Normothermic Perfusion in Renal Transplantation

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by

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Abstract

One of the main causes of early graft dysfunction in kidney transplantation is ischaemia reperfusion (I/R) injury. This unavoidable event occurs immediately when oxygenated blood is re-introduced into the transplanted kidney. Its severity is influenced by many predetermined factors. However, the condition under which an organ is preserved has a significant bearing on the outcome. Traditionally, organs are preserved using hypothermic temperatures, to reduce metabolism and the requirement for oxygen. Although practical and simple, hypothermic conditions are not entirely favourable and over time the depletion of energy substrates causes substantial cellular injury. This is thought to be a particular problem in kidneys from marginal donors, which are often exposed to a period of warm ischaemia (WI) prior to retrieval.

The aim of this thesis was to determine the effects of varying degrees of the combined insults of warm and cold ischaemic injury and to develop a technique of normothermic perfusion (NP) to reduce ischaemic injury. The effects were assessed using *ex-vivo* and *in-vivo* porcine kidney models before translation of NP into clinical practice for marginal donor kidneys.

This research demonstrated that prolonging the hypothermic preservation period after a minimal and a substantial degree of WI injury increased the severity of acute I/R injury and graft dysfunction. A short period of NP after hypothermic preservation was able to resuscitate the kidney, replenish ATP and reverse some of the detrimental effects of cold ischaemic injury. When translated into an autotransplant model, NP was found to be a safe and feasible method of preservation. NP was then adapted for use in clinical practice for kidneys from marginal donors. This first in man clinical series of 15 cases has demonstrated the safety and feasibility of NP for marginal kidneys. Although, the high rate of initial graft function is notable, further comparative studies are required to assess the effects on delayed graft function.

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Original Publications

The following work has been published from this thesis

Original manuscripts

1 Hosgood SA, Bagul A, Yang B, Nicholson ML. The relative effects of warm and cold ischaemic injury in an experimental model of nonheartbeating donor kidneys. *Transplantation.* 2008; 85 (1):88-92.

2 Hosgood SA, Hunter JP, Nicholson ML. Early urinary biomarkers of warm and cold ischaemic injury in an experimental kidney model. *Journal of Surgical Research* 2012.174 (2): e85-90.

3 Bagul A, Hosgood SA, Kaushik M, Kay MD, Waller HL, Nicholson ML. Experimental renal preservation by normothermic resuscitation perfusion with autologous blood. *British Journal of Surgery*. 2008.95(1):111-8**.**

4 Hosgood SA, Barlow AD, Yates PJ, Snoeijs MG, van Heurn EL, Nicholson ML. A pilot study assessing the feasibility of a short period of normothermic preservation in an experimental model of non heart beating donor kidneys. *Journal of Surgical Research*. 2011; (1): 283-90.

5 Hosgood SA, Nicholson ML. First in man renal transplantation after *ex vivo* normothermic perfusion. *Transplantation.* 2011. 92 (7):735-8.

Review articles

6 Hosgood SA, Nicholson ML. Normothermic kidney preservation. *Current Opinion in Organ Transplantation*. 2011. 16 (2):169-73. (Review).

7 Hosgood SA Nicholson HF, Nicholson ML, Oxygenated kidney preservation techniques. *Transplantation* 2012; 93(5):455-9. Review.

Prizes

The ‘Clinical Medawar Medal and Prize’. The British Transplant Society (BTS) February 2012. ‘The first clinical series of normothermic perfusion in marginal donor kidney transplantation’.

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List of Abbreviations

AP-1	Activator protein 1
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
Ala-AP	Alanine aminopeptidase
ALT	Alanine transaminase
AST	Asparatate transaminase
ATM	Atmospheres
ATN	Acute tubular necrosis
ATP	Adenosine triphosphate
AUC	Area under the curve
CAPD	Continuous ambulatory peritoneal dialysis
CI	Cold ischaemia
CMV	Cytomegalovirus
COPD	Chronic obstructive pulmonary disease
CPDA-1	Citrate phosphate dextrose adenine
Cr	Creatinine
CrCl	Creatinine clearance
CS	Cold storage
DCD	Donation after circulatory death
DDFPe	Dodecafluoropentane
DBD	Donation after brain death
DGF	Delayed graft function
ECD	Extended criteria donor
ECMO	Extracorporeal membrane oxygenation
ELISA	Enzyme linked immunosorbant assay
E-selectin	Endothelial leukocyte adhesion molecule
ESRF	End stage renal failure
ET-1	Endothelin 1
EVLP	*Ex-vivo* lung perfusion

Fr Na+	Fractional excretion of sodium
GGT	Gamma-glutamyltransferase
GFR	Glomerular filtration rate
GST	Glutathione *S*-transferase
HD	Haemodialysis
HES	Hydroxyethyl starch
H-FABP	Heart-type fatty acid binding protein
HLA	Human leukocyte antigen
HMP	Hypothermic machine perfusion
HOC	Hyperosmolar citrate
HSP	Heat shock protein
HTK	Histidine-tryptophan-ketoglurate
ICAM-1	Intercellular adhesion molecule-1
IGF	Initial graft function
IL-1ß	Interleukin-1ß
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IRR	Intra-renal resistance
I/R	Ischaemia reperfusion
KIM-1	Kidney injury molecule 1
LDB	Leukocyte depleted blood
LDH	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen-1
L-selectin	Leukocyte endothelial adhesion molecule
M101	Hemarina-M101
MAC-1	Macrophage-1 antigen
MAP	Mean arterial pressure
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases

MnSOD	Manganese superoxide dismutase
MPO	Myeloperoxidase
NAG	N-acetyl-β-D-glucosaminidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NFkB	Nuclear factor-kappa B
NGAL	Neutrophil gelatinase-associated lipocalin
NHBD	Non heart beating donor
NIPAG	New interventional procedures advisory group
NP	Normothermic perfusion
OCS	Organ care system
RBF	Renal blood flow
PBS	Phosphate buffered sucrose
PEG	Polyethylene glycol
P-selectin	Granule membrane protein 140
PFI	Perfusion flow index
PHP	Pyridoxalated haemoglobin polyoxyethylene
PFC	Perfluorocarbon
PNF	Primary non function
PRBC	Packed red blood cells
SGF	Slow graft function
T_{crit}	Time to critical level
T_{max}	Time to maximum level
TNFα	Tumour necrosis factor alpha
TLM	Two layer method
UW	University of Wisconsin
VCAM-1	Vascular adhesion molecule-1
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor
WI	Warm ischaemia

Chapter 1

Introduction

1.11 Introduction

One of the fundamental requirements of transplantation is the ability to preserve an organ outside the body from the time of retrieval until transplantation. Since the 1970s organ preservation has relied on hypothermic conditions to reduce metabolism and maintain tissue viability for an extended period of time. This allows the organ to be allocated nationally to the most suitable and best immunologically matched recipient. However, the boundaries of kidney transplantation are increasingly being extended and there is more reliance on the use of so called marginal donors to accommodate the increasing demand. These kidneys present with higher incidences of early graft dysfunction and reduced survival compared to the traditional donor sources. The inferior outcome of these kidneys may be influenced by the hypothermic temperatures during preservation. This raises the question of whether hypothermic preservation is the most suitable method of preservation. An alternative is to use normothermic temperatures, to support circulation and metabolism and prevent cellular deterioration. Previous attempts at normothermic preservation in past centuries were relatively unsuccessful. However, with improved technology, normothermic preservation may now be a more realistic concept for modern day kidney transplantation.

1.12 Kidney transplantation

Chronic kidney disease is a major health problem that affects approximately 3 million people in the UK (1). Approximately 19,000 of those people have end stage renal failure (ESRF) and are treated with dialysis. Kidney transplantation is considered the best treatment for ESRF with longer life expectancy and superior quality of life compared to dialysis therapy (2-4). However, a major constraint to transplantation is the lack of suitable organ donors. During the past decade, the number of brain death donors has declined in the UK due to a reduction in deaths from trauma and changes in neurosurgical practice. In this time period, the number of people waiting for a kidney has increased by approximately 50% and currently only one third of those on the waiting list will receive a transplant each year (1). To increase the number of available organs there has been an initiative to use alternative sources of organ donors, in addition to kidneys from the traditional living and brain death donors (5-8).

1.13 Donation after circulatory death donors

Donation after circulatory death (DCD) formally known as non heart beating (NHB) donors are donors from which the organs are retrieved after the heart has stopped. These organs are regarded as marginal organs due to the warm ischaemic (WI) insult that they receive before the onset of preservation. This WI interval causes a degree of injury that can lead to irreversible damage, resulting in an unfavourable outcome after transplantation. NHB donors were used in early transplantation however with the introduction of brain death donors in 1968 their use fell from favour. It wasn't until 1995, after a formal workshop held in Maastricht to address the growing demand for organs, that their use was again encouraged. The aim of the workshop was to establish a uniform criteria by which NHB donor organs could be identified and address the ethical issues involved, thereby encouraging the establishment of NHB programmes around the world. Four classifications of NHB donors were categorised depending on the circumstances of death and when the organs were retrieved (9, 10). These criteria were later modified to include controlled and uncontrolled donors (Table 1.13). Since this time the number of NHB donors, or now the more commonly accepted term DCD donors, used for kidney transplantation has increased by almost 90% in the UK (1).

Category	Definition	Type
1	Dead on arrival	Uncontrolled
2	Unsuccessful resuscitation	Uncontrolled
3	Awaiting cardiac arrest	Controlled
4	Cardiac arrest while brain death	Controlled/uncontrolled

Table 1.13 Maastricht categories of donation after circulatory death donors.

1.14 Uncontrolled/controlled donation after circulatory death donors

Maastricht type 1 and 2 donors are patients who have died suddenly from a cardiac event or trauma and therefore are usually based in the Accident & Emergency department. After a failed resuscitation, the patient is pronounced dead and a 5 minute 'hands off' period allowed to lapse. The organs are perfused *in-situ* through aortic cannulas inserted through the femoral artery (11).

Maastricht type 3 and 4 are patients who are based on an intensive care unit after a severe brain injury. There may still be brain function and the patient will maintain spontaneous ventilation and therefore not meet the criteria for brain stem death. Under controlled conditions with no possibility of recovery withdrawal of treatment is planned. After the cessation of the heartbeat the patient is transferred to the operating theatre and the kidneys retrieved after *in-situ* cooling. In the uncontrolled situation an

unexpected cardiac arrest follows brain stem death. The WI time is usually within the region of 15 minutes for controlled donor but can be considerably longer in the uncontrolled situation. In the UK, a significant portion of kidneys are from controlled DCD donors (Maastricht type 3) (12).

1.15 Extended criteria donors

Kidneys from extended criteria donors (ECD) are also increasingly being utilised to meet the shortfall in demand (13). ECD kidneys are defined as any brain dead donor aged ≥ 60 years or over 50 years with ≥ 2 of the following conditions; Hypertension, terminal serum creatinine equal or greater than 130µmol/L or death resulting from an intracranial haemorrhage.

1.16 Clinical outcome

Renal function post transplant is influenced by many factors. However, donor and recipient age, creatinine clearance, history of hypertension, poor human leukocyte antigen (HLA) matching, cause of death, ethnicity and the cold ischaemic time have been described as major determinants of graft survival (14,15). As a result of these factors, kidneys from DCD and ECD donors have reduced kidney function after transplantation ((16-21). Acute kidney injury is typically measured by rates of delayed graft function (DGF). DGF is typically defined as the requirement for dialysis within the first week after transplantation. This is based on a rising serum creatinine,

increasing metabolic instability and reduced urine output. However, it is subject to varying medical judgement and does not directly measure kidney injury (22). Nonetheless, DGF is commonly used to determine early graft function and is associated with complications such as acute rejection, increased fibrosis and the risk of reduced long term graft survival (23, 24). DGF also has a significant economic cost and can prolong hospital stay (20). Rates of DGF typically range from 22% to 84% in DCD kidneys compared to 14% to 40% in brain death donors (7, 8, 25-27). Evidence suggests that the outcome of kidneys from uncontrolled DCDs is poorer when compared to the controlled DCDs with significantly higher rates of DGF, as a response to the longer duration of WI under the uncontrolled situation (15). Nonetheless, a recent study by Hoogland *et al* (28) found no difference in rates of DGF between uncontrolled and controlled DCD transplants (61% vs 56%). However, despite the different donor types, similar WI intervals were recorded in both groups (25 minutes) which may have influenced this outcome.

Kidneys from ECDs have up to a 70% increased risk of graft loss and a higher rate of DGF (13, 25, 29) compared to younger donors. The prognosis is even poorer in DCD kidneys from older donors (over 50 years) with the risk of graft failure rising to 80% (25).

In addition to DGF, a small but significant proportion of kidneys from DCD donors also have primary non function (PNF), this is defined as the kidney never functioning. Rates have been reported to range from 4 to 22% amongst transplant centres over the last 30 years (30, 31). PNF is particularly detrimental as the patient is exposed to

surgery and immunosuppression therapies without benefit. Furthermore, they may become sensitized to donor antigens, reducing the opportunity for future transplants. Despite the high incidence of early graft dysfunction, the impact of DGF on long term graft survival in DCD kidneys does not appear to be as detrimental as in DBD donation (20, 34). Recent evidence from clinical DCD and DBD programmes have reported similar rates of graft survival after 5 and 10 years (26, 28, 32-35). In the series of uncontrolled NHBD kidneys at Leicester from 1992-2003, DGF rates were 84% compared 22% in brain dead donors (32). Nevertheless, the graft survival rates were similar in both groups of patients, 69.3% versus 75.5% at 5 years and 50.3% versus 57.9% at 10 years, respectively.

Although long term graft survival is important and the results from DCD transplantation extremely encouraging, it is still crucial to improve the early graft function. Reducing the rates of DGF and PNF in DCD and ECD kidneys would have a significant benefit to the patients and healthcare system and perhaps prolong graft survival further. The majority of the factors that influence the outcome of DCD and ECD kidneys are unavoidable. However, there is potential to reduce the severity of ischaemic injury to improve graft outcome.

1.2 Ischaemic Injury

1.21Warm and cold ischaemic injury

Ischaemic injury in DCD transplantation occurs in four phases. 1: After cardiac death; warm ischaemic (WI) injury. 2: Under hypothermic conditions during preservation; cold ischaemic (CI) injury. 3: WI injury; during anastomosis of the graft. 4: Upon reperfusion immediately after transplantation; ischaemia reperfusion (I/R) injury. Ischaemic injury is a complex series of events that is triggered by the depletion of energy substrates and hypoxic conditions (36). The depletion of adenosine triphosphate (ATP) due to the inhibition of oxidative metabolism induces a shift to adenosine monophosphate (AMP) as the predominant nucleotide (37). This increases levels of adenosine, inosine and hypoxanthine leading to the formation of lactic acid within the cell. This lowers the intracellular pH causing lysosomal instability with the activation of lytic enzymes (38, 39). The depletion of ATP also reduces a large number of cellular processes. Inactivation of Na+/K+ ATPase pumps allows the accumulation of calcium, sodium and water within the cell causing cellular swelling (39). Fatty acids, lysophospolipids and diacylglycerol also accumulate within the cell.

The binding of transition metals such as iron to their carrier proteins (transferrin, ferritin) is also inhibited which increases the intracellular concentration of free iron (40, 41). This is a strong catalyst for the generation of oxygen free radicals which promotes the production of other free radicals (38) (Figure 1.21).

