01)	7.28 (0.02)
6.	WB + SNP	7.5 (0.07)	7.26 (0.05)	7.28 (0.04)
7.	LDB + SNP	7.43 (0.05)	7.31 (0.04)	7.32 (0.09)

4.4.4.6 Oxygen consumption

Oxygen consumption was seen to increase in the WB+1400W group between the beginning and end of the reperfusion period. Oxygen consumption in the LDB+1400W group was much lower than in the other groups. This group was not analysed statistically. The oxygen consumption in the WB+1400W group was initially significantly lower than in the LDB+SNP group, but by the end of the reperfusion period was significantly higher. These data are shown in table 33. Mann-Whitney test between groups: 60min; WB+1400W vs LDB+1400W p=0.013, WB+1400W vs WB+SNP p=0.015, WB+1400W vs LDB+SNP p=0.004, LDB+1400W vs WB+SNP p=0.013, LDB+1400W vs LDB+SNP p=0.013, WB+SNP vs LDB+SNP p=NS. 120min; WB+1400W vs LDB+1400W p=0.023, WB+1400W vs WB+SNP p=NS, WB+1400W vs LDB+SNP p=NS, LDB+1400W vs WB+SNP p=0.013, LDB+1400W vs LDB+SNP p=0.013, WB+SNP vs LDB+SNP p=NS.

Table 33. Table showing mean oxygen consumption (kPa.ml/min/100g) at 60 and 180 minutes post-reperfusion in the WB+1400W, LDB+1400W, WB+SNP and LDB+SNP groups. Values shown are mean ±SD.

		Mean Oxygen Consumption (SD)	
	Group	**1hr**	**3hr**
4.	WB + 1400W	24.1 (11)	36.4 (22.8)
5.	LDB + 1400W	1.1 (2.2)	3.9 (7.9)
6.	WB + SNP	44.1 (11.7)	40.9 (16.9)
7.	LDB + SNP	50 (14)	48.4 (12.6)

5 Discussion

5.1 Methods

5.1.1 The model

5.1.1.1 Animal choice

The use of porcine kidneys as a model for human transplantation is well established. Porcine organs are anatomically and physiologically analogous to human kidneys and as such present a better model than other mammals [195, 196]. The pigs used in these experiments were of such an age and size so as to provide organs of a weight comparable to human organs. Pig kidneys of this weight have been used extensively with the NPS in the published literature [185, 186, 189].

5.1.1.2 Normothermic perfusion system

The NPS has been used as a model for reperfusion in a number of studies and it is a well established model for human transplantation [185, 189, 191]. The NPS apparatus and the constituents of the perfusate, detailed in the methods chapter, have undergone no significant alteration since the systems inception.

5.1.2 Experimental design

Sample size analysis using the NPS model has been performed previously. These analyses demonstrated that six organs *per* group provide an adequate sample size for statistical analysis [188, 189].

This study was designed to model the effects of interventions on the reperfusion of organs from DCD donors. An accurate model for DCD organs and controls had to be derived. Two aspects were considered to be of particular importance in deriving organs analogous to DCD kidneys: pre-perfusion parameters, warm and cold ischaemic times; and reperfusion parameters, perfusion pressure and duration. Evidence from *in vivo* experiments and experience from the use of the NPS was then used to design the experimental conditions.

5.1.2.1 Pre-perfusion parameters

The physiological similarities between porcine and human kidneys have been described above, thus the WIT and CIT endured by human DCD kidneys were used as a baseline for these experiments. Many studies have outlined the WIT and CIT that DCD kidneys are exposed to [19, 23, 197]. From the published data a WI insult of 25 minutes, followed by a SCS period of 18 hours was decided upon. A preliminary study was performed to assess the differential effects of WIT and CIT on renal injury during reperfusion [190]. This study demonstrated that 25 minutes WIT and 18 hours CIT was necessary to produce a level of acute renal injury significantly worse than organs subjected to 10min WIT and 18 hr CIT or 25 minutes WIT and 2hrs CIT. Of note was that kidneys subjected to 25 minutes WIT and 2hr CIT did not demonstrate significant functional differences to organs exposed to 10 minutes WIT and 18 hrs CIT during the

initial reperfusion period. This finding lead to the use of a 25 minutes WIT and 2hr CIT group as the control (group 1) in part one of this study.

5.1.2.2 Reperfusion parameters

The most important reperfusion parameter is the choice of reperfusion blood pressure. Some studies have advocated the use of sub-physiological perfusion pressures in order to minimize endothelial injury and thus allow prolonged perfusion, but at the expense of initial renal function [179, 198, 199]. The use of physiological pressures is associated with better initial renal function but higher degrees of organ injury that restrict the duration of perfusion [188, 200, 201]. During the development of the NPS model various perfusion pressures were trialed [188]. This study demonstrated that although organ viability was maintained at sub-physiological pressure (55mmHg) renal function was impaired. At higher perfusion pressures (95mmHg), renal function was improved initially, but towards the end of perfusion there was evidence of acute tubular injury both functionally and histologically. It was therefore decided that perfusion blood pressure would be between 75 and 95 mmHg. In human studies there is a well-established association between lower systolic and, even more so, lower diastolic blood pressure at the time of initial reperfusion and the development of DGF [202, 203]. The high incidence of DGF associated with DCD kidneys, independent of reperfusion blood pressure, guided the decision to choose a reperfusion pressure of 85mmHg.

5.1.3 Leukocyte depletion

Leukocyte recruitment, adhesion, infiltration and release of chemokines are important steps in the generation of RI. The pathophysiology underlying these steps is detailed in the introduction section. Previous work in a DBD model, that is short WIT and short CIT, demonstrated improved renal function when grafts were reperfused with LDB versus WB [189]. In this study renal haemodynamics and urine output were enhanced in the LDB versus the WB reperfused group. Furthermore, functional measures such as creatinine clearance, fractional excretion of sodium and acid-base balance were superior.

It was hypothesized that the use of LDB in the reperfusion of DCD kidneys would improve renal haemodynamics sufficiently to reduce the severity of RI. Minimisation of RI could confer some protection to DGF that would be detectable by analysis of functional parameters.

5.1.4 Inhibition of iNOS and NO donation

NO is an important monovalent gas that regulates vascular tone, inhibits platelet aggregation and leukocyte adhesion, scavenges ROS and maintains vascular homeostasis by preserving normal vascular permeability, inhibiting smooth muscle proliferation and stimulating endothelial regeneration. NO is generated constitutively by eNOS and, in response to stress, by iNOS. Following ischaemia and reperfusion, the excess of NO generated by iNOS may interact with ROS to form deleterious peroxynitrite that contributes to RI.

There is a wealth of published literature concerned with the modulation of NO levels by use of NO donors and NOS inhibitors/inducers. Administration of NO donors is associated with amelioration of RI associated vasospasm and maintenance of endothelial function [158, 204]. Inhibition of iNOS is reported to reduce tissue damage after ischaemia and reperfusion [162, 163].

The are many potential NO donors however SNP was chosen as it has current clinical use in humans, demonstrating its physiological safety *in vivo,* and it has been used previously in experiments using the NPS model [189]. The short half life of SNP necessitated a continuous infusion.

There are a number of potential inhibitors of iNOS, however only 1400W has been shown to be highly specific for the inducible isozyme of NOS and non-specific for eNOS [161]. Furthermore, 1400W remains highly bound to iNOS for a period beyond the duration of reperfusion in these experiments [161]. In rats 1400W is reported to have a wide therapeutic window between 0.3mg/kg and 25mg/kg, with toxicity reported only at levels approaching 50mg/kg [161]. No study has assessed the effect of 1400W on renal RI in swine. A number of studies have used 1400W to assess iNOS blockade in renal RI in rats. In all these studies a standard dose of 10mg/kg was used [162, 163, 205]. A swine model for endotoxaemia studying the impact of 1400W has been used. In these studies a dose of 0.5mg/kg/hr was used [206, 207]. Taking into account the wide therapeutic interval of 1400W and the experimental evidence a preliminary dosing study was performed. These data, presented in the results section figures 12-16, demonstrated no significant difference in haemodynamic and functional parameters between a dose of 1mg/kg and 10mg/kg of renal weight. It was therefore

decided that a dose of 10mg/kg of renal weight would be used as the dose of 1400W in this study.

5.2 Results

5.2.1 Leukocyte Counts

As expected the white cell count (WCC) observed in filtered blood was significantly lower than that in whole blood in all LDB groups. The difference in leukocyte count observed in pre and post-reperfusion samples is worthy of further discussion.

5.2.1.1 Decrease in leukocyte count in WB groups pre-and post-reperfusion

In the WB perfused groups WCC was lower in post-perfusion samples when compared to pre-perfusion samples. This reduction in WCC reached statistical significance in the 2hr control, 18hr control and WB+1400W groups but not in the WB+SNP group.

As described in the Methods chapter the perfusate is augmented by various supplements and urine output replaced to maintain overall circulating volume throughout the reperfusion period. Despite meticulous fluid management the lower WCC could thus be explained by dilution of the WB perfusate. A dilutional cause for the decrease in WCC is less likely when it is noted that there was no significant difference in haematocrit level pre- and post-reperfusion in any of the experimental groups.

It is well established, and expounded in the Introduction chapter, that in response to reperfusion injury there is leukocyte recruitment and infiltration [33]. The

extravasation into the interstitium and tethering of leukocytes to the endothelium could account for the reduction in WCC.

5.2.1.2 Increase in leukocyte count in LDB groups pre- and post-reperfusion

It was observed that the WCC in the LDB perfused groups increased following reperfusion. This increase only reached statistical significance in the LDB group.

It would be reasonable to expect that the WCC in the LDB groups would reduce further as a result of leukocyte tethering and infiltration. The fact that the WCC actually increases implies that leukocytes numbers are augmented by the end of the reperfusion period.

All donor organs contain passenger leukocytes that are able to enter the circulation following reperfusion. Studies in liver transplantation have shown that donor leukocytes can contribute up to 20% of the WCC immediately following transplantation [208]. It is therefore probable that the rise in WCC observed in LDB groups is due to release of passenger leukocytes into the perfusate.

5.2.2 Platelet count

In addition to the reduction in WCC count the platelet count (PC) also decreased following leukocyte filtration. Although the degree of platelet depletion was 5-fold less than the leukocyte depletion the reduction was statistically significant in all LDB groups. There was no difference in PC pre- and post-reperfusion.

This finding has important implications when interpreting the results from these experiments. Platelets play an important role in the tethering of leukocytes to the

damaged endothelium via P-selectin [137]. Platelet adhesion to the endothelium contributes to the no-reflow phenomenon encountered in RI, and is modulated by the expression of a host of adhesion molecules [135, 137]. Although leukocytes are the most important effector cells in RI, platelets are a pivotal modulator, working independently and synergistically with leukocytes, in the generation of RI. The potential impact of lower numbers of circulating platelets must be considered when discussing the haemodynamic effects of leukocyte depletion.

5.2.3 Kidney weight

There was a significant difference in pre-SCS kidney weight between different groups. At the time of culling there were slight differences in the age of animals, which had an effect on the weight of the animal the kidneys retrieved. Furthermore, as each animal provided two kidneys for experimentation any difference in organ weight would be exaggerated between groups comprised of six organs.