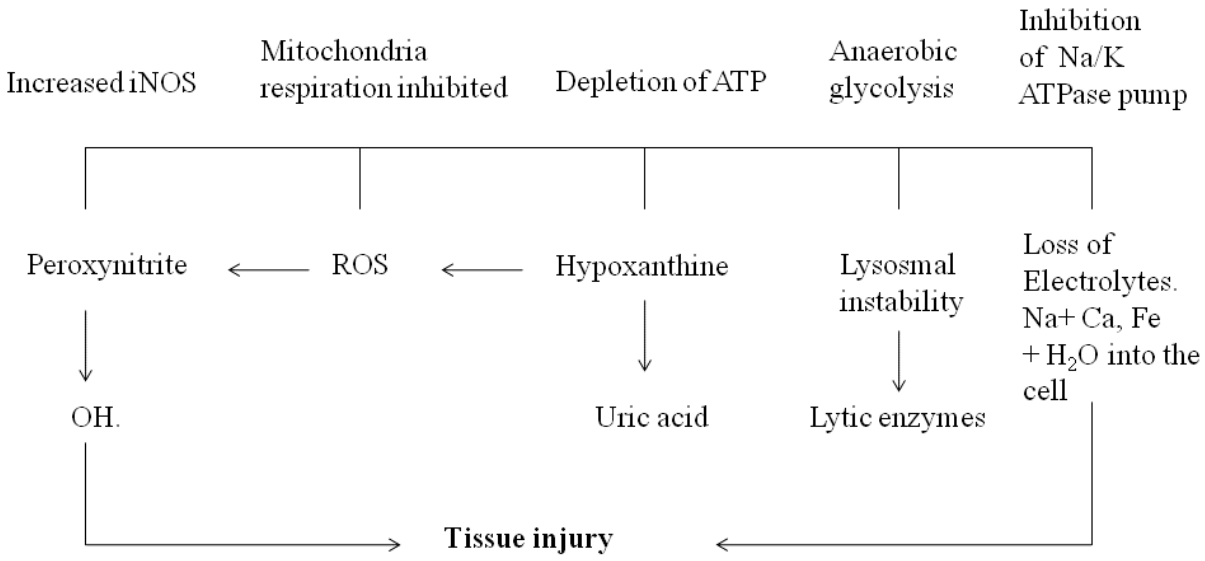


Figure 1.21 A schematic diagram of the effects of warm and cold ischaemic injury. The depletion of ATP leads to dysfunction, the aggregation of many cellular proteins including ion pumps and cytoskeletal and contractile structures. Adensoine, triphosphate (ATP), hydroxyl radical (OH.), inducible nitric oxide (iNOS), reactive oxygen species (ROS).

Hypothermic temperatures act to inhibit the enzymatic processes and there is a 2-3 fold decrease in metabolism for every 10°C reduction in temperature (42, 43). This slows the depletion of ATP and also inhibits the degrading processes (phospholipid hydrolysis). This allows an organ to be stored without oxygen for a significant period of time and therefore, the impact of CI is less severe than WI. Nonetheless, under hypothermic condition the metabolic rate remains at about 10% and therefore, over time the hypoxic conditions cause substantial injury (43). This is particularly detrimental to graft function and survival.

The CI time alone is an independent risk factor for graft failure and is directly associated with DGF (44). It also significantly increases allograft immunogenicity provoking acute and chronic rejection (45, 46). Studies have shown a significant reduction in the rate of DGF when the CI time was reduced. In an analysis of a series of DBD donor transplants, the risk of DGF was found to increase by 23% for every 6 hours of CI (47) and Locke *et al* found that limiting the CI time to less than 12 hours reduced the risk of DGF by 15% (25). Several experimental models also suggest that even after 6 hours of CI significant injury occurs (48, 49) However, other studies have found the CI period to have less of an impact. A multicentre analysis of kidney preservation found that only when the preservation period exceeded 18 hours was the CI time associated with reduced graft survival even in kidneys from ECD and DCD donors (50). Furthermore, Taylor *et al* found no change in the rate of DGF in kidneys from DCD donors when the CI time was reduced from a median of 15 to 12 hours (51). Athough it is difficult to compare these studies there is a general consensus to reduce

the CI time and in the UK the average CI time for DCD and DBD kidneys is now approximately 16-18 hours.

It is thought that the severity of CI injury during hypothermic preservation is enhanced by a period of WI injury prior to retrieval and it is therefore logical to assume that reducing the preservation period would lessen the injury. WI injury in DCD transplantation is an unavoidable event that is difficult to control. However, the impact of different durations of WI and CI injury on kidney injury has yet to be determined.

1.22 Acute kidney injury

The re-introduction of oxygenated blood during transplantation after a period of ischaemia initiates a complex series of actions (Figure 1.22). The downstream effects of ischaemia reperfusion (I/R) injury results in the impairment of blood flow to the kidney and reduced urine output after transplantation (40). I/R injury is a major determinant of acute kidney injury and DGF (22, 40, 52). This also has important consequences on long-term graft survival (22). The severity is influenced by many predetermined factors such as donor and recipient characteristics but also the variable injury incurred before retrieval and during the preservation period.

Ischaemia Reperfusion Injury

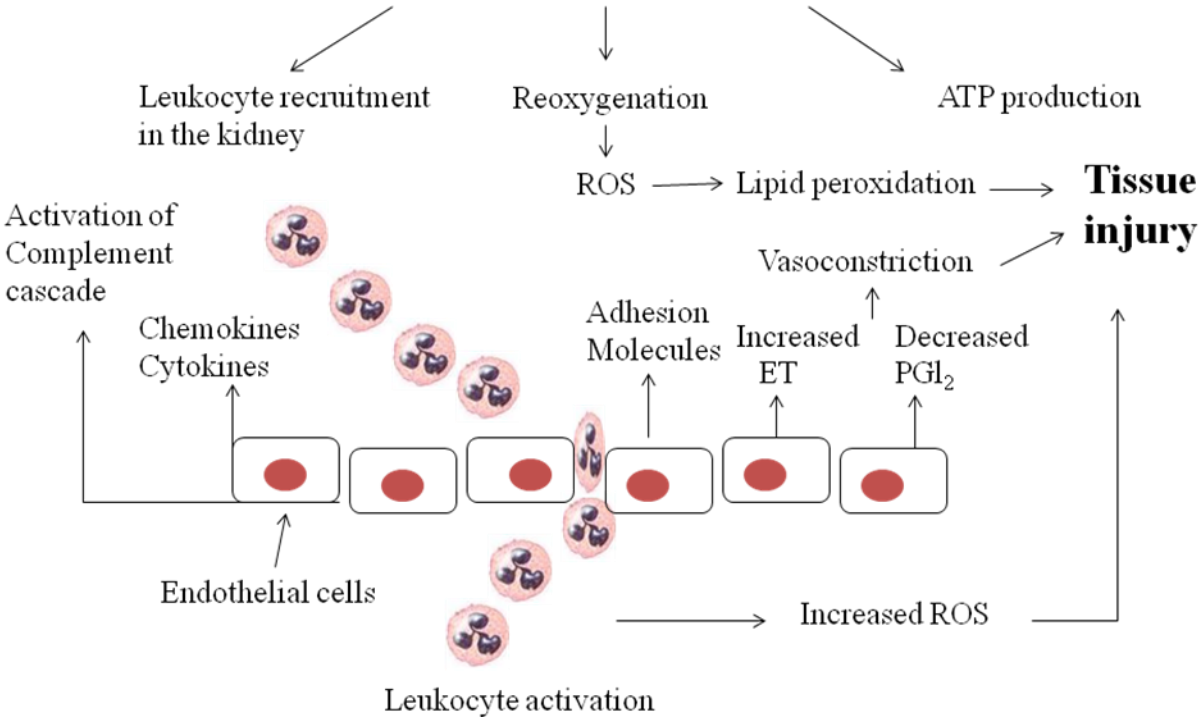


Figure 1.22 A schematic diagram of the complex mechanisms of ischaemia reperfusion (I/R) injury after transplantation. The re-introduction of oxygenated blood mediates a series of injury processes involving activated endothelial cells causing vasoconstriction, the recruitment of leukocytes to the injured tissue, release of pro-inflammatory cytokines and chemokines, activation of the complement system and production of reactive oxygen species (ROS) causing free radical damage. These events cause significant tissue injury which results in acute kidney injury. Endothelin-1 (ET-1), prostacyclin (PGI_2).

1.23 Ischaemia reperfusion injury

Endothelial dysfunction

Severe endothelial cell injury due to ischaemic damage ultimately results in reduced perfusion of the kidney (53). The increase in renal vascular resistance and afferent arteriolar resistance subsequently reduces the glomerular filtration rate. The blood flow to the capillaries and tissues is also reduced. Stiff activated leukocytes, swollen and partly detached endothelial cells and interstitial oedema caused by leukocyte dependant enhancement of protein and fluid leakage in the post-ischaemic venules leads to compression of the microstructure (54). Swelling of the tubular epithelial cells can also reduce the blood flow through the vasa recta ultimately leading to further ischaemic injury and the classically termed 'no-flow' phenomenon (53, 55).

Endothelial cell injury also reduces the bioavailability of agents such as prostacyclin and nitric oxide and increases the production of endothelin and thromboxane A_2 which also causes vasoconstriction (56, 57). Vasoconstriction also occurs from severe injury to the proximal tubular cells which results in transtubular backleak of filtrate and impaired proximal sodium reabsorption (58). The back pressure from congested tubules that have been obstructed with cellular debris due to ischaemic injury also contributes to this dysfunction (53). This is classically known as acute tubular necrosis (ATN) (52). The membrane potential is also altered disrupting cellular ions, increasing the intracellular volume, decreasing membrane fluidity and impairing the cytoskeletal organization (53).

The endothelium is the source of haemostatic regulatory molecules such as von Willebrand factor (vWF). vWF participates in platelet adhesion and plays a role in thrombus formation. Plasma levels have been shown to be a reliable marker of endothelial injury (59-61).

Inflammatory response

The acute inflammatory response to reperfusion and tissue injury occurs rapidly after transplantation. Activation and injury are mediated by neutrophils, pro-inflammatory cytokines, chemokines and other soluble mediators such as the kinin system, thrombin, phospholipids and the complement system (53, 62). They produce vasoactive and cytotoxic effects in response to injury by directly targeting the endothelium and activating the signalling pathways. These are initiated by receptors followed by a series of intracellular signalling reactions which include cytosolic calcium, protein kinase, tyrosine kinase, Rho GTPase and cAMP.

Adhesion molecules

One of the first inflammatory responses is the infiltration of neutrophils into the tissue. Cell adhesion molecules are used by leukocytes to interact with tissue cells to allow the movement of molecules to the injury site. This is mainly mediated through the up-regulation of endothelial adhesion molecules (ICAM-1, VCAM-1 and E-Selectin) (63-65). The synthesis of adhesion molecules is stimulated by histamine, oxygen-derived free radicals, thrombin, platelet-activating factor, activated complement fragments, damaged endothelium, endotoxins and inflammatory cytokines (63).

There are three structural families of adhesion molecules responsible for leukocyte adhesion, penetration of the vessel wall and transendothelial migration into the tissue (Table 1.23).

Super family	Molecule	Location
Immunoglobulins	ICAM-1, VCAM-1	Endothelium
Integrins	MAC-1, LFA-1	Polymorpho-nuclear leukocytes
Selectins	E-selectin	Endothelium
	L-selectin	Polymorphonuclear leukocytes, monocytes, lymphocytes
	P-selectin	Endothelial cells and platelets

Table 1.23 Adhesion molecules; intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), macrophage-1 antigen (MAC-1), lymphocyte function-associated antigen-1 (LFA-1), endothelial leukocyte adhesion molecule (E-selectin), leukocyte endothelial adhesion molecule (L-selectin) and granule membrane protein 140 (P-selectin).

Soluble forms of adhesion molecules can be detected in the circulating blood (VCAM-1, ICAM-1) in response to increased endothelial injury. ICAM-1 and VCAM-1 are particularly important in the attachment and transendothelial migration of leukocytes. The role of ICAM-1 in renal injury has been demonstrated by experiments in which the administration of ICAM-1 antibody was found to be protective (66, 67).

Leukocyte extravasation

The infiltration and migration of leukocytes involves a process called extravasation. Neutrophils infiltrate into the tissue early during reperfusion. After rolling along the activated endothelium they are activated, attach to the surface of the endothelial cells and finally migrate transendothelially. Once attached they release superoxide radicals, proteolytic enzymes and cytokines and when in the tissues they also express increased levels of receptors for chemoattractants such as complement products, fibrinopeptides, prostaglandins and leukotrienes (Figure 1.23). Macrophages are recruited 5 to 6 hours after the inflammatory response. These increase the release of inflammatory mediators and cytokines to sustain the inflammatory response (68).

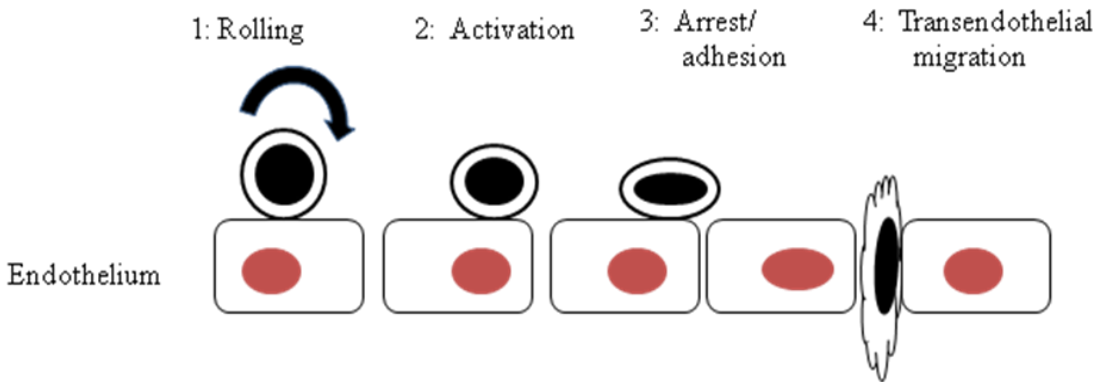


Figure 1.23 The four steps in leukocyte extravasation. (1) the initial rolling by binding of selectin molecules (2) activation by chemoattractant stimuli (3) arrest and adhesion mediated by integrins and (4) transendothelial migration.

Cytokines

Cytokines are a group of cell derived polypeptides which to a large extent orchestrate the inflammatory response. They are major determinants of the cellular infiltrate, cellular activation, and the systemic responses to inflammation. Pro-inflammatory cytokines such as interleukin 1 (IL-1), tumour necrosis factor alpha (TNFα), interleukin 6 (IL-6), interleukin 11 (IL-11) and interleukin (IL-8) are amongst some of the cytokines involved in acute inflammation. They are involved in extensive networks that exhibit both negative and positive regulatory effects on various target cells (69). IL-6 is produced by a variety of cells and is released in the kidney during the acute reperfusion phase. It has been described as having both pro- and anti-inflammatory properties (69-71). The protective role of IL-6 has been demonstrated in studies of liver and kidney I/R injury. In the liver, IL-6 reduced cell damage and stimulated hepatocyte proliferation to protect against I/R injury (72, 73). In the kidney, Nechemia-Arbely *et al* found that IL-6 first mediates early kidney damage by instigating an inflammatory response (74). Subsequently, it is then responsible for resolving the tissue damage. Pro-inflammatory cytokines such as TNFα and IL-1 play a major role in augmenting endothelial permeability and facilitating leukocyte infiltration. TNFα binds to common receptors on the surface of target cells and exhibits several common biological activities. It is up-regulated in response to renal ischaemic injury and is thought to be induced by activation of the p38 mitogen-activated protein kinase (MAPK) pathway via enhanced tyrosine phosphorylation (39, 75-77). It regulates essential biological

functions such as an immune response, cell proliferation, survival, differentiation and apoptosis and has a strong link with the production of oxidative stress (78).