All kidneys were observed to increase in weight following the reperfusion period. The highest weight gain was observed in the 18hr control, WB+1400W and LDB+1400W groups. These groups demonstrated the poorest renal blood flow characteristics during the reperfusion period. Previous lapine normothermic perfusion studies have demonstrated a close correlation between kidney weight gain and impaired tubular function and overall renal injury [200].

5.2.4 Histology

Statistically significant changes in histological appearance were confined to tubular dilatation and tubular vacuolation. Although differences were observed pre and post perfusion within the same groups, there was no difference in histological appearance between groups. Other markers of function and injury demonstrate a vast difference between certain groups, such as the LDB and LDB+1400W groups.

Biopsies were taken during the reperfusion period, which was limited to only 180 minutes. It is thus likely that most of the histological features associated with acute kidney injury would not have become manifest in this short time frame.

5.3 Functional data

5.3.1 Haemodynamics

5.3.1.1 Part1.

RBF was initially poor in all groups. After approximately 30 minutes of reperfusion the RBF trends diverged, with the flow in the 2hr control and LDB groups improving significantly when compared to the 18hr control group. The RBF in the LDB group was higher throughout the latter stages of reperfusion when compared to the 2hr control group, but this did not reach statistical significance (p=0.09). As expected the IRR was significantly lower in the LDB and 2hr control groups when compared to the 18hr control group.

Previous studies using the NPS model have demonstrated a similar trend in RBF [189, 192]. Furthermore these studies demonstrated that leukocyte depletion was associated with improved blood flow after 30 minutes of reperfusion [189]. The observed improvement in RBF between the 18hr and 2hr WB perfused groups represents the severity of damage incurred by a combination of 25 min WIT and 18hr SCS compared to 25 min WIT but only 2 hr SCS in the 2hr control group. The duration of ischaemia leads to a higher degree of endothelial dysfunction and enhanced adhesion of leukocytes, which results in leukocyte adhesion and the release of deleterious cytokines [57, 58, 209]. The improvement of RBF and reduction in IRR in the LDB group clearly reinforces the importance of leukocytes (and platelets) in the propagation of reperfusion injury in this model.

5.3.1.2 Part 2

The initial AUC RBF, 5-30 minutes, observed in the WB+1400W and LDB+1400W groups showed variation to that in the 18hr control and LDB groups. For this reason AUC RBF during the initial 30 minutes of reperfusion and the subsequent 150 minutes were analysed separately.

During the initial 30 minutes of reperfusion there was no significant difference in RBF between the 18hr control vs LDB groups, or the WB+1400W vs LDB+1400W groups. The AUC RBF was significantly different between the 18hr control vs WB+1400W, 18hr control vs LDB+1400W, LDB vs WB+1400W and LDB vs LDB+1400W groups. Inhibition of iNOS thus has an immediate effect upon the RBF in both LDB and WB perfused groups suggesting a mechanism independent of leukocyte mediated processes, such as no-reflow phenomenon or inflammatory cytokine production.

Renal expression of eNOS and iNOS differs in response to ischaemia. It has been shown that iNOS expression may increase four-fold during warm ischaemia and eight-fold during the first 30 minutes of reperfusion up to a sixteen-fold maximal increase at 60 minutes post-reperfusion [210]. Conversely, eNOS expression halves during ischaemia and remains reduced until 180 minutes post-reperfusion [210]. Thus the immediate reduction in RBF in the WB+1400W and LDB+1400W groups may be explained by a reduction in NO mediated vasodilatation as a result of iNOS inhibition.

During the period 30-180 minutes post-reperfusion there was a marked divergence in the RBF in the WB+1400W and LDB+1400W groups. The RBF in the LDB+1400W group became statistically lower than the 18hr control, LDB and WB+1400W groups, and showed no recovery during the reperfusion period. The RBF in the WB+1400W group improved markedly such that there was no statistical difference with the RBF in group 18hr control vs WB+1400W during the period 30-180 minutes post-reperfusion.

The observation that RBF improves in the WB+1400W group to control levels implies that RBF in the period 30-180 minutes post-reperfusion is influenced by mechanisms other than NO mediated vasodilatation. As discussed in the introduction chapter, iNOS not only catalyses the formation of NO, but also superoxide [153, 154]. The improvement of RBF in the WB+1400W group could thus be related to lower levels of superoxide production. This hypothesis is reinforced when the levels of 8-isoprostane after 180 minutes of reperfusion are noted. These data demonstrate a significantly lower level of 8-isoprostane, and thus oxidative stress, in the WB+1400W group compared to the 18hr control and LDB groups (the LDB+1400W group was not analysed statistically because of limited sample numbers). The importance of

superoxide reduction by the inhibition of iNOS and the maintenance of vascular tone has also been suggested in the published literature [211]. The impact of NO mediated vasodilatation, although not of paramount importance during this phase of reperfusion, should not be ignored. The abrogation of iNOS derived NO means that vasodilatation is dependent upon eNOS derived NO. The endothelium is the primary source of eNOS, however eNOS is also expressed on platelets and neutrophils [212, 213]. The improvement in RBF could thus be influenced by the increasing expression of eNOS from the injured endothelium in addition to that expressed by circulating neutrophils and platelets.

The finding that the RBF is catastrophically reduced in the LDB+1400W group was unexpected. The removal of leukocytes and eradication of leukocyte mediated cytokine release, superoxide formation and no-reflow phenomenon would be expected to improve the RBF. Theoretically, the absence of eNOS derived NO from leukocytes, platelets and the ischaemic endothelium, coupled with the blockade of iNOS by 1400W, reduces NO levels to a degree incompatible with adequate perfusion. In this scenario RBF is impaired to such a degree that the kidney remains ischaemic and damage is exacerbated. This can be observed in the particularly high IRR in the LDB+1400W group coupled with poor markers of function and high levels of urinary NO and isoprostane.

5.3.1.3 Part 3

RBF during the initial 30 minutes of reperfusion was statistically higher in the SNP supplemented groups when compared to the 18hr control group, but no different to the LDB group. This finding suggests that NO donation induces a rapid vasodilatation

that reduces IRR and improves RBF. This effect on RBF is demonstrated to be equivocal to the benefits of leukocyte depletion, but not synergistic with them.

During the period 30 – 180 minutes post-reperfusion the initial improvement in RBF is maintained, with RBF in the LDB, WB+SNP and LDB+SNP groups being significantly higher than the 18hr control group. There was observed to be no synergistic benefit on RBF of leukocyte depletion and SNP supplementation together when compared to LDB only and SNP supplemented only groups.

In addition to NO dependent vasodilatation it is well established that NO donors potently inhibit leukocyte and platelet endothelial adhesion [214]. NO scavenges superoxide, thus removing a potent stimulator for leukocyte adhesion [215, 216]. The inhibition of leukocyte adhesion to the injured endothelium would reduce the degree of no-reflow phenomenon and leukocyte infiltration, giving similar effects to leukocyte depletion itself.

5.3.2 Creatinine Clearance

The CrCl offers a simple method by which the glomerular filtration rate can be measured. Unfortunately, CrCl can be skewed by up to 60%, in the damaged kidney, by tubular secretion of creatinine [217, 218]. Despite this CrCl has been shown to correlate with inulin-clearance GFR [219].

5.3.2.1 Part 1.

CrCl was markedly significantly higher in the 2hr control group compared to the 18hr control and LDB groups for all time points following reperfusion. Similarly the CrCl was

higher in the LDB group compared to the 18hr control group at all time points. Intra-group variation in CrCl at different time-points was negligible. Although the renal tubule secretes creatinine, the CrCl is more specifically a marker of glomerular function. These data thus demonstrate that glomerular function is initially much better in the short CIT group compared to the other groups and remains better throughout reperfusion. Moreover these data also show that there is little improvement in CrCl in any group throughout reperfusion. Importantly, despite RBF being higher in the LDB group than the 2hr control group, CrCl was significantly lower. This demonstrates that the insult of a prolonged CIT has a deleterious impact on CrCl that improved RBF is unable to reverse after 180 minutes of reperfusion.

5.3.2.2 Part 2.

Despite a lower AUC RBF during 5-30 minutes of reperfusion, the CrCl in the WB+1400W group was significantly higher than that in the 18hr control group. This trend was maintained throughout the reperfusion period. This finding implies that, despite initially poor RBF, glomerular damage is restricted in the WB+1400W group. Inhibition of iNOS results in reduced superoxide and NO formation and subsequently reduced peroxynitrite production as discussed below. Peroxynitrite has a multitude of deleterious effects upon post-ischaemic tissues [220].

The CrCl in the LDB+1400W group was negligible throughout reperfusion and is consistent with the massive reduction in RBF and ongoing ischaemic damage suggested in the haemodynamics section of this discussion.

5.3.2.3 Part 3.

The CrCl at 60 minutes post-reperfusion was statistically higher in the LDB, WB+SNP and LDB+SNP groups when compared to the 18hr control group. This finding is not surprising given the statistically higher RBF and lower IRR observed during initial reperfusion.

Beyond 60 minutes of reperfusion CrCl in the SNP supplemented group falls, whereas in the LDB group it is maintained. Statistically, the CrCl in the WB+SNP group is lower than in the LDB and LDB+SNP groups at both 120 and 180 minutes post-reperfusion. The reduction in CrCl beyond 60 minutes of reperfusion in the SNP supplemented groups is thus not related to RBF.

Typically there are polar views as to whether the effects of NO in reperfusion injury are beneficial or detrimental. Recent evidence suggests that the differing effects of NO are temporal and superoxide dependent. Early in reperfusion NO dependent vasodilatation and inhibition of leukocyte adhesion leads to improvement in RBF with a consequent improvement in CrCl. However, as reperfusion continues the degree of iNOS expression increases and hence the levels of superoxide also increase [210]. High concentrations of superoxide in association with endogenously produced NO and NO supplementation leads to an abundance of peroxynitrite [221]. Excessive peroxynitrite is a potent inducer of cell damage and death, leading to worsening glomerular function and so reduction in CrCl [220].

5.3.3 Urine output

5.3.3.1 Part 1.

Urine output was found to be significantly higher at all time points in the 2hr control and LDB groups when compared with the 18hr control group. Although severe oliguria or anuria are specific indicators of acute renal failure, the urine output *per se* is a poor marker of renal function, being neither sensitive nor specific [222].

5.3.3.2 Part 2.

The urine output results mirrored those of the CrCl.

There was no statistical difference between urine output between the LDB and WB+1400W groups. Initially, there was no statistical difference in urine output between the 18hr control and WB+1400W groups, however at the end of the reperfusion period total urine output was found to be statistically higher in the WB+1400W group.

The urine output in the LDB+1400W group was significantly lower than all the other groups.

5.3.3.3 Part 3.

Urine output mirrored the CrCl results. Urine output was initially statistically higher in the LDB, WB+SNP and LDB+SNP groups. At 150 and 180 minutes of reperfusion the urine output in the WB+SNP group was significantly lower than that in the LDB group, and lower than the LDB+SNP group at 180 minutes.