Chemokines

Chemokines are a superfamily of small polypeptides that control the adhesion, chemotaxis and activation of many leukocyte populations. They are induced by inflammatory cytokines such as TNFα. They are expressed soon after reperfusion and mediate the early recruitment of leukocytes (69).

Oxidant injury

Oxygen free radicals are generated immediately after reperfusion. They are thought to be derived from three sources, the xanthine oxidase system, mitochondrial dysfunction and activated neutrophils (40, 79). Xanthine oxidase is an important source of endothelial cell derived superoxide and hydrogen peroxide. The reaction of xanthine oxidase and hypoxanthine from the breakdown of ATP results in the production of superoxide (O^{-}_{2}) and hydrogen peroxide (H_2O_2). It is thought that hypoxanthine is responsible for free radical damage within the first few minutes of reperfusion (40). Superoxide is also produced by NADPH oxidase in lysozymes and the electron transport chain in the mitochondria. This undergoes rapid uncoupling with hydrogen ions in the presence of superoxide dismutase (SOD) to form hydrogen peroxide (80).

Adherent neutrophils are thought to account for greater oxidant stress in the later stages of I/R injury.

During reperfusion H_2O_2 may also react with free iron to form the hydroxyl radical ($OH^{\cdot}$) and superoxide with nitric oxide to produce peroxynitrite ($ONOO^-$). Hydroxyl radicals are responsible for the majority of oxidative mediated damage, causing lipid peroxidation and oxidation of proteins and DNA (58, 81).

Oxidative stress and the production of reactive oxygen species (ROS) have also been implicated in the initiation and progression of the inflammatory response to I/R injury by the up-regulation of leukotriene B_4, thromboxane A_2 and again endothelial adhesion molecules (82,83). Oxidative stress also increases the production of VEGF and up-regulates complement activation which in turn activates pro-inflammatory cytokines, leukocytes and endothelial cells. O^-_2 and H_2O_2 rapidly initiate an increase in the inflammatory state by inducing platelet activating factors via phospholipase activation, promoting the activation and deposit of complement on the surface of the endothelial cells and by mobilizing the stored pool of P-selectin to the endothelial cell surface, thus mediating leukocyte rolling (68). After several hours of reperfusion genes that encode the adhesion molecules are activated. The expression of oxidant dependant activated genes is mediated by nuclear transcription factors such as NFkB and AP-1 which in turn are activated by oxidant stress. This ensures that the inflammatory response lasts several hours after reperfusion.

Matrix metalloproteinases (MMPs) are also significantly up-regulated in response to oxidant stress and contribute to I/R injury through increased endothelial cell permeability. They are associated with degradation of extracellular matrix components, reduced cell-matrix interaction (cell detachment) and subsequently cause apoptosis (53).

I/R injury is also often accompanied by intracellular calcium stress and accumulation of ROS which can disrupt endothelial cell-cell junctions and cause microvascular hyperpermeability (40).

Mitochondrial dysfunction

The mitochondria play a critical role in energy production and under ischaemic conditions generate ROS and initiate apoptosis (42). Ischaemic injury causes alterations to the mitochondrial electron transport chain and the activity of the complexes (I and IV) is reduced resulting in decreased ATP production (84). Impairment also leads to leakage and reduction of oxygen causing the formation of superoxide radicals. It also results in the liberation of ferrous iron an important mediator in the formation of hydroxyl radicals as previously described. Furthermore, there is reduced activity of antioxidant enzymes such as manganese superoxide dismutase (MnSOD) and glutathione which causes more oxidative stress and injury. Excess production of ROS and a decrease in production of ATP during reperfusion leads to further impairment of the mitochondria (85, 86). Lipid damage and oxidative

stress affects membrane permeability resulting damage to the mitochondria DNA and resulting in cell death by apoptosis and necrosis (78).

Nitric oxide-superoxide interaction

Reperfusion induces the impairment of endothelium derived dependant vasodilatation by altering the balance between nitric oxide (NO) and superoxide in the endothelial cells (40). Under normal conditions nitric oxide scavenges superoxide, reduces arteriolar tone via guanylate cyclase activation in smooth muscles, prevents platelet aggregation and thrombus formation and minimizes the adhesive interaction between leukocytes and the endothelial cell surface. I/R injury causes a biological imbalance, with increased production of superoxide and reduced bioavailability of nitric oxide. This causes a loss in endothelium dependant vasodilatation resulting in vasoconstriction. The accumulation of superoxide in the absence of nitric oxide also causes enhanced generation of hydrogen peroxide (40, 87).

Complement

Complement is recognized as an important mediator of innate immune defense and inflammation. The most important action of complementing antibodies is the killing of bacteria (88). However, it has become apparent that it has many other functions. Complement activity plays a major role in I/R injury as previously described. Its activation is initiated through the classical, alternative or mannose-binding lectin pathways that result in the production of C3 convertase (89). Activation of this system results in the recruitment of a large number of inflammatory cells leading to cell injury and cell death. The inhibition and deficiency of C3 and C5 is known to protect against I/R injury (89).

The importance of each pathway in I/R injury remains controversial. However, the original view that the classical pathway was responsible for the majority of injury has been challenged by recent studies examining the effects of the alternative and lectin pathway (90).

Immunogenicity

Ischaemic injury is also thought to increase the risk of acute rejection in response to the effects of innate and adaptive immunity (52). After a period of ischaemia there is an increased expression of major histocompatibility complex (MHC) class I and II molecules which may be related to the immunogenicity of the graft (52). MHC antigens elicit a strong immune response via direct recognition of MHC alloantigen molecules on the surface of the kidney or through indirect recognition of peptides derived from these molecules presented to the recipients' immune system by antigen-presenting cells. The increased expression is a result of increased transcription induced by cytokines (interferon γ, interleukin 10 and TGFβ) and expression of adhesion molecules in response to ischaemic injury (68) .

Necrosis and apoptosis

Severe injury leads to two types of cell death, necrosis and apoptosis. Necrosis is a passive process, beginning with the loss of homoeostatic processes, cell swelling and ending in cell lysis. This can trigger a damaging inflammatory response (91).

Programmed cell death or apoptosis is an active cellular event aimed to control cell turnover and homeostasis. The DNA is broken into distinct mono- and oligosoma fragments. Increased apoptosis as a result of I/R injury results in severe organ dysfunction. It is the result of the activation of a cascade of intracellular proteins (caspases). There are two major pathways of caspase activity, extrinsic and intrinsic (91). The extrinsic pathway is triggered by binding of extracellular ligands to the cell surface death receptors. The intrinsic pathway is activated by numerous intracellular stimuli including anoxia, DNA damage and growth factor deprivation. These stimuli target the mitochondria by triggering mitochondrial membrane permeabilization (opening of the pores) resulting in the release of caspase activation factors, for example cytochrome c (92). Regulation of the intrinsic pathway is controlled by a balance of pro and anti-apoptotic (Bax and Bcl-2) proteins (92, 93). Unlike necrosis, apoptosis does not induce a local inflammatory response.

Chapter 2

Kidney Preservation

2.11 The history of kidney preservation

Organ preservation was first proposed by the French physician, scientist and philosopher, Julien Jean Cesar le Gallois in 1813 (94). He stated that

'If one could replace for the heart some kind of injection of artificial blood, either natural or artificially made… one could succeed easily in maintaining alive indefinitely any part of the body'

In 1849, Loebell devised an isolated organ perfusion system to preserve organs at warmer temperatures (95). This concept of preservation was pursued by Alexis Carrel in the early nineteen hundreds (96). Carrel studied the effects of tissue preservation at both hypothermic and normothermic temperatures (97-101). He discovered that cooling allowed tissues to retain viability for longer periods compared to normal temperatures and demonstrated the ability to transplant blood vessels after several days when stored in a specially designed solution (Locke's solution). However, he soon realized that cold conditions had their limitations and with time viability was lost. In an attempt to improve the condition of the tissues by maintaining them in a functional state he sought the help of Charles Lindbergh, an engineer and pilot, to develop the first organ perfusion device (1935) (101). The system was capable of maintaining a sterile, pulsating circulation of fluid through a living organ. It was made up of three glass chambers, an organ chamber at the top, a pressure equalizing chamber in the middle and fluid chamber on the bottom. A mixture of oxygen, carbon dioxide and nitrogen was pulsated by compressed air into the perfusate through the equalizing

chamber and into the organ. They perfused a variety of organs such as the thyroid, suprarenal gland, ovary, spleen, heart and kidney from adult fowls and cats. The organs were perfused with a nutrient fluid designed to culture the organs which often contained blood, serum or solutions containing protein split products such as hemin, cysteine, insulin, thyroxine, glutathione, vitamin A and ascorbic acid. This perfusion system allowed the organs to be studied in an *ex-vivo* environment to acquire knowledge on the metabolic processes and nutrient requirement of individual organs (Figure 2.11).

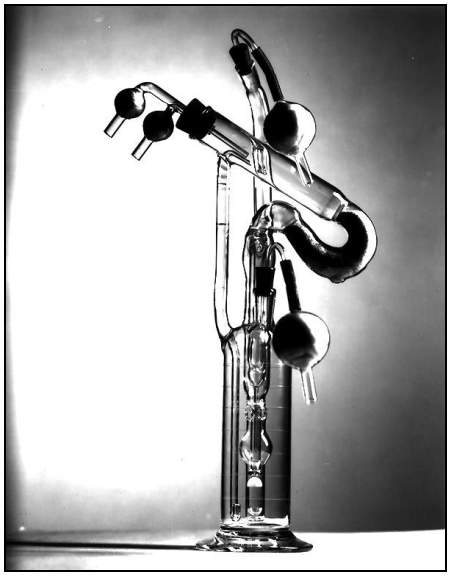


Figure 2.11 The first organ perfusion pump developed by Carrel and Lindbergh (101).

2.12 Transplantation (1960s)

It wasn't until the 1960s with the introduction of immunosuppressive therapies that transplantation became more successful, and the interest in, and true development of preservation techniques began. Until this time without proper preservation conditions, kidneys were transplanted as soon as possible after retrieval to minimize the injury. It was then recognized that in order to improve the outcome of transplantation, better methods of preservation were required. Supported by experimental studies in the 1950s by Lapchinsky (102) in the Soviet Union and the early work by Carrel and Lindbergh, which showed that ischaemic injury could be minimized by reducing the temperature (101), in 1963, Calne *et al* used the concept of hypothermic temperatures to extend the preservation time and successfully transplanted canine kidneys after 12 hours of storage (103). This led to the application and development of preservation techniques and solutions that are used today.

2.13 Machine perfusion (1960-1980)

Another well renowned pioneer of organ perfusion who led the clinical application of hypothermic machine perfusion (HMP) for kidneys was Folkert O Belzer. The development of HMP coincided with the evolution of other clinical technologies such as cardio-pulmonary bypass and renal haemodialysis. The technique involved the continual re-circulation of a soultion through the kidney at a low temperature. Belzer was the first to develop a portable HMP system (104,105). However, the systems were

complex, incorporating roller pumps to produce a pulsatile flow, membrane oxygenators for oxygenation, filters and heater/coolers to maintain temperature. Diluted blood was first used in these systems however problems with increased viscosity and thrombus formation led to the development of a cryoprecipitate solution (106). Plasma was frozen to -20°C, followed by rapid thawing to produce a precipitate of the lipoprotein fraction which could be removed by filtration. Serum albumin based solutions were also developed and on occasion supplemented with dextran to increase the oncotic pressure and reduce interstitial oedema. Due to risk of infection and lack of availability, these protein based solutions fell from favour.

2.14 Preservation solutions (1970-2010)

The introduction of machine perfusion techniques also coincided with the development of hypothermic preservation solutions. There was a recognition of a need to develop a preservation solution that could efficiently flush blood from the organ and prevent the harmful effects of fluid shifts during hypothermic storage. Solutions were developed to include an impermeant to counteract swelling and to provide stability of the ultra structure of the cell. A buffer to prevent the build up of intracellular acidosis and a balanced electrolyte composition with either a high or low Na+ / K+ ratio, again to counteract cellular swelling. Solutions with a high potassium concentration are classified as intracellular and those with a high sodium as extracellular solutions (Table 2.14). A high potassium concentration is thought to be important in preventing potassium loss during cold storage. It also prevents the build up of intracellular calcium

during ischaemia but can cause vasoconstriction, reducing the efficiency of the initial flush and cooling of the organ.

Components	
Impermeants	glucose, lactobionate, mannitol, raffinose, sucrose
Colloid	hydroxyethyl starch (HES), polyethylene glycol (PEG)
Buffers	citrate, histidine, phosphate
Electrolytes	calcium, chloride, magnesium, magnesium sulphate, potassium, sodium
Anti-oxidants	allopurinol, glutathione, mannitol, tryptophan, histidine
Additives	adenosine, glutamic acid, ketoglutarate

Table 2.14 Components commonly used in organ preservation solutions

Euro Collins

In 1969, Geoffrey Collins developed the first acellular preservation solution (Collins solution) containing a high concentration of potassium and glucose (107). The kidney was simply flushed with cold preservation solution then stored in solution surrounded by crushed ice. This led to the introduction of static cold storage (CS) as a method of organ preservation.This practical method of preservation grew in popularity throughout the transplant community and was quickly adopted by the majority of transplant centres. The introduction of cross matching techniques encouraged the allocation of kidneys to the most suitable recipient and therefore a technique of preservation that facilitated the simple transportation of the kidneys was a major breakthrough. Furthermore, CS proved to have an equivalent outcome compared to HMP with similar graft survival rates at that time. Collins solution was later modified omitting some of the ingredients such as magnesium, heparin and procain and replacing glucose with mannitol to provide better osmotic properties and lower the viscosity (108-110) (Table 2.141). It was renamed Euro Collins solution and was widely used amongst the transplant community.

Hyperosmolar citrate (HOC)

Hyperosmolar citrate (HOC) or more commonly known as Soltran or Marshall's solution was first developed in the 1970s as an alternative to Collins solution (111,112). It is a high potassium, intracellular solution containing basic ingredients and using citrate as a buffer. Citrate also binds with calcium to prevent accumulation within the cell. Its hypertonicity is designed to prevent fluid entry into cells (Table 2.141). It is a relatively inexpensive, non-viscose solution that is used throughout the UK in kidney transplantation. However, it is not recommended for DCD or marginal kidneys, despite the fact that there is little evidence to support this view.