The initial high urine output in the SNP supplemented groups is representative of the improved RBF observed in these groups secondary to NO mediated vasodilatation. By

120 minutes of reperfusion the RBF in the LDB group converges with that in the WB+SNP and LDB+SNP groups, resulting in improved urine output. Potentially increased levels of peroxynitrite secondary to the aforementioned high availability of NO and superoxide in the SNP supplemented groups results in the reduction in urine output in these groups. The absence of leukocytes and platelets, and thus leukocyte/platelet eNOS derived NO/superoxide, could ameliorate the oxidative stress in the LDB+SNP group, explaining the higher urine output when compared to the WB+SNP group.

5.3.4 Fractional excretion of sodium

FE_{Na} is a marker of tubular function. Clinically the FE_{Na} has been shown to have a specificity and sensitivity of 75% and 78% respectively for acute kidney injury [223]. Experimentally, the FE_{Na} *in vivo* and during extra-corporeal perfusion have been shown to be equivocal in kidneys subjected to similar insults [224]. FE_{Na} has been used extensively in experimental models of ischaemic/reperfusion injury to assess tubular function [225, 226].

5.3.4.1 Part One

FE_{Na} was statistically better in the 2hr control group compared to the 18hr control and LDB groups. Statistically there was no difference in FE_{Na} between the 18hr control and LDB groups, although FE_{Na} was lower in the 18hr control group. All groups demonstrated an improvement in FE_{Na} throughout the reperfusion period, though this was most marked in the 2hr control group. FE_{Na} is an indicator of tubular function; these results demonstrate, therefore, that the 2hr control group has the best initial

tubular function. The finding that the FE_{Na} in the 18hr control and LDB groups did not diverge during the reperfusion period indicates that leukocyte depletion and superior RBF alone do not improve the rate of tubular functional recovery.

5.3.4.2 Part Two

The FE_{Na} results concurred with other functional data that function was maintained to a higher degree in the WB+1400W group when compared to the 18hr control and LDB groups despite initial poor RBF, though this did not reach statistical significance. The severity of injury and anuria observed in the LDB+1400W group precluded statistical analysis of FE_{Na}.

5.3.4.3 Part Three

The FE_{Na} was better in the LDB, WB+SNP and LDB+SNP groups when compared with the 18hr control group, though this did not reach statistical significance. The FE_{Na} was lowest in the SNP supplemented groups after 60 minutes of reperfusion and continued to reduce throughout the remainder of the reperfusion period. This observation countered the finding that CrCl reduced in the SNP supplemented groups after 60 minutes of reperfusion. This may a preservation of tubular function despite increasing glomerular damage in the SNP supplemented groups and warrants further delineation.

5.3.5 Hydrogen ion concentration

5.3.5.1 Part One

The pH demonstrated no difference in pre-perfusion samples taken from the primed circuit. After 60 minutes of reperfusion the pH in all groups had fallen. Statistically the

pH in the 2hr control group was higher than in both the 18hr control and LDB groups. At the end of reperfusion the pH in the 2hr control group had retuned to pre-perfusion levels and remained significantly higher than both the 18hr control and LDB groups. There was no significant difference in pH between the 18hr control and LDB groups at 60 or 180 minutes of reperfusion. Interestingly after 180 minute of reperfusion the pH in the LDB group demonstrated a resolving acidosis whereas that in the 18hr control group continued to increase.

In this model the severity of the ischaemic insult leads to induction of an anaerobic metabolism and production of a lactic acidosis [227]. Thus the degree of acidosis and the speed at which it is reversed is indicative of both the severity of the initial ischaemic insult and the ability of reperfusion to restore normal aerobic metabolism.

5.3.5.2 Part Two

Hydrogen ion concentration results showed a worsening acidosis in the 1400W supplemented groups, which was equivalent to that observed in the 18hr control group. This finding confirmed an initial ischaemic lactic acidosis that did not recover during the reperfusion period.

5.3.5.3 Part Three

The pH in the SNP supplemented groups increased after 60 and 180 minutes of reperfusion. This result implies that despite improvement in RBF there is ongoing acidosis in the WB+SNP and LDB+SNP groups.

5.3.6 Oxygen consumption

5.3.6.1 Part One

Oxygen consumption was initially significantly higher in the 2hr control and LDB groups when compared to the 18hr control group. At the end of the reperfusion period the oxygen consumption was significantly higher in the LDB group compared to both the 2hr control and 18hr control groups. These findings represented an increased in oxygen consumption in the 18hr control and LDB groups throughout the reperfusion period, but a relatively static consumption in the 2hr control group.

The oxygen consumption of the normal kidney is dependent upon RBF and, beyond basal metabolic requirements, is directly proportional to the tubular reabsorption of sodium [228, 229]. The oxygen consumption observed in this model is thus dependent upon restitution of blood flow and the restoration of normal aerobic metabolism in the renal tubule, both of which are dependent upon the degree of initial ischaemia. The higher oxygen consumption in the 2hr control and LDB groups after 60 minutes of reperfusion is reflective of higher blood flow in these groups and, in the case of the 2hr control group, the less severe ischaemic insult. The improvement in oxygen consumption observed in the 18hr control and LDB groups, absent in the 2hr control group, may be explained by the continued improvement in RBF between 60 and 180 minutes observed in these groups. The improvement in oxygen consumption in the LDB group may also be related to improved sodium reabsorption, as reflected in the $FE_{Na.}$ It is worthy of note that the oxygen consumption levels observed are significantly below what would be expected in a non-injured kidney. It has been shown experimentally that oxygen consumption is significantly reduced for many days post-

ischaemic injury [230]. This finding is due to a combination of impaired renal function due to ischaemic injury, observed as reduced GFR and FE_{Na}, and impaired oxygen uptake due to depletion of ATP and cell death, as expounded upon in the introduction chapter.

5.3.6.2 Part Two

The oxygen consumption levels observed at 180 minutes post-reperfusion were consistent with the RBF. The higher than expected oxygen consumption in the WB+1400W group at 60 minutes reperfusion infers, as does the CrCl, that metabolic function in the tubule is maintained, in spite of reduced RBF. This may be due to reduced oxidative damage as a result of iNOS inhibition and subsequent reduced superoxide and peroxynitrite formation.

5.3.6.3 Part Three

Oxygen consumption was statistically higher in the LDB, WB+SNP and LDB+SNP groups when compared to the 18hr control group. The oxygen consumption in the SNP supplemented groups declined, though not significantly, throughout the reperfusion period. Reduction in the metabolic requirement of the SNP supplemented kidneys at 180 minutes of reperfusion implies ongoing damage to the kidney associated with augmented peroxynitrite production.

5.3.7 Urinary quantification of NO

It is well established that NO is produced by the vascular endothelium both constitutively and in response to injury. It has also been shown experimentally that the renal tubule and glomerulus have the ability to produce NO in response to injury [59,

60, 231]. In humans higher levels of urinary NO have been correlated to proximal tubular injury [232, 233]. Although in these studies the insult was endotoxaemic rather than ischaemic.

Measurement of plasma levels of NO could quantify the degree of endothelial injury but would not detect alterations in NO production in the glomerulus or renal tubule in response to injury. As discussed in the introduction the renal tubule is particularly susceptible to acute ischaemic injury. Furthermore there is a wealth of evidence that extra-corporeal perfusion *per se* can cause endothelial activation and alter the production of NO [234-237].

Urinary quantification of NO was therefore chosen as a preferable assay to plasma quantification in this study.

5.3.7.1 Part One

There was no significant difference between NO levels in the 2hr control, 18hr control and LDB groups after 60 minutes of reperfusion. After 180 minutes of reperfusion the NO levels in the 2hr control group were significantly lower when compared to the 18hr control and LDB groups. This finding represents recovery of tubular and glomerular function in the 2hr control group when compared to the 18hr control and LDB groups. This is reinforced by the CrCl and FE_{Na} data.

5.3.7.2 Part Two

Urinary NO levels were significantly higher in the WB+1400W and LDB+1400W groups compared to the 18hr control and LDB groups after 60 minutes of reperfusion. This finding was unsurprising given the reduction in RBF encountered during the initial 60

minutes of reperfusion in the 1400W supplanted groups and the consequential ischaemic injury sustained by the kidney.

Urinary levels of NO after 180 minutes of reperfusion remained essentially the same as after 60 minutes of reperfusion. A statistically higher NO level in the LDB+1400W group would be expected as a sequela of persistent low RBF, however the observation that NO levels remained high in the WB+1400W group, despite a marked improvement in RBF, were unexpected. Studies have shown that iNOS induction in the kidney, following ischaemia, is almost immediate and remains elevated beyond 180 minutes of reperfusion [210]. Thus, despite improved RBF, NO levels may still be elevated because of persistent iNOS expression.

5.3.7.3 Part Three

The finding of equivalent urinary NO levels between the 18hr control, LDB and LDB+SNP groups at both 60 and 180 minutes of reperfusion are consistent with CrCl and FE_{Na} data. Although leukocyte depletion and SNP supplementation augment haemodynamic parameters they have little effect on functional markers.

The urinary NO levels observed in the WB+SNP group, statistically higher than the 18hr control, LDB and LDB+SNP groups at 180 minutes of reperfusion, are consistent with SNP supplementation and increasing induction of iNOS and production of peroxynitrite towards the end of reperfusion. This result is consistent with the declining CrCl in the WB+SNP group, but not with maintained FE_{Na}.

5.3.8 Urinary quantification of 8-isoprostane

Isoprostanes are a family of eicosanoids produced by the oxidation of tissue phospholipids by ROS. 8-isoprostane or, more accurately, 8-iso-$PGF_{2\alpha}$ belongs to the F_2 family of isoprostanes. The precise mechanisms by which the F_2-isoprostanes are formed are still debated [238, 239]. Essentially, arachidonic acid undergoes oxidation by ROS to form endoperoxide. Endoperoxide is then reduced to form the stable F_2-isoprostanes, further oxidation may occur forming other isoprostanes [240].

F_2-isoprostanes are expressed constitutively in all bodily fluids [241]. Recently, a large study compared the sensitivity of different markers of lipid peroxidation in response to oxidative stress. This study demonstrated F_2-isoprostane quantification in both urine and plasma as the superior marker of oxidative stress [102]. 8-isoprostane, specifically, has been shown to be a urinary marker for oxidative stress [242].

Studies have demonstrated that metabolized-8-isoprostane levels increase in plasma and urine in a closely correlated manner [243]. There is, however, poor correlation between plasma and urine levels of unmetabolised-8-isoprostane [243]. This finding highlights the importance of local production of 8-isoprostane in the kidney, and also that local production is only reflected in the urine levels of 8-isoprostane and not plasma levels.

Urinary quantification of 8-isoprostane was therefore chosen as a more accurate marker of oxidative stress in this model compared to plasma measurement.

5.3.8.1 Part 1

Isoprostane levels were significantly lower in the 2hr control group when compared to the 18hr control group. Although, the levels in the LDB group were higher than in the

2hr control group this did not reach significance. This finding indicates the lower degree of oxidative stress in the 2hr control group, a finding that would be expected given the shorter SCS time. The levels in the LDB group, although not statistically significant, do demonstrate that perfusion with LDB, as a consequence of improved RBF and amelioration of leukocyte dependent inflammatory changes, does appear to reduce oxidative stress.