University of Wisconsin (UW) solution

By the 1980s CS was the most accepted and commonly used method of preservation. Originally, Belzer and Southard developed a preservation solution for HMP that contained gluconate, a high sodium concentration, adenine, glucose and ribose (104). However, due to the popularly of CS they modified the solution by removing some of the ingredients (113). University of Wisconsin (UW) solution for CS is an intracellular solution with a high potassium concentration to maintain the intracellular ionic balance. It is a more complex preservation solution compared to Euro Collin and HOC, containing trisaccharide raffinose and the anion lactobionate as osmotic impermeants, a phosphate buffer, antioxidants (glutathione) to scavenge oxygen free radicals, allopurinol to block the activity of xanthine oxidase and adenosine, an ATP precursor (Table 2.141). It also contains the colloid hydroxyethyl starch (HES), to prevent cellular swelling (114). However, it is debatable whether this is it necessary in a static storage solution and there is some evidence showing that HES can increase tubular damage and cause red blood cell aggregation (114). Another potential disadvantage of UW solution is the high concentrations of potassium. Although thought important in the prevention of the build up of intracellular calcium, as mentioned previously, potassium can cause vasoconstriction. Furthermore, it induces cellular depolarization, reduces cellular 5'-triphosphate content and activates voltage-dependent channels, such as calcium channels (115). Nonetheless, due to its composition, UW solution had, and still has, a significant advantage over other preservation solutions enabling kidneys to

be stored for longer periods with better function and less histological injury after transplantation. It is still considered the ‘gold standard’ preservation solution today.

Histidine-tryptophan-ketoglutarate (HTK)

HTK is used throughout Europe and the US. It was originally developed as a cardioplegic solution but because of its low viscosity was quickly adopted for clinical preservation of the kidney, pancreas and liver (116-118). It is an extracellular solution and uses the impermeant mannitol and histidine as a buffer. It also contains 2 amino acids, tryptophan, to stabilize cellular membranes and prevent oxidant damage and ketoglutararate, a substrate to support anaerobic metabolism (Table 2.141). Nonetheless, recent concerns have been raised regarding its use for marginal and DCD kidneys or for kidneys with prolonged storage times (118-120). Some clinical studies have associated its use with the increased risk of PNF and early graft loss (121).

Celsior solution

Celsior is an extracellular solution and was initially designed for heart transplantation. It contains a high sodium concentration with histidine as a buffer, lactobionate and mannitol to prevent oedema and glutathione as an antioxidant. The solution has proved beneficial in heart, liver, pancreas and in kidney transplantation (122-125).

Phosphate-buffered sucrose (PBS 140)

PBS 140 was developed in the UK as an inexpensive alternative to UW solution. It is a basic solution with the 140 referring to the concentration of sucrose. It contains sodium but no potassium and uses phosphate as a buffer (126). It has not been used in clinical practice although experimental studies have shown its benefits in reducing cellular oedema and tubular injury (127).

Components	HOC	HTK	UW	KPS-1
Sodium	84	15	25	
Potassium	80	9	120	25
Citrate	54			
Magnesium	41	4		
Sulphate	41			
D-glucose				10
Calcium chloride		0.015		0.5
Histidine		18		
Trytophan		2		
Potassium citrate	8.6g /L			
Sodium citrate	8.2g/L			
Mannitol	33.8g/L	30		30
Magnesium sulphate	10.0g/L			
Magnesium gluconate				5
Sodium gluconate				80
HEPES (Free acid)				10
Poly (0-2 hydoxyethyl starch)			50 g/L	50g/L
Potassium phosphate				25
Potassium hydrogen 2-ketoglurarate		1		
Potassium hydroxide			100	
Sodium hydroxide			27	0.7
Adenosine			5	5
Allopurinol			1	
Potassium dihydrogen phosphate			25	
Magnesium sulphate			5	
Lactobionic acid			100	
Raffinose			30	
Glutathione			3	3
Ribose				5
pH	7.1	7.4 – 7.45	7.4	7.4
Osmolarity	486 mOsmol/L	310mOsmol/L	320mOsmol/L	300mOsmol/L

Table 2.141 Composition of the commonly used static storage preservation solutions, hyperosmolar citrate (HOC), histidine-tryptophan-ketoglutarate (HTK), university of wisconsin (UW), and kidney preservation solution-1 (KPS-1) used for hypothermic machine perfusion (HMP).

2.15 Hypothermic machine perfusion versus static cold storage

Since the introduction of CS techniques in the 1970s there has been much debate about whether CS or HMP is the best method of kidney preservation. Undoubtedly, the simplicity of CS has a significant advantage over HMP. However, HMP is it thought to be a better method of preservation in that it allows a continual flush of the microcirculation, preventing the accumulation of waste products, sustaining a higher metabolic rate, protecting against depolarization of the endothelial cell membrane and reducing free radical formation (128).

With the recent development of new simpler and portable systems such as the Lifeport Kidney Transporter (Organ Recovery System, US) and the increasing use of DCD and ECD kidneys, there has been renewed interest into the use of HMP over the last decade. However, the evidence for determining the best method of preservation remains controversial. Many experimental studies have found HMP to improve preservation (43,129) and recently a European multicentre clinical trial found that HMP reduced the risk of DGF compared to CS (130). However, these findings were contradicted by a smaller UK trial that found no advantage with the technique (131). Furthermore, HMP techniques are still open to criticism with the suggestion of increased endothelial injury, as found in a recent study of porcine livers (132), risk of trauma to the vessels and the question of cost effectiveness compared to static storage techniques (133). As such it is not routinely used in the UK.

2.16 Viability assessment

Measuring the viability of an organ before transplantation would be advantageous in that the suitability of the kidney for transplantation could be pre-determined. This would be particularly beneficial for marginal kidneys and avoid the likelihood of PNF. Viability is normally assessed by numerous factors including donor history, duration of cardiac arrest, quality of *in-situ* perfusion, CI interval and visual inspection of the kidney. Ultimately this relies on the judgement of an experienced surgeon. As such, many kidneys are deemed unsuitable for transplantation and are discarded (28). HMP has been used to determine viability. Two aspects can be measured; Firstly, the continuous recirculation of preservation solution through the kidney allows the perfusate flow to be measured and intra-renal resistance to be calculated. Secondly, the perfusate can be sampled to measure cellular injury.

The Leicester group has previously demonstrated that early intra-renal resistance during HMP was reflective of the degree of WI and CI injury in porcine kidneys (134). Clinically, the perfusion flow index (PFI) has been used as a measure of flow and resistance (135,136). This is based on a minimum flow being obtained for a given pressure. The Transplant Group at Newcastle, UK recommend that a PFI of greater than 0.6ml/min/mmHg/100 gram of kidney is needed for a kidney to be deemed suitable for transplantation (137). However, the evidence for the ability of these parameters to predict DGF or PNF in clinical practice is limited. Jochman *et al* recently reported that although renal resistance (RR) at the end of HMP was an independent risk factor for DGF and reduced 1 year graft survival, it had a low predictive power and

could not be relied on as a sole measure of viability (138). This is in agreement with other small clinical studies by Sonnenday (139) and Guarrera (140) *et al* that showed that kidneys with poor perfusion parameters had a similar outcome to those with good parameters.

Injury can also be measured by sampling the perfusate for biomarkers of cellular injury. Markers such as redox free iron, glutathione S-transferase (GST), total glutathione S-transferase (tGST), lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAG), heart-type fatty acid binding protein (H-FABP) and alanine aminopeptidase (Ala-AP) have all been used to determine injury (136-138,141). There is little information on their predictive value. However, Jochman *et al* recently published the results from the European HMP trial in which perfusate samples were taken for the assessment of biomarkers at the end of HMP (138). GST, NAG, and H-FABP were found to be independent predictors for DGF but not for graft survival in the first year after transplantation. LDH, ASAT, and Ala-AP were found to have no predictive potential for post transplant outcome. Furthermore, the biomarkers did not correlate with intra renal-resistance. The evidence suggests that viability assessment during HMP cannot be used independently but may be used collectively with the kidney characteristics and donor demographics to determine the suitability of a kidney for transplantation.

2.2 Oxygenation during preservation

2.21 Introduction

Hypothermic preservation is based on the presumption that by lowering the temperature the metabolic demand is extremely low and therefore the requirement for oxygen to support aerobic metabolism is negligible. Nonetheless, the need to provide oxygen during hypothermic preservation has been deliberated since the introduction of hypothermic techniques in the 1960s. Historically, oxygenation was considered an essential component of kidney preservation in order to support mitochondrial resynthesis of ATP and to delay the injury process. However, with the introduction of the modern day preservation solutions, and the rapid adoption of simple CS techniques, oxygen was not thought to be a vital ingredient and as such is not commonly applied in the clinical setting. Furthermore, in a highly oxygenated environment there is the risk that there will be an increase in the production of ROS causing further oxidant stress and tissue injury (43). Various techniques have been used to apply oxygen under CS and HMP conditions.

2.22 Retrograde persufflation

Retrograde oxygen persufflation is a simple technique whereby filtered and humidified oxygen is bubbled directly through the renal vasculature during CS. The gas is then allowed to escape through small perforations in the surface of the organ. Reports of its application date back to the 1970s. The technique was successful when 100% oxygen was applied at either high (50-60mmHg) or lower (11mmHg) pressures (142,143). Experiments showed that persufflation could effectively deliver oxygen to the organ and support aerobic metabolism with evidence of adenine nucleotide generation during preservation (143,144). In 1989, after a series of successful experiments in warm ischaemially damaged canine kidneys and based on the previous work by Fischer *et al* and Ross *et al* (142,143,145), Rolles *et al* translated the technique into clinical practice (146) . The pilot study of 10 kidneys showed that graft function could be improved after significant WI injury. However, there were no further reports of the application in clinical practice after this time, presumably due to the introduction of the modern day preservation solutions and more simple approaches to kidney preservation. Experimentally, the technique of persufflation has since been revisited and a study by Yin *et al* in the 1990s found that although it could reduce the loss of adenine nucleotides in rat kidneys during 24 hours hypothermic storage, it did not improve the function of the grafts (147). There has been greater development of the technique for liver preservation (148-150) which led to its application in a small series of patients in 2008 (151). Furthermore, the same authors have recently applied the technique to the kidney. Using porcine kidneys, Treckmann *et al* found that persufflation had a

beneficial effect on graft function when compared to CS and HMP techniques (152,153). They used a similar technique as Rolles *et al,* administering oxygen at a pressure of 18mmHg in kidneys that had 30 minutes of WI injury but modified the conditions by adding oxygen scavengers to prevent lipid peroxidation, based on their previous work in the liver (154).

2.23 Hyperbaric oxygenation

Hyperbaric oxygenation is the delivery of oxygen under increased atmospheric pressure under CS or HMP techniques. It is used to treat decompression sickness, carbon monoxide poisoning, gas embolism, circulatory disorders and to promote wound healing (155-157). Under normal atmospheric pressure there is a limit to the amount of oxygen that can be carried in the blood. Increasing the atmospheric pressure at which it is delivered, increases the amount of dissolved oxygen in the plasma allowing deeper penetration into the tissue (Henry's Law). Therefore, tissues can be adequately oxygenated in the absence of a blood flow, a particular advantage in organ preservation (155,156).

Historical research into the use of hyperbaric oxygenation in kidney preservation has had positive implications, although there was some doubt whether oxygen could penetrate deep enough into the renal parenchyma to be of benefit (158-161). In an attempt to extend the preservation interval beyond a matter of hours, in the 1960s and 70s, hyperbaric oxygenation was combined with the concept of hypothermic static storage and perfusion techniques. The kidney was immersed in preservation solution

and placed in a highly pressurised chamber to allow the delivery of oxygen under a set atmospheric pressure whilst refrigerated at 4ºC. Ladaga *et al*, found that indeed the combination of the two methods optimised postoperative kidney function (162). Other studies also noted that hyperbaric oxygenation improved the protective effect of hypothermia (160,163,164). The effects of different atmospheric pressures were also studied. In 1967, Rudolf and Mandel intermittently perfused canine kidneys with a low molecular weight dextran and balanced salt based solution at 3, 8 and 15 atmospheres (atm) at 2ºC (159). Only kidneys that were preserved at 3 atm functioned after transplantation. It is now understood that the optimal atmospheric pressure for oxygen delivery is 2.5 atm and that a partial pressure of above 500mmHg can cause vasoconstriction possibly due to the reduced availability of endogenous nitric oxide (155).

Hyperbaric oxygenation has since been developed as a therapy and there are isolated cases of proven benefit both before organ retrieval and during reperfusion (165,166). Although it has not been used as a method of kidney preservation since the 1970s, it has been used with some success for the preservation of the liver and small bowel in the experimental setting (167-169).

2.24 Hypothermic machine perfusion

At present, HMP is not supplemented with oxygen based on the presumption that air equilibration in perfusates sufficiently supports energy metabolism and that oxygen consumption at 4ºC is only approximately 5% of that found at body temperature (170). However, conditions may be improved with the addition of oxygen.

There are no studies directly assessing the effects of continuous oxygenation during machine perfusion in kidney transplantation, although experimental studies that have used oxygenated machine perfusion, have found a beneficial effect compared to static storage conditions (43). An early study by Pegg *et al* (171) showed that the ATP: ADP ratio could be restored to half the normal level with the addition of oxygen and energy substrates during perfusion.

Although it is difficult to determine the importance of oxygenation, it does appear to be particularly beneficial when organs have been subjected to a period of WI injury. A recent study by Buchs *et al* (172) aimed to establish whether oxygenated hypothermic perfusion was preferable to CS in preserving kidneys from DCD donors. They found that levels of ATP were restored in porcine kidneys with 30 minutes of WI injury. However, oxygenation had no added benefit in kidneys without injury.

Short periods of oxygenated perfusion after CS have also been used to resuscitate and condition organs, correcting ATP loss, reducing levels of oxidative stress and improving organ viability (173). Koetting *et al* demonstrated the advantage of adding oxygen to recover organs after ischaemic injury (174). Porcine kidneys were subjected to 90 minutes of hypothermic reconditioning by HMP either with or without

oxygenation of the perfusate, having first been flushed and cold stored for 18 hours. Oxygenation of the perfusate resulted in a threefold improvement of renal clearances of creatinine and urea. The study concluded that continuous aerobiosis during graft storage effectively prevented energetic breakdown and deterioration of mitochondrial redox homeostasis. A short period of oxygenation is also supported by studies in the liver. De Rougemont *et al*, found that porcine livers perfused for 60 minutes by hypothermic oxygenated extracorporeal machine perfusion (HOPE) after 60 minutes of warm ischemia and 6 hours cold storage had less injury and lower levels of oxidative stress compared to statically stored livers (175).

In contrast to the benefits of oxygenation, a highly oxygen concentrated environment may support the generation of oxygen free radicals causing further oxidative injury. The addition of free radial scavengers such as superoxide dismutase (SOD) to the preservation solution has been found to be beneficial (154,173). Although there are no reports of the detrimental effects of high concentrations in kidney preservation, liver models provide us with more information. ‘t Hart *et al* found that although oxygenation during preservation supported a higher production of ATP and urea synthesis and resulted in less cellular injury, the higher concentrations (95%) increased the generation of reactive oxygen species during preservation. They concluded that lower concentrations (21%) were probably optimal (176). However, studies are inconsistent and others support the use of high concentrations. Luer *et al* found that it was more beneficial to perfuse rat livers with 100% oxygen, with less oxidative injury and

improved bile production compared to room air or non oxygenated perfusion (177). The disparity between the studies highlights the need for more research in this area.

2.25 Artificial oxygen carriers

Oxygen can also be effectively administered during preservation by the use of artificial oxygen carriers. Perfluorocarbons (PFC) are inert solutions that have a high capacity for dissolving oxygen. They release oxygen down a concentration gradient creating a highly oxygenated environment which is not affected by temperature (178,179). They can be added simply during CS in a technique called the two layer method (TLM). The density of the PFC allows two layers to be formed, PFC on the bottom and the preservation solution on top. The organ is placed in the solution and remains between the two layers. Oxygen can be continuously added allowing adequate diffusion through the organ. TLM has been particularly beneficial for pancreas preservation, allowing a sufficient amount of ATP to be generated to improve organ viability (178,180). The use of the TLM has shown potential in other organs but has failed to gain much support as the ability of oxygen to penetrate deep into tissue in more densely capsulated organs has been questioned. In the kidney its beneficial effect was found in a rat model, however, when applied in a porcine model the results showed no advantage (178,181-183).

PFC can also be formulated as an emulsion for continuous perfusion and was applied during early attempts at machine perfusion (183-186). However, the instability and

adverse effects of the emulsions at that time prevented their continued application (178).

Other novel oxygen carriers have recently been applied in kidney preservation. Hemarina-M101 (M101) is a respiratory pigment derived from a marine invertebrate, *Arenicola marina* (187) . Is has an extremely high affinity for oxygen and functions over a large range of temperatures (4-37ºC) releasing oxygen against a gradient. Using a porcine kidney model, Thuiller *et al* recently showed that adding M101 to UW or HTK solution during CS for 24 hours improved renal function and reduced fibrosis after transplantation. Micro-bubbles derived from Dodecafluoropentane (DDFPe) are also being investigated as oxygen replacement therapies and may in the future be applied during organ preservation (188,189).

2.26 Outcome

At present the evidence comparing oxygenated and non-oxygenated preservation techniques is sparse. However, it appears that oxygenation may be particularly beneficial in kidneys that have undergone a period of warm or cold ischaemic injury. It has been successfully applied in the experimental setting using techniques such as retrograde persufflation and machine perfusion. Oxygenation during HMP perhaps hold more promise as more robust, portable machines that have the capacity for oxygenation are being developed. Other techniques such as the addition of artificial oxygen carriers may be applied more readily in the future.

It is apparent that the benefits of oxygenation are not uniform and that in kidneys with minimal injury, oxygenation may hold no benefit. As such a careful balance of preservation conditions is necessary and the addition of oxygen may be used selectively. However, more evidence is needed to directly compare oxygenated and non oxygenated preservation techniques in kidney transplantation.

Chapter 3

Normothermic Preservation

Chapter 3: Normothermic preservation

3.11 Introduction

In the modern day with the increasing reliance on DCD and ECD kidneys, it is apparent that alternative methods of preservation may be needed to improve outcomes. Hypothermia has been described as ‘a compromise between the benefits and detriments of cooling’ (129). Although there appears to be a beneficial effect of oxygenated hypothermic conditions in organs with WI injury, resuscitation under normothermic conditions is a more attractive concept.

The innovated vision of Le Gallois and Alex Carrel in maintaining an organ in a functional state is perhaps now a much more logical and feasible approach to organ preservation. The continuous perfusion at warmer temperatures with the delivery of nutrients and oxygen has the advantage of avoiding hypothermic injury and hypoxia. In addition, it also may aid recovery and prevent further injury.

Early attempts at normothermic preservation were generally unsuccessful due to the inability to maintain cellular integrity and support renal metabolism. However, advances have been made over the last few decades with the use of technology borrowed from cardiac surgery. The development of less traumatic perfusion pumps and the recognition of the necessity for the delivery of nutrients and oxygen to achieve successful perfusion has made normothermic preservation a realistic contender in clinical transplantation. Nonetheless, despite the potential benefits, normothermic preservation is logistically difficult to carry out requiring technical support and

expensive perfusion systems. This has undoubtedly hindered its popularity and advancement in clinical practice (Table 3.1).

Normothermic preservation

Advantages	*Disadvantages*
Aerobic metabolism	Technical support/equipment
Restoration of function	Logistics of transportation
Organ assessment	Cost
Resuscitation	Risk of infection
Regeneration and repair	Potential for further damage
Treatment and modification	

Table 3.1 Advantages and disadvantages of normothermic preservation

3.12 Normothermic solutions

The composition of the perfusate is vital to ensure adequate delivery of nutrients and oxygen to maintain cellular integrity and vascular processes during perfusion. Blood based solutions were previously considered to have their limitations for prolonged preservation because it led to haemolysis, platelet activation and degradation of products during perfusion causing increased resistance and tissue oedema (190). However, with the use of centrifugal pumps, which reduce the risk of stress and haemolysis and membrane oxygenators enabling filtration and improved oxygenation, the use blood base perfusates is a feasible option. Blood based solutions are convenient and provide good oxygenation and stable renal function (191-193).

An alternative to blood is to use an artificial blood substitute with the capacity to carry oxygen (194,195). This may be more beneficial for oxygen delivery and for prolonged periods of perfusion. In addition to hypothermic conditions, perfluorochemical and haemoglobin solutions can also be used to deliver oxygen at normothermic temperatures (196). Brasile *et al* originally developed an acellular normothermic solution based on a modified cell culture medium and PFC emulsion (Perflubron) (197). The perfusate was made up of a highly enriched tissue culture-like medium containing essential and non-essential amino acids, lipids and carbohydrates.

Historically, haemoglobin based solutions such as Stroma-free haemoglobin had their difficulties because of toxic effects on the kidney. However, a newly developed solution, pyridoxalated haemoglobin-polyoxyethylene (PHP) has been deemed to be a more stable solution (196). Brasile *et al* have since replaced the PFC in their perfusion

medium with pyridoxylated haemoglobin (198,199). Nonetheless, the use of these normothermic perfusates in clinical practice is still awaited. New more stable 2nd and 3rd generation PFCs are being developed and several are undergoing clinical trials to assess their safety. Humphreys *et al* recently used a commercially made PFC 'Oxygent' to provide oxygenation and reduce ischaemic injury to the kidney during WI by retrograde infusion through the urinary collecting system (200). These new solutions may hold more promise for future development of normothermic preservation perfusates.

Other solutions such as Lifor, a new artificial preservation medium containing a non protein oxygen carrier that can be used at room temperature may also be used for preservation (201). A recent study by Gage *et al*, showed that during perfusion porcine kidneys perfused with Lifor had higher flow rates and lower levels of resistance compared to kidneys perfused with UW at room temperature or 4ºC (201). There was no information on the outcome however the higher perfusion flows suggested better preservation of the kidney. Lifor has also been found to be beneficial in reducing apoptotic cell death after cold ischaemic injury (202).

3.13 Technology

There is a great deal of variation in the type of systems used for normothermic perfusion. Early systems incorporated animal hearts and lungs to ensure adequate oxygenation and improve vascular tone and perfusion (203). Later blood pump oxygenation systems were used to perfuse the kidney with whole blood oxygenated with air (204). Normothermic systems have more recently been developed to study renal physiological parameters in response to different perfusion conditions and pharmacological interventions in addition to being used as a method of preservation (205-209). Some have adapted hypothermic perfusion systems (210) or used custom made systems with roller pumps and dialysis circuits (205) to obtain optimal renal function. The normothermic system reported by Brasile *et al*, exsanguinous metabolic support (EMS), used a pressure controlled perfusion system including an oxygenator and pulsatile pump with controllers to maintain PaO_2, $PaCO_2$, pH and temperature (197,211). Kidneys were perfused at a subnormal temperature of 32ºC and mean arterial pressure of approximately 35 mmHg.

3.14 Techniques of normothermic preservation

The concept of NP can be applied in different ways as follows. Before the kidneys are removed from the donor, to flush an organ, for prolonged preservation or in combination with hypothermic techniques.

3.15 Normothermic recirculation

Traditionally, organs from deceased and DCD donors are flushed *in situ* with cold preservation to reduce the temperature and to lower the metabolism as quickly as possible to prevent cellular injury. However, the concept of extracorporeal membrane oxygenation (ECMO) to maintain extracorporeal circulation at normal room or body temperature with hyperoxygenated blood can be used to maintain tissue perfusion after the heart has stopped. Normothermic recirculation has proved beneficial in the retrieval of hearts, lungs and abdominal organs. Valero *et al* assessed the effects of implementing this technique in clinical practice in small group of DCD donors (212). Circulation was maintained for 60 minutes before total body cooling. The incidence of DGF and PNF was reduced after normothermic recirculation compared to standard *in situ* or total body cooling. Gravel *et al* described a DGF rate of 11% in controlled NHB donors (91) and Lee *et al* found similar 5 year graft survival rates to heart beating and living donors (213). Maintaining circulation before retrieval is also thought to condition the organs by up-regulating adenosine receptors which may protect against preservation injury (212). Reznik *et al*, recently reported the application of

extracorporeal normothermic recirculation in uncontrolled DCD donors using leukocyte depleted blood (214,215). Initial kidney graft function was achieved in 6 out of the 16 patients. In the kidney, more evidence is needed to determine how normothermic recirculation before retrieval correlates with early and longer term graft function.

3.16 Normothermic flush

The initial flush of an organ removes the blood, perfuses the microcirculation and cools the kidney to slow metabolism. However, the cold conditions can cause vasoconstriction thus reducing the effectiveness of the perfusion. Normothermic temperatures have been used to directly flush the kidney in an attempt to improve microperfusion and reduce vasoconstriction. Das *et al* (1979) found that normothermic flushing of canine kidneys prior to hypothermic flush and cold preservation eliminated vasoconstriction (216). However, perfusion volumes exceeding 100ml caused endothelial injury. Rat kidneys flushed with UW at normal body temperature supported a higher level of metabolism after transplantation and showed lower serum creatinine, urea and had less histological change compared to kidneys flushed with Euro Collins, saline or Ringer's lactate (217). The most recent experimental study showed that no detrimental effect was found in kidneys flushed with the novel non phosphate buffered solution 'AQIX RS-I®' at a normothermic temperature (218). AQIX RS-I® is a newly developed normothermic preservation solution that is capable of carrying oxygen and also reflects physiologic ionic concentrations, osmolarity, and ion conductivity to

maintain the ionising status of the cell membrane and enzymatic function. A previous study from Leicester showed that AQIX RS-I® could sufficiently maintain viability in kidneys statically stored for 2 hours at 32ºC in the pre-oxygenated solution (219).

3.17 Short term normothermic preservation

In consideration of the logistical problems of prolonged preservation a great deal of research has focused on using normothermic preservation in combination with hypothermic techniques. One of the early aims of kidney preservation was to extend the preservation period to a matter of days to facilitate cross matching and antibody screening. van Der Wijk *et al* was able to preserved canine kidneys for 144 hours using a combination of hypothermic and normothermic preservation techniques (220). Half way through the hypothermic preservation period kidneys were perfused at a normothermic temperature, with blood, for periods of 1- 4 hours before returning to hypothermic preservation. 3 and 4 hours of normothermic perfusion were deemed necessary to reverse the ischaemic damage. Rijkmans *et al* extended the preservation period to 6 days with a 3 hour period of normothermic preservation before and after 3 days of hypothermic preservation (204). This intermediate period of normothermic preservation restored energy metabolism with replenishment of adenosine levels, effectively 'resuscitating' the organ and retaining improved viability compared to kidneys stored under hypothermic conditions. Other groups followed with this 'intermediate' normothermic preservation again finding improved survival compared to hypothermic conditions (221,222).

The concept of resuscitating the kidney has since been adapted for kidneys from marginal and DCD donors. Brasile *et al* found that a period warm *ex-vivo* perfusion at the end of the preservation period could resuscitate the kidney after warm and cold ischaemic injury (17,223). More prolonged normothermic preservation periods have also been more beneficial than hypothermic techniques (198,224).

3.18 Viability assessment

With the restoration of metabolism normothermic preservation also offers the potential to assess viability before transplantation. Various functional tests have been used during normothermic preservation or isolated kidney perfusion to assess viability after different preservation conditions or after ischaemic injury (81,207,225-230) (Table 3.18). In general, low levels of renal function and oxygen consumption were associated with increased ischaemic injury in these experimental models. Normothermic temperatures may allow a more comprehensive assessment compared to hypothermic conditions. The functional measures could be combined with injury markers to predict renal function and avoid the use of non functioning kidneys. Potential parameters are listed in table 3.18.

Viability assessment

Perfusion parameters	*Renal function*	*Tubular function*	*Intracellular enzymes/ molecular markers*
Renal blood flow	Urine output	Filtration fraction	AST, GGT, GST, LDH
Intra-renal resistance	Creatinine levels	Fractional excretion Na+	Free iron
Oxygen consumption	Creatinine clearance	Total protein	KIM-1, NGAL
	Acid base balance		Oxidative damage

Table 3.18 Potential parameters to assess viability during normothermic kidney perfusion.
Aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), glutathione *S*-transferase (GST), lactate dehydrogenase (LDH), Kidney Injury Molecule 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL).

3.19 Kidney manipulation/conditioning

Another clear advantage of restoring metabolism during preservation is the opportunity to manipulate the kidney before transplantation. Brasile *et al* found that the protective gene hemeoxygenase-1 could be up-regulated during warm perfusion after canine kidneys sustained both WI and CI injury (231). They demonstrated that cellular processes should be supported and that kidneys could be 'repaired' after 2 hours of WI with the addition of fibroblast growth factors during EMS perfusion (232). Protein synthesis was also evident during 24 hours of *ex-vivo* perfusion of non transplanted human kidneys.

3.2 Normothermic preservation of other organs

3.21 Heart

Immediate recovery of myocardial function after preservation is essential in heart transplantation. The duration of cold preservation must be limited to 4 to 6 hours before irreversible damage ocurrs. Several groups have developed normothermic preservation techniques for the heart and in one case this has been translated into clinical application. Hassanein *et al* first described the continuous perfusion of porcine donor hearts in a beating state in 1998 (233). They were able to extend the preservation time to 12 hours demonstrating complete preservation of the contractile, metabolic and vasomotor function compared to hearts that were statically stored in UW solution for the same period. They used a leukocyte depleted blood based solution supplemented with a preservation based solution. Hearts were perfused on an extracorporeal circuit and maintained in a beating state. This research group has gone on to develop a commercially available device, The Organ Care System (OCS) (Transmedics US). Phase I clinical trials are ongoing mainly in Europe and the US (234,235). Results so far have shown good patient survival with the use of this technique.

Other research groups have carried out experimental studies examining perfusion pressures (40-50mmHg) and have avoided the use of cardioplegia and hypothermia altogether. This has allowed more prolonged preservation with less damage to the endothelium when compared with higher perfusion pressures (80-90mmHg) and hypothermic storage (236).