5.3.8.2 Part 2

Urinary 8-isoprostane levels were higher in the LDB+1400W group, though sample size precluded statistical analysis. This finding was unsurprising given the persistently low RBF and thus the continuation of the ischaemic insult.

Induction of iNOS expression in the glomerulus and tubule, explaining the elevated NO levels in this group, would lead to the conclusion that 8-isoprostane levels would also be elevated. Counter-intuitively the 8-isoprostane levels in the WB+1400W group were statistically lower than in the 18hr control, LDB and LDB+1400W groups.

Urinary 8-Isoprostane quantification measures global oxidative stress, only a portion of which will be iNOS dependent. Furthermore, urinary isoprostane levels quantify the production of isoprostanes from glomerular and tubular sources and also from the endothelium. Endothelial iNOS expression, and so endothelial oxidative stress, is attenuated due to iNOS inhibition in the WB+1400W group, but not in the 18hr control and LDB groups. Glomerular and tubular iNOS expression are however not attenuated by 1400W supplementation, such that urinary NO levels may be elevated despite a reduction in global oxidative stress as measured by 8-isoprostane levels.

5.3.8.3 Part 3

The was no statistical difference in 8-isoprostane levels between the 18hr control, LDB, WB+SNP and LDB+SNP groups. Expression of iNOS and subsequent NO/superoxide production, coupled with SNP supplementation, increases deleterious peroxynitrite formation and would be expected to increase oxidative stress, and consequently 8-isoprostane expression, after 180 minutes of reperfusion. The finding that there was no difference in 8-isoprostane expression was therefore surprising. The absence of leukocyte and platelet derived iNOS in the LDB+SNP group could explain an overall reduction in oxidative stress and would be consistent with the functional data.

Despite a reduction in function in the WB+SNP group, due to enhanced peroxynitrite production, the 8-isoprostane levels are similar to those in the LDB+SNP group. This result remains unexplained, especially in view of the markedly elevated urinary NO levels observed in this group.

6 Conclusion

Reperfusion injury is a complex multifaceted entity involving many pathophysiological pathways. The kidney is a heterogenous organ consisting of a number of diverse tissues, such as endothelium, glomeruli and tubules, all of which exhibit different physiological characteristics. Invariably these constituent tissues will respond differently to ischaemic and reperfusion injury.

This study demonstrated that, irrelevant of the degree of injury, renal haemodynamics show a significant variation within the first three hours of reperfusion.

6.1 Part 1

Kidneys subjected to 18 hours cold ischaemia demonstrated significantly worse blood flow than those undergoing only 2 hours of cold ischaemia. However, when these kidneys were perfused with leukocyte depleted blood the flow characteristics were equivalent to the 2hr control group. This improved blood flow was attributable to abrogation of no-reflow phenomenon, whereby the adherence of leukocytes to the injured endothelium restricts RBF. The functional improvement in the LDB group was not as marked as the haemodynamic improvement. Functional parameters in the LDB group were generally intermediary between the function seen in the 2hr control group and that seen in the 18hr control group.

The absence of infiltrating leukocytes explains the improved function when compared to the 18hr control group. The fact that function was not restored to the levels observed in the 2hr cold stored group is indicative of the degree of ischaemic injury

imparted by 18hrs SCS. Furthermore these results demonstrate that restoration of tubular and glomerular function is not just dependent upon RBF or leukocyte dependent factors. The functional results were corroborated by quantification of urinary damage markers.

6.2 Part 2

1400W exhibits high affinity and specificity for iNOS. The reperfusion of kidneys with LDB and WB supplemented with 1400W gave some unexpected results. Poor RBF was observed during the initial 30 minutes of reperfusion in both 1400W supplemented groups. This was consistent with reduced NO production by the endothelium and circulating leukocytes in response to inhibition of iNOS, with a subsequent impaired vasodilatation. After 30 minutes blood flow improved in the WB+1400W group but remained poor in the LDB+1400W group. This can be explained by the up-regulation of eNOS on circulating leukocytes in the WB+1400W group, but not in the LDB+1400W group. The observed poor blood flow in the LDB+1400W group suggests that NO mediated vasodilatation is pivotal in initial reperfusion.

Despite the absence of the no-reflow phenomenon the severity of the initial ischaemic insult and the absence of sufficient NO result in the observed poor RBF throughout reperfusion in the LDB+1400W group. The WB+1400W group demonstrated generally improved functional parameters when compared to the 18hr control group. This was explained by reduced oxidative stress and superoxide formation secondary to iNOS blockade. Urinary nitric oxide levels were far higher in the 1400W supplemented groups at 60 minutes post-reperfusion, as a result of reduced RBF. The urinary NO

level remained high in the LDB+1400W group because of consistently poor RBF and ongoing damage. The unexpected higher NO levels observed at 180 minutes in the WB+1400W group represent persistent iNOS activity in the renal tubule despite improved RBF and function. The low 8-isoprostane values observed in the WB+1400W group was consistent with reduced oxidative stress subsequent to iNOS inhibition.