3.22 Lung

Lung transplantation is restricted by the lack of suitable organs with only 20% of all potential donor lungs being transplanted. To meet the growing demand many transplant centres have extended the criteria and considered lungs from marginal and DCD donors. Normothermic *ex-vivo* lung perfusion has been particularly beneficial in re-conditioning and assessing the function of lungs that were deemed unsuitable for transplantation (237). The first reported *ex-vivo* re-conditioning of a NHB donor lung followed by transplantation was carried out by Steen *et al* in Lund, Sweden in 2000 (238). Lungs were perfused with Steen solution (Vitrolife, Uppsala, Sweden), a physiological electrolyte solution containing human serum albumin and dextran mixed with ABO compatible erythrocytes that had been irradiated, leukocyte-filtered and washed. Imipenem, insulin and heparin were added and isotonic trometamol was used as a buffer to adjust the pH to 7.4. The perfusion system (Medtronics) incorporated a centrifugal pump, oxygenator and ventilator. The system is used in conjunction with hypothermic preservation and serves to provide a period of resuscitation and assessment to determine the viability and judge the suitability for transplantation. Other research groups have used a similar technique called *ex-vivo* lung perfusion (EVLP) (239-242). Their research has concentrated on extending the preservation time up to 12 hours. Previous attempts at prolonged NP resulted in the inability to maintain normal integrity and barrier functions of the vasculature and epithelial beds leading to progressive deterioration in the vascular flow and increased oedema. The development of the technology and use of Steen solution has allowed the lung to be perfused successfully without deterioration for these more prolonged periods. In clinical

practice, several series of *ex-vivo* lung perfusion have been carried using short periods of perfusion (2-4h) as a means to recover and assess function prior to transplantation (242). The commercially available OCS developed for the heart has recently been extended for use in the lung also (243).

3.23 Liver

The liver can tolerate periods of hypothermic preservation for up to 18 hours however this is significantly reduced in DCD donor organs. Alternative methods of preservation may therefore be particularly important to reduce injury and expand the donor pool. There is some experimental evidence to suggest that normothermic liver preservation has an advantage compared to hypothermic techniques. Porcine livers have successfully been perfused after warm ischaemic injury and the preservation period extended beyond the limits of cold preservation. (190,244). In one study, function and viability was maintained over a 72 hour period (245).

The technology used for normothermic liver perfusion is again reliant on cardiopulmonary bypass technology. The system reported by Friend *et al* uses two parallel circuits to perfuse the liver under a physiological environment (190, 246). Other research groups have conditioned the liver with a short period of normothermic perfusion (30 minutes) to increase mitochondrial ATP (247) . Longer periods of 3 to 4 hours of perfusion have also been shown to resuscitate the liver after warm ischaemic injury (248). However, the application of normothermic perfusion in clinical liver transplantation is still awaited.

3.24 Kidney

Normothermic kidney preservation has been dominated by the work of Lauren Brasile and colleagues since the late 1990s as described in previous chapters. This research group has successfully used a technique of normothermic preservation to recover damaged kidneys using short resuscitation and prolonged periods of NP using an acellular tissue culture like medium (198,231,232,249-251). They have also used the system to deliver therapies and promote repair. Despite the abundance of research, normothermic preservation has not yet been used in clinical kidney transplantation. The use of a blood based perfusate and ulitilsation of cardiac bypass technology may be a more feasible approach to translating the technique into clinical practice.

3.25 Outcome

The development of cardiac bypass technology has encouraged the adaptation of normothermic techniques which have been applied to the heart, liver and lung. Experimentally, these normothermic techniques have proved beneficial in reducing ischaemic injury. The clinical application in the heart and lung has preceded the liver and kidney. This is perhaps due to the sensitivity of the heart and lung to hypothermic injury, whereas the liver and kidney are considered more robust and tolerate longer periods of hypothermic preservation. Nonetheless, with increasing reliance on organs from DCD and ECD donors, preservation of all organs for transplantation needs to be improved. Normothermic kidney preservation offers many potential advantages over hypothermic conditions. Whether applied before retrieval, for the duration of the preservation period or in combination with hypothermic techniques, it can be used to limit the injury caused by preservation.

Chapter 4

Hypothesis and Aim

Chapter 4: Hypothesis and Aim

4.1 Hypothesis

Some of the detrimental effects of warm and cold ischaemic injury in kidney transplantation can be ameliorated by a short period of normothermic perfusion.

4.2 Aims

I: To assess the effects of varying periods of warm and cold ischaemic injury on the acute phase of reperfusion using an *ex-vivo* kidney model.

II: To develop a normothermic kidney perfusion technique and compare its effects to the traditional static cold storage and hypothermic machine perfusion techniques using an *ex-vivo* model.

III: To examine the effects of normothermic perfusion on kidney graft survival and function in an *in-vivo* large animal model.

IV: To translate normothermic perfusion into clinical practice for kidneys from marginal donors and to assess the safety and feasibility of the technique.

4.3 Summary of experimental design

The research is divided into four experimental projects.

I: An *ex-vivo* model to assess the effects of varying periods of warm and cold ischaemic injury.

II: An *ex-vivo* model to assess the effect of normothermic perfusion.

III: An autotransplant model to examine the effects of normothermic perfusion on graft survival and function.

IV: A clinical series of normothermic perfusion for kidneys from marginal donors.

The experimental design including warm and cold ischaemic intervals and preservation conditions are outlined in Table 4.3.

4.3 Summary of experimental design

Study	WI (minutes)	CI (hours)	Preservation solution	Preservation technique
Ex-vivo reperfusion model (n = 6 kidneys per group)				
I Effects of WI and CI	0, 10, 25	2, 18	HOC	CS
II Effects of NP	10	2, 18	HOC KPS-1 LDB	CS HMP NP
Autotransplant model (n = 6 animals per group)				
III Feasibility of NP	30	22	KPS-1 LDB	HMP HMP (20h) +NP (2h)
Human Kidneys (n = 15)				
IV NP in clinical practice	(0,17, 30)	(9 – 20.10)	PRBC	NP (35m-100m)

Table 4.3 Experimental design. *Ex-vivo* reperfusion model **I** The effects of warm and cold ischaemia **II** The effects of NP. Autotransplant model **III** feasibility of NP. Human kidneys, **IV** NP in clinical practice.
Warm ischaemia (WI), cold ischaemia (CI), hyperosmolar citrate (HOC), cold storage (CS) and hypothermic machine perfusion (HMP), kidney perfusion solution 1 (KPS-1), normothermic perfusion (NP), leukocyte depleted blood (LDB), packed red blood cells (PRBC).

Chapter 5

The effects of warm and cold ischaemic injury in an *ex-vivo* model of kidney transplantation

Chapter 5: The effects of warm and cold ischaemic injury in an *ex-vivo* model of kidney transplantation

5.1 Introduction

Kidneys from DCD donors represent a valuable additional source of organs for transplantation and encouragingly, graft survival at 1, 5, and 10 years post transplant has been found to be comparable to those of DBD kidneys (32, 34). However, high rates of both DGF (40 –85%) (7, 8, 16, 25, 26) and PNF (9 –22%) (28, 30, 252) complicates the early management of these patients. Renal transplantation from any donor source can result in slow initial graft function, DGF, or even PNF; this escalating sequence of outcomes is undoubtedly related to the duration, and therefore severity, of ischaemic injury. Nonetheless, the relative importance of warm and cold ischaemia is still not fully determined and we do not know how much WI the human kidney can tolerate without loss of viability. In the UK the average duration of WI in the controlled DCD donor is approximately 12 to 19 minutes and in the uncontrolled 25 minutes (15, 16). The average preservation period is approximately 17.7 hours (14.5-21.5 hours) (15). The combined effects of hypothermic preservation after a period of WI are still incompletely understood. The aim of this study was to determine the effects of combining varying degrees of warm and cold ischaemia injury.

5.12 Experimental design (Figure 5.12)

A porcine heart beating model was used for retrieval of kidneys without WI injury and a DCD model for kidneys subject to 10 and 25 minutes of WI. Kidneys were flushed *in-situ* and retrieved with 0 minute WI, or removed 10 or 25 minutes after death, before being flushed with cold preservation solution. Kidneys were then stored on ice for either 2 or 18 hours. After CS, kidneys in all groups were reperfused *ex-vivo* with autologous blood at normal body temperature on an isolated organ perfusion system for 3 hours to assess the effects of acute reperfusion injury (Figure 5.12)

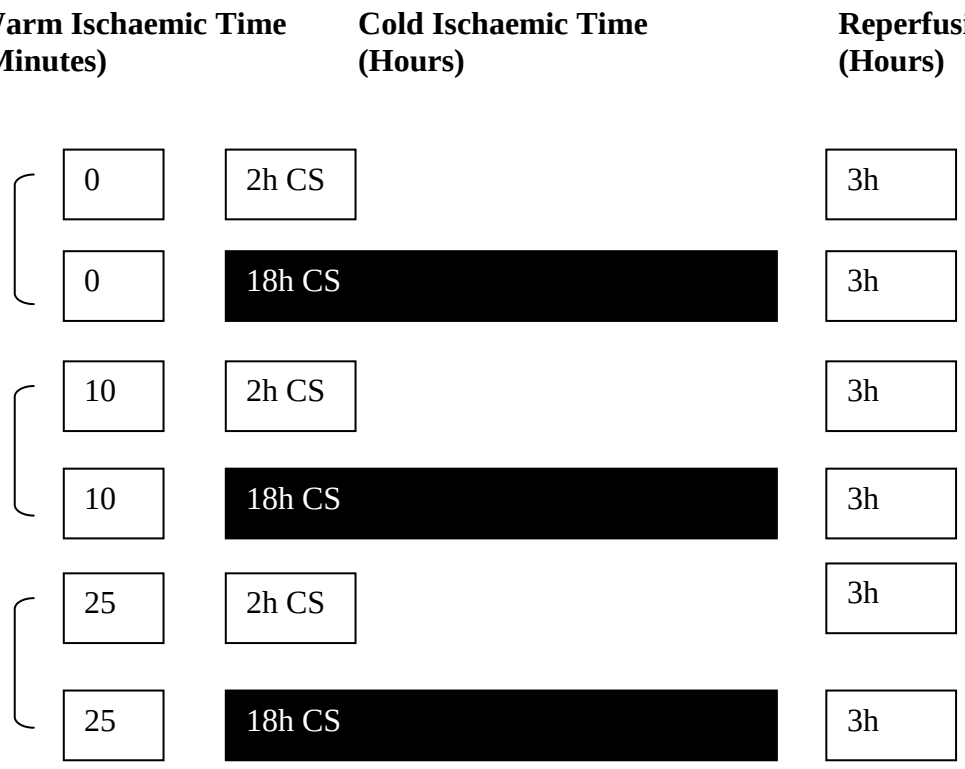


Figure 5.12 Experimental design. The effects of warm and cold ischaemic injury (n = 5 kidneys in the 0 2h and 0 18h CS groups and n = 6 in the other groups) [0 (2h), 0 (18h), 10 (2h), 10 (18h), 25 (2h), 2 (18h)].

5.2 Methods

5.21 Heart beating model of the deceased donor

Kidney Retrieval

Under the Home Office Scientific Procedures Act (1986), large white pigs weighing 40–50kg were sedated then anesthetized with sodium thiopentone (5 mg/kg; Vetofol, Norbrook, United Kingdom), intubated and ventilated with oxygen and isoflurane. A central line was surgically placed in the left external jugular vein for vascular access. One litre of Hartmann's solution (Baxter Healthcare. Thetford, UK) was administered followed by the withdrawal of 1 litre of blood directly into citrate-phosphate-dextrose-adenine (CPDA)-1 blood collection bags (Baxter Healthcare) and stored at 4ºC.
A midline laparotomy was performed and the kidneys and large vessels exposed. A double-balloon catheter (Porges, France) was inserted into the iliac artery and advanced into the aorta and inflated. An isolated segment of aorta was then perfused by gravitational a hydrostatic pressure of 100cmH_2O with hyperosmolar citrate solution, (HOC) (Soltran; Baxter Healthcare) at 4°C, allowing rapid perfusion of the kidneys. A large vessel cannula was inserted into the vena cava to allow venous drainage. The animal was then exsanguinated via the caval cannula and the kidneys removed *en bloc*. The kidneys were placed in a sterile bag with approximately 300ml of preservation solution (HOC) and stored in a polystyrene organ retrieval box and packed in crushed ice.

5.22 Donation after circulatory death model

Kidney Retrieval

All experiments were in accordance with the Home Office animals (Scientific Procedures) Act 1986. Under schedule 1 method, large white pigs (60-70kg) were sacrificed by electrocution followed by exsanguination. Approximately 2 litres of blood was collected from the cervical incision into a sterile receptacle containing 25,000 units of heparin (Multiparin®; CP Pharmaceuticals, Wrexham, UK). The blood was then transferred back to the laboratory and placed into CPDA-1 blood bags (Baxter Healthcare) for storage at 4ºC.

Immediately after death a midline incision was made and the kidneys dissected from their retroperitoneal space. The ureter was dissected and ligated leaving a length of approximately 6cm. The renal artery and vein were exposed and dissected distally to the aorta and vena cava. The kidneys were removed *en bloc* with a patch of aorta and vena cava before dissection on the back table. The kidneys were immersed in iced HOC solution. A Tibbs cannula (Bolton Surgical, Sheffield, UK) attached to a blood giving set (Coden 4mm) and preservation solution at 4ºC was placed and secured in the renal artery. The kidneys were then flushed with the 500ml of HOC solution infused at a hydrostatic pressure of 100 cmH_2O. The WI interval was standardized to either 10 or 25 minutes and the kidneys left in the abdomen for this period. After flushing the kidneys were placed in a sterile bag with approximately 300ml of preservation solution

(HOC) and stored in a polystyrene organ retrieval box and packed in crushed ice for transportation back to the laboratory.

5.23 *Ex-vivo* Reperfusion model

An isolated organ perfusion system was used to reperfuse the kidneys after the preservation period to assess the effects of acute reperfusion injury.

The system was designed using paediatric cardiopulmonary bypass technology (Bioconsole 550, Medtronic, Watford, UK) (Figure 5.23a,b) adapted to allow the isolated perfusion of a single kidney. The system incorporated a paediatric centrifugal pump, flow probe (BP50), pressure transducer, heat exchanger (Grant Instruments, Cambridge, UK) and temperature probe (Acorn, Oakton Instruments,Vernon Hills, IL, USA). Two Gemini PC-2 infusion pumps (Imed, San Diego, CA. USA) were used to infuse a nutrient solution, Ringer's solution and 5% glucose into the system (Table 5.24).

The disposable perfusion set was customised for kidney perfusion (Medtronic). It incorporated the biohead pump, venous reservoir, polyvinyl chloride (PVC) tubing, and membrane oxygenator (minimaxplus, Medtronic). A custom made glass chamber (University of Leicester) was used to house the kidney during perfusion.

Isolated Organ Perfusion System

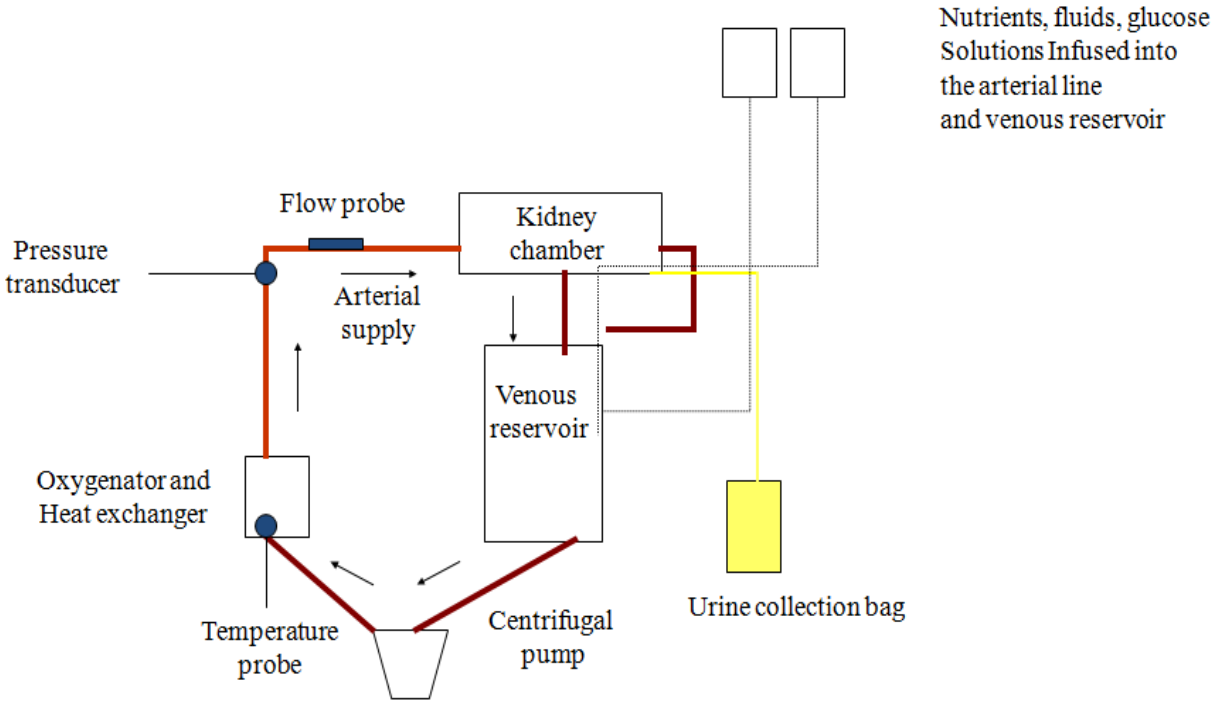


Figure 5.23a Schematic diagram of the isolated organ perfusion system. The system hardware was based on paediatric cardiopulmonary bypass technology. The renal artery, vein and ureter were cannulated and the kidney placed in the kidney chamber. The perfusate was then pumped from the venous reservoir via the centrifugal pump into the membrane oxygenator and heat exchanger before it entered the arterial arm of the circuit. The oxygenated and warmed blood entered the kidney via the renal artery and then was allowed to drain from the vein back into the reservoir were it was re-circulated. The arterial pressure was fixed allowing the kidney to autoregulate its own blood flow. The urine was collected into a urinometer bag. Nutrients, fluids and glucose were continually infused into the circuit at set rate.

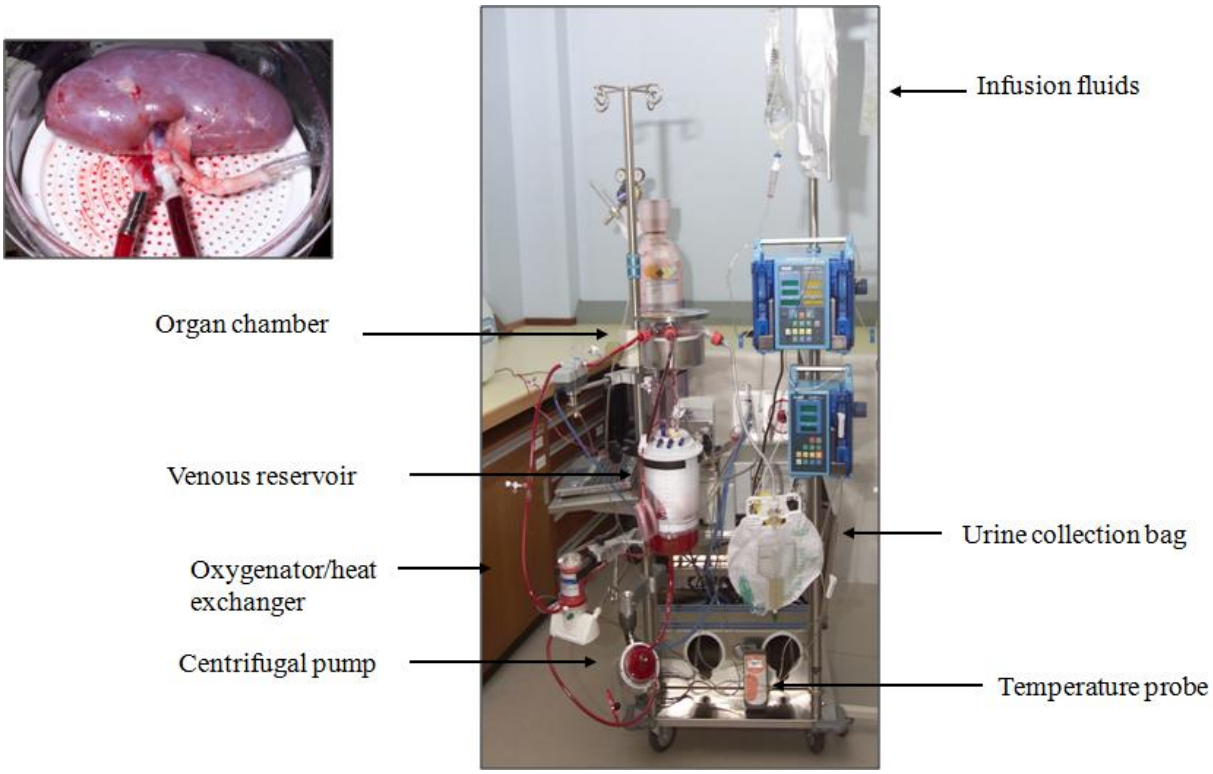


Figure 5.23b Photograph of the isolated organ perfusion system with the main component parts highlighted and the kidney pictured in the perfusion chamber with the renal artery, vein and ureter cannulated.

5.24 Kidney preparation and perfusion system

The renal artery, vein and ureter were cannulated with soft silastic catheters (Pennine, UK).The kidney was placed in the organ perfusion chamber. The circuit was primed with 500ml of perfusate solution followed by the addition of 500ml of autologous whole blood (Table 5.24) to give a total circulating volume of 1 litre. Dilution of the blood with an isotonic solution reduced the viscosity of the perfusate and improved capillary blood distribution in this model. The perfusate was oxygenated with 95% O_2/ 5% CO_2 at 0.5L/min and allowed to circulate at a temperature of 38ºC. Kidneys were perfused at a set mean arterial pressure of 85mmHg and supplements were infused into the venous reservoir and arterial arm of the circuit for resemble reperfusion (Table 5.24). The system had no ability to metabolically produce creatinine, therefore 1000µmol/L creatinine (Sigma-Aldrich, Steinheim, Germany) was added to the circuit so that serum creatinine fall and creatinine clearance could be measured as markers of renal function. Urine was collected after each hour and the volume replaced with Ringer's lactate solution.

Components	
Reperfusion mode	
Autologous whole blood	500 ml
Ringer's lactate solution (Baxter Healthcare, Thetford, UK)	500 ml
Mannitol 10% (Sigma-Aldrich)	5g
Cefuroxime 750mg (Stragen, Reigate, UK)	5 ml
Sodium bicarbonate 8.4% (Fresenius Kabi, Cheshire, UK)	12 ml
Heparin 1000iu/ml (CP Pharmaceuticals, Wrexham, UK)	2 ml
Creatinine (Sigma)	1000μmol/L
Supplements	
Nutriflex infusion (B Braun, Sheffield, UK) with the following added;	20 ml/hr
Insulin (Novo Nordisk, Denmark)	100 units
Sodium bicarbonate 8.4% (Fresenius Kabi)	25 ml
Glucose 5% (Baxter Healthcare)	7 ml/hr
Ringer's lactate solution to replace urine output ml for ml	

Table 5.24 Components of the perfusate solution and supplements added to the circuit during *ex-vivo* reperfusion.

5.25 Reperfusion outcome measurements

- Renal blood flow (RBF) and mean arterial pressure (MAP) were recorded continuously and intra-renal resistance (IRR) calculated (MAP/RBF). Urine output was also measured hourly during reperfusion.

- Plasma levels of sodium, potassium, creatinine, glucose, asparatate transaminase (AST) and lactate dehydrogenase (LDH) were measured by the Biochemistry Department (University Hospital of Leicester NHS Trust).

- Urine levels of sodium, potassium, creatinine, glucose were also measured by the Biochemistry Department (University Hospital of Leicester NHS Trust).

- Blood gas analysis (Blood Gas Analyser; Rapidlab 248, Bayer Corp, East Walpole, MA. USA) was used to record arterial and venous partial pressure of oxygen (P_aO_2) carbon dioxide (P_aCO_2), oxygen saturation (SO_2), pH, bicarbonate and base excess.

Biochemical analysis of plasma and urine samples where used to measure the following:

- Serum creatinine fall
- Creatinine clearance (urinary creatinine x urinary volume / plasma creatinine),
- Fractional excretion of sodium [(urinary sodium x urine volume)/(creatinine clearance x plasma sodium) x 100)]
- Oxygen consumption [(arterial P_aO_2 – venous P_vO_2) x flow rate/weight]

Plasma samples were taken pre and post 3 hours of reperfusion and urine samples after 3 hours of reperfusion. Samples were centrifuged at 1000g for 15 minutes at 4ºC, the supernatant collected and either sent for biochemical analysis or stored at -80ºC until analyses of the following injury parameters (Summary; Appendix 10.1).

5. 26 Injury markers

Lipid peroxidation (8-isoprostane)

Urine levels of 8-isoprostane were determined by ELISA (Cayman Chemical Co, MI, USA). Samples were defrosted and centrifuged at 10,000g for 2 minutes and the supernatant taken for analysis. Urine samples were diluted 10 fold prior to analysis. The samples and standards were added in duplicate to the ELISA plate together with an 8-isoprostane-acetylcholinesterase (AChE) conjugate and incubated for 18 hours at 4ºC. During incubation any 8-isoprostane present in the sample competed with the 8-isoprostane AChE conjugate for the 8-isoprostane rabbit antiserum binding sites on the pre-coated plate. The plate was then washed and developed by the addition of the substrate to AChE. The plate was read at 405nm after colour development for 90 minutes.

Inflammation (Interleukin-6 and Tumour Necrosis Factor alpha)

Urine levels of interleukin 6 (IL-6) and tumour necrosis factor alpha (TNFα) were determined by the quantitative sandwich enzyme immunoassay technique (R&D Systems. Minneapolis, MN, USA). The samples and standards were added in duplicate to the pre coated ELISA plate and incubated for 2 hours (IL-6; polyclonal antibody specific for porcine IL-6 or TNFα polyclonal antibody specific for porcine TNFα). After washing an enzyme-linked polyclonal antibody specific to IL-6 or TNFα was added. After a further incubation period of 2 hours the plate was washed and developed

with the addition of a substrate solution. A stop solution was then added and the plate read at 450nm.

Endothelial cell injury (Endothelin- 1)

Urine levels of endothelin 1 (ET-1) were determined using an enzyme immunometric assay (EIA) kit (Assay Designs, Michigan. USA). The samples and standards were added in duplicate to the pre-coated plate and incubated for 1 hour at room temperature (monoclonal antibody specific for ET-1). After washing, the HRP labelled monoclonal antibody to ET-1 was added and incubated for 30 minutes at room temperature. A substrate solution was added after washing then incubated for a further 30 minutes at room temperature. A stop solution was added to stop the substrate reaction and the optical density read at 450nm using a spectrophotometer. The concentration was calculated using the standards.

5.27 Tissue samples

Wedge biopsies were taken post-storage and after 3 hours of reperfusion. Samples were either fixed in 10% formal saline or snap frozen in liquid nitrogen (Summary; Appendix 10.1).

Formalin fixed tissue

Tissue was fixed in 10% formal saline for 24 hours, paraffin processed, embedded in wax then sectioned (4μm) and mounted onto slides. The slides were stained with Haematoxylin & Eosin as follows:

Haematoxylin & Eosin

1: De-paraffinize and hydrate sections to deionized water

5 min Xylene, 5 min Xylene, 3 min 99% industrial myelated spirits (IMS), 3 min 95% IMS, 3 min 90% IMS, 5 min Tap water

2: 1 min Hematoxylin (Sigma Aldrich, Dorset, UK)

3: Rinse with tap water until clear

4: 1 min Eosin (Sigma Aldrich, Dorset, UK)

5: Rinse with tap water until clear

6: Dehydrate: 3 min 90% IMS, 3 min 95% IMS, 3 min 99% IMS, 3 min Xylene, 3 min Xylene

9: Mount slides with Xylene based mounting media

5.28 Morphological injury

Sections were evaluation using light microscopy. They were scored over ten fields, assessing changes in the following morphological variables; tubular dilation, tubular epithelial loss, tubular debris, vacuolation, and glomerular shrinkage. Samples were semi-quantitatively scored by two blinded assessors from 0 to 3 according to the level of damage; 0 representing normal, 1 mild, 2 moderate and 3 severe morphological changes. The mean of each sample was then recorded.

5.29 Statistics

Group size was determined by power calculations based on previous *ex-vivo* experiments and statistical advice. A pilot study was carried out in which significant differences in multiple parameters were seen between organs perfused with leukocyte depleted and normal blood using groups of 8 kidneys. Creatinine clearance is one of the key functional assessment parameters of renal viability. In the pilot study creatinine clearance in the leukocyte depleted group was 10.6 ± 2.8 ml/min/100g compared with 1.9 ± 1.0 ml/min/100g in the normal blood group (P = 0.002). Power analysis indicated that 6 kidneys in each group are required to have an 80% chance of detecting a difference in mean glomerular filtration rate of 1ml/min/100g at the level of 5% level of significance using an unpaired t-test.

Values are presented as mean ± S.D. The area under the curve (AUC) for individual perfusion experiments was calculated using Excel® software (Microsoft, Reading, UK) and Graphpad Prism (GraphPad Software, San Diego California USA).
Mean values and mean AUC values were compared using the Kruskal-Wallis test with Dunn's post-test or Mann Whitney U test were appropriate (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, California USA). $P<0.05$ was taken as statistically significant. Correlations between reperfusion parameters and WI time were made with Spearman's non-parametric rank correlation.

5.3 Results

5.31 Reperfusion

Haemodynamics

No significant differences in the renal blood flow or intra-renal resistance were found in kidneys with 0, 10 and 25 minutes of WI after 2 hours of CS (RBF P = 0.071, IRR P > 0.05; Figure 5.31a,b). However, after 18 hours of CS the renal blood flow was significantly reduced throughout reperfusion in the kidneys with 10 and 25 minutes WI compared to kidneys without WI (P = 0.0001; Figure 5.31a). AUC Levels of intra-renal resistance were significantly higher in kidneys with 25 minutes WI after 18 hours of CS compared to kidneys without WI injury (P = 0.0015; Figure 5.31b).

Oxygen consumption was slightly reduced in kidneys with 10 and 25 minutes WI after 18 hours CS. There was a significant reduction in the levels of oxygen consumption in kidneys with 10 minutes WI after 18 hours CS compared to those stored for 2 hours (P = 0.004; Table 5.31).

Renal Function

Serum creatinine fell to a similar level in kidneys with 0, 10 and 25 minutes of WI after 2 hours of CS (P = 0.151; Figure 5.31c). However, after 18 hours of CS serum creatinine levels were significantly higher in the kidneys with 10 and 25 minutes WI compared to kidneys without WI (P = 0.0002; Figure 5.31c).

There were no significant differences in the levels of creatinine clearance after 2 hours of CS between the groups, although levels were numerically lower in kidneys with 25 minutes WI ($P > 0.05$; Figure 5.31d). After 18 hours of CS creatinine clearance was significantly reduced after 10 and 25 minutes WI compared to the kidneys without WI ($P = 0.0001$; Figure 5.31d).