6.3 Part 3

Haemodynamically, SNP supplementation improved RBF to the level observed in the 2hr control group. This demonstrates that NO mediated vasodilatation, at least in the immediate reperfusion period, is vital for adequate blood flow restoration, and is more important than abrogation of leukocyte adhesion and the no-reflow phenomenon during this phase. Statistically, this trend continued to the end of the perfusion period, though the improvement in flow plateaued when compared to the continual increase observed in the LDB group. Functional parameter improvement was variable in the SNP supplemented groups, for example initial CrCl was improved but later during reperfusion returned to control levels, whereas FE_{Na} was improved throughout the reperfusion period. This was related to the initial benefit of NO mediated vasodilatation on blood flow, but the later adverse effects of enhanced peroxynitrite formation due to higher NO levels and the increased expression of NOS. The high urinary NO levels observed after 180 minutes of reperfusion are consistent with the reduced CrCl, both representing glomerular injury and release of NO. This finding would not account for the improvement in FE_{Na}, which remains a somewhat unexplained result. Urinary Isoprostane levels were essentially equivalent between the

groups. The disparity between isoprostane and NO levels in the SNP supplemented groups cannot be readily explained.

6.4 Proof of hypotheses

This study confirmed the first hypothesis that leukocyte depletion did influence initial graft function. RBF was significantly improved by leukocyte depletion, and to a lesser extent functional parameters were also improved.

The second hypothesis of this study was also confirmed. Specific inhibition of iNOS showed a marked deterioration in RBF, followed by an improvement in functional parameters over controls in the WB+1400W group. NO supplementation demonstrated a marked improvement in RBF, but a variable effect upon functional parameters, with an initial improvement but later deterioration, when compared to controls. In summary, supplementation of NO showed early improvement in function but later deterioration; whilst inhibition of iNOS demonstrated early deterioration in function, but later improvement.

6.5 Future directions

6.5.1 Warm ischaemic times

Clinical practice is evolving to utilise donor kidneys with longer and longer warm ischaemic times. The NPS model could thus be used to assess kidneys with warm ischaemic times in excess of the 25 min WI used in this study.

6.5.2 Leukocyte depletion

Specific investigation of the effects of depletion upon 'no-reflow' and leukocyte infiltration, using, for example, immunohistochemistry would delineate the underlying pathophysiology of the effects noted in this study. Furthermore, analysis of non-mechanical methods of leukocyte depletion, with monoclonal/polyclonal antibodies, which may be more clinically relevant, would be worthwhile.

6.5.3 Platelet depletion

The role of platelets in reperfusion injury is well established. There is however a relative paucity of research concerned with the non-haemostatic properties of platelets in RI. Experiments using the NPS model with specific depletion of platelets, either mechanically or chemically, would further define potentially pivotal non-haemostatic roles.

6.5.4 Nitric oxide modulation

Further investigation of the effects of NO supplementation and iNOS inhibition at different time-points post-reperfusion would aid the characterisation of the precise role of NO in real-time reperfusion injury in the kidney.

7 Abbreviations

1400W	N-(3-(Aminomethyl)Benzyl)Acetamidine
A/VBG	Arterial/Venous Blood Gas
AChE	Acetylcholinesterase
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
AP	Arterial Pressure
AP_{aO_2}	Arterial Partial Pressure of Oxygen
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BH_4	Tetrahydrobiopterin
BSD	Brain-stem Death
cCrCl	Corrected creatinine clearance
CIT	Cold Ischaemic Time/s
CPDA-1	Citrate Phosphate Dextrose Adenine - 1
DBD	Donation after Brain Death
DBTLC	Double-Balloon Triple Lumen
DCD	Donation after Cardiac Death
DGF	Delayed Graft Function
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
ESRD	End-stage Renal Disease

FBC	Full Blood Count
FE_{Na}	Fractional Excretion of Sodium
GTP	Guanosine Triphosphate
HD	Haemodialysis
HMP	Hypothermic Machine Perfusion
HO-1	Haemoxygenase-1
ICAM-1	Intracellular Adhesion Molecule-1
IL	Interleukin
INFγ	Interferon-γ
iNOS	Inducible Nitric Oxide Synthase
IRR	Intra-renal Resistance
K-W test	Kruskal-Wallis ANOVA test
LD	Live donor/s
LDB	Leukocyte Depleted Blood
LFA-1	Lymphocyte Function Antigen-1
L-NAME	N^{w}-Nitro-L-Arginine Methyl Ester
L-NMMA	N-Methyl-L-Arginine Acetate
MAC	Membrane Attack Complex
Mac-1	Macrophage-1 Antigen
MAP	Mean Arterial Blood Pressure
MCP-1	Monocyte Chemotactic Protein-1
MPTP	Mitochondrial Permeability Transition Pore
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide-Adenine-Dinucleotide Phosphate

NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NP	Normothermic Preservation
NPS	Normothermic Perfusion System
PAF	Platelet Activating Factor
P_{Cr}	Plasma creatinine
PD	Peritoneal Dialysis
PLA_2	Phospholipase A_2
P_{Na}	Plasma Sodium
PNF	Primary Non-function
ppm	Patients Per Million
RB	Renal Biopsy
RBF	Renal Blood Flow
RI	Reperfusion Injury
ROS	Reactive Oxygen Species
RRT	Renal Replacement Therapy
SCS	Static Cold Storage
SD	Standard Deviation
SFP/U	Snap Frozen Blood/Urine
SNP	Sodium Nitroprusside
TEM	Trans-endothelial migration
TGF-β	Transforming Growth Factor-β
TNFα	Tumour Necrosis Factor-α
UC	Urine Chemistry

U_{Cr}	Urinary creatinine
uCrCl	Uncorrected creatinine clearance
UE	Urea and Electrolytes
U_{Na}	Urinary Sodium
VEGF	Vascular Endothelial Growth Factor
VO_2	Oxygen Consumption
VP_{aO_2}	Venous Partial Pressure of Oxygen
WB	Whole Blood
WIT	Warm Ischaemic Time

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