Tubular Function

Low levels of fractional excretion of sodium were recorded in all of the kidneys stored for 2 hours. However, after 18 hours of CS excretion levels were significantly higher in kidneys with 10 and 25 minutes WI compared to the kidneys without WI ($P = 0.0001$; Figure 5.31e).

There was no significant difference in the total amount of urine produced in any of the groups of kidneys that underwent 2 hours CS ($P > 0.05$). However, after 18 hours CS, kidneys with 10 and 25 minutes of WI produced significantly less urine during reperfusion ($P = 0.001$; Figure 5.31f).

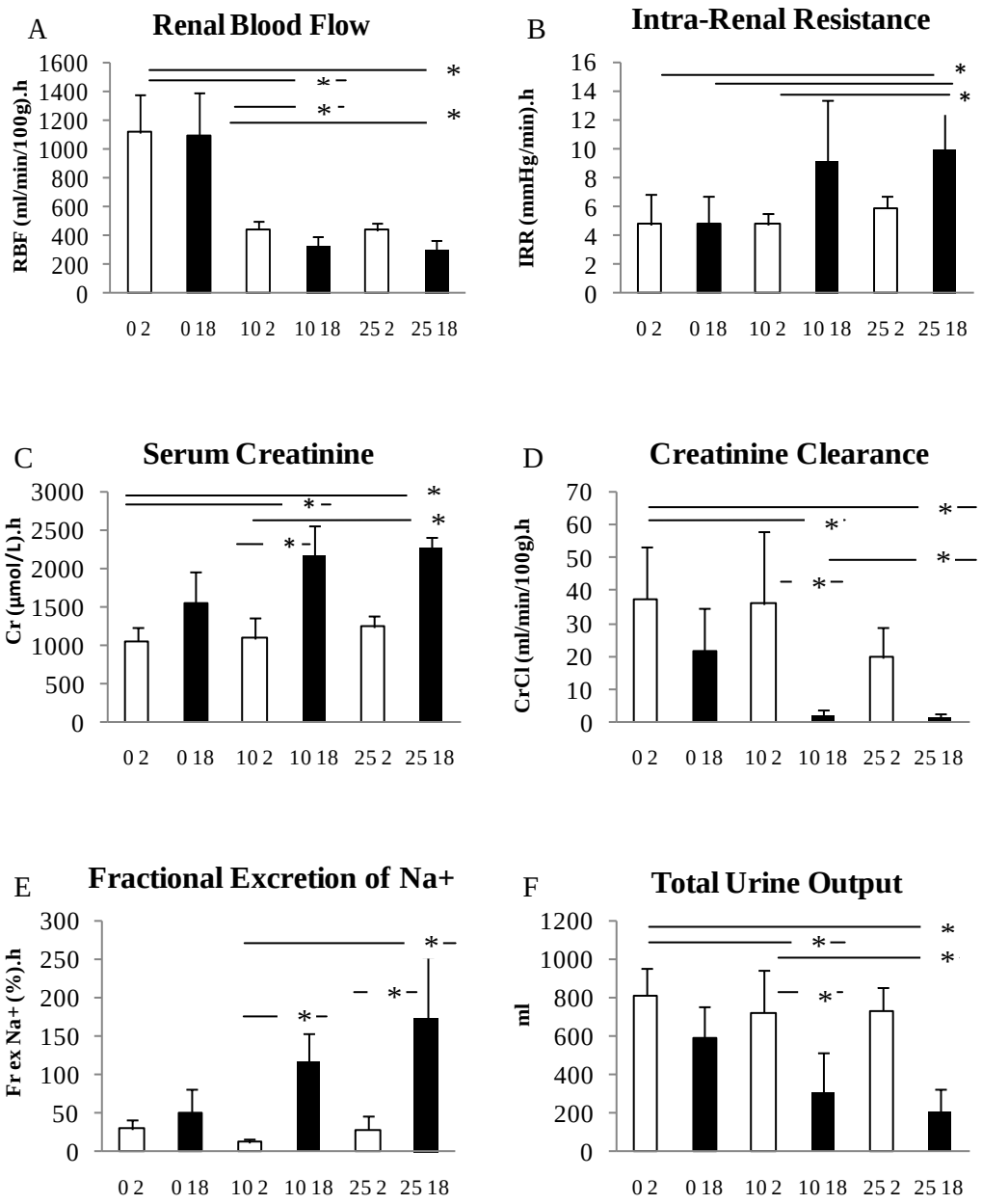


Figure 5.31 Outcome of kidneys undergoing 3 hours of *ex-vivo* reperfusion with oxygenated autologous blood on the isolated organ perfusion system after warm ischaemic (WI) periods of 0, 10 and 25 minutes and 2 and 18 hours of cold storage (CS). Values are mean ± SD and presented as area under the curve (AUC); (A) renal blood flow (B) intra-renal resistance (C) serum creatinine (D) creatinine clearance (E) fractional excretion of sodium and (F) total urine output. (Kruskal Wallis with post test) (**P<0.05,10 +25 (18) vs 0 (2) + 0 (18), figures a,c,d,e,f) (**P <0.05 25 (18) vs 0 + 10 (2) + (18), figure b).

Acid base balance

Kidneys cold stored for 2 hours maintained a relatively normal level of pH and acid base homeostasis during the 3 hours of reperfusion. However, when the CS time was extended to 18 hours, kidneys with 25 minutes WI showed a significant reduction in pH and bicarbonate levels indicating acidosis (Table 5.31).

Serum potassium levels increased steadily during reperfusion in kidneys with 10 and 25 minutes WI after 18 hours of CS ($P = 0.0002$; Table 5.31).

Cellular Injury

Serum aspartate aminotransferase (AST) levels increased during 3 hours of reperfusion in all kidneys after both 2 and 18 hours of CS. Levels were significantly higher in kidneys with 10 and 25 minutes WI after 18 hours CS ($P = 0.001$; Table 5.31).

Serum levels of lactate dehydrogenase also increased during reperfusion in all groups of kidneys but no statistical differences were found between any of the groups (Table 5.31).

Perfusion parameters after 3 hours of reperfusion in each of the groups

Parameters at 3h reperfusion:	0 (2)	0 (18)	10 (2)	10 (18)	25 (2)	25 (18)	P value
O_2 (ml/min/g)	75.0 ± 34.8	102.4 ±46.9*	40.0 ± 9.4	23.7 ± 11.9	43.1 ± 10.8	32.5 ± 13.6	0.004
pH	7.41 ± 0.04	7.37 ± 0.06**	7.40 ± 0.08	7.37 ± 0.07	7.49 ± 0.03	7.31 ± 0.09	0.002
Bicarbonate (mmol/L)	21.8 ± 2.0	20.9 ± 3.0¶	23.0 ± 4.9	21.6 ± 3.7	26.2 ± 3.9	17.6 ± 3.5¶¶	0.001
Potassium (mmol/L)	3.8 ± 1.0***	4.5 ± 1.5	4.7 ± 0.7	10.0 ± 0	4.6 ± 0.9	10.0 ± 2.5	0.001
AST (iu/L)	39.6 ± 13.2†	38.2 ± 6.1††	57.5 ± 11.5	125.3 ± 40.1	127.5 ± 37.3	142.2 ± 39.9	0.001
LDH (iu/L)	323.2 ± 35.7	361.4 ± 41.6	331.2 ± 38.2	381.5 ± 28.2	371.5 ± 20.3	361.3 ± 37.5	0.085

Table 5.31 Parameters after 3 hours of *ex-vivo* reperfusion with oxygenated autologous blood on an isolated organ perfusion system after 0, 10 and 25 minutes of warm ischaemia (WI) and 2 and 18 hours of cold storage (CS). Oxygen consumption (O_2), pH, bicarbonate, serum potassium, asparatate transaminase (AST) and lactate dehydrogenase (LDH) levels. Value are mean ± SD (Kruskal Wallis with post test) * P < 0.05 0 (18) vs 10 (18), ** P < 0.05 0 (18) vs 10 (18) and 0 (18) vs 10 (2), ¶ P = < 0.05 0 (18) vs 10 (2), ¶¶ P < 0.05 25 (18) vs 10 (2), 10 (18), *** P < 0.05 0 (2) vs 10 (18) and 25 (18), † P = < 0.05 0 (2) vs 10 (18), 25 (2) and 25 (18). †† P < 0.05 0 (18) vs 10 (18) and 25 (18).

Correlations

The level of renal blood flow, intra-renal resistance and creatinine clearance correlated significantly with the duration of WI injury (Table 5.311).

	RBF	**IRR**	**Cr**	**CrCl**	**Fr Na+**
WI	r = 0.6404	r = 0.5269	r = 0.312	r = -0.4360	r = 0.2310
P value	< 0.0001	0.001	0.072	0.01	0.189

Table 5.311 Correlations between the WI (WI) time and AUC renal blood flow (RBF), intra-renal resistance (IRR), serum creatinine (Cr), creatinine clearance (CrCl) and fractional excretion of sodium (Fr Na+). Correlations between reperfusion parameters and WI time were made with Spearman's nonparametric rank correlation.
$P < 0.05$ was taken as significant.

5.32 Injury markers

Oxidative damage/Inflammation

There were no significant differences in the urine levels of 8-isoprostane, a marker of lipid peroxidation after 3 hours of reperfusion between the groups (P = 0.222; Figure 5.321a). Endothelial injury measured using urine levels of ET-1 were significantly higher in kidneys with 25 minutes WI after 18 hours of CS compared to kidneys without WI injury after 2 and 18 hours of CS (P = 0.002; Figure 5.321b).

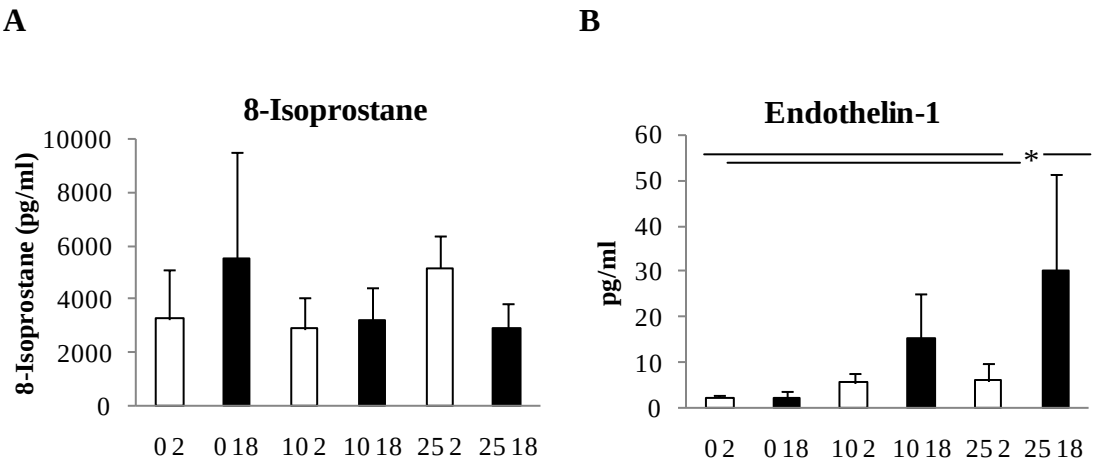


Figure 5.321 Urine levels of (A) 8-isoprostane and (B) endothelin 1 (ET-1) measured by ELISA after 3 hours of *ex-vivo* reperfusion in each of the experimental groups. Values are mean ± SD. (Kruskal Wallis with post test). (B) [**P = 0.002, 25 (18) vs 0 (2) and (18)].

Inflammation was also more severe in kidneys with 10 and 25 minutes WI and 18 hours CS with significantly higher levels of TNFα in the urine after 3 hours of reperfusion compared to kidneys without WI and 10 and 25 minutes of WI and just 2 hours CS (P = 0.001; Figure 5.322a). Levels of IL-6 after 3 hours of reperfusion were lower in kidneys with 10 minutes WI and 2 hours CS compared to kidneys with 10 and 25 minute WI and 18 hours CS (P = 0.001; Figure 5.322b).

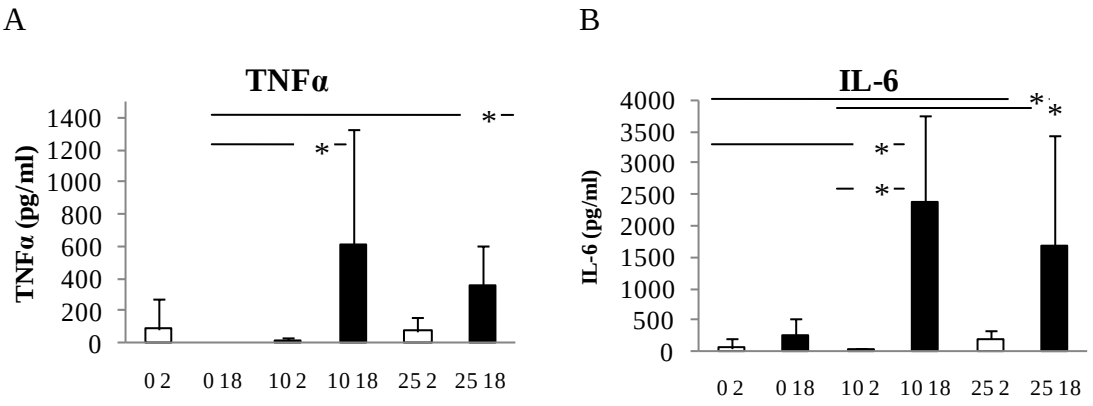
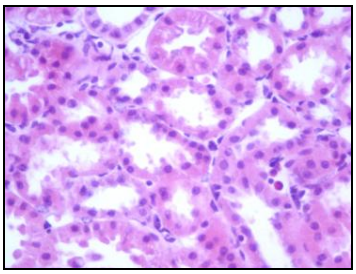


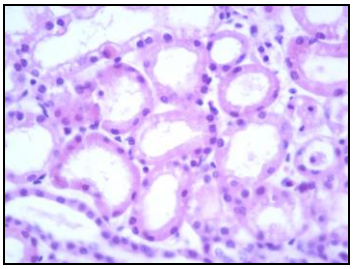
Figure 5.322 Urine levels of (A) TNFα and (B) IL-6 measured by ELISA after 3 hours of *ex-vivo* reperfusion in each of the experimental groups. Values are mean ± SD. (Kruskal Wallis with post test). (A) (**P = 0.001, 10 (2) vs 10 and 25 (18). (B)[**P = 0.001, 10 + 25 (18) vs 0,10, 25 (2) and 0 (18)].

Histology

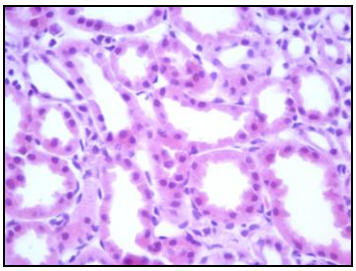
There were no significant differences in the morphology in the post-storage biopsies ($P < 0.05$; Appendix 10.2; Table 10.2a; Figure 5. 323a-l).). There was an increase in cellular injury in all groups after reperfusion ($P < 0.05$) with a significant increase in tubular debris and vacuolation in the 25 min WI 2h CS group compared to the 10 min WI 2h CS group ($P = 0.037, 0.003$; Appendix 10.2; Table 10.2b; Figure 5.323a-l).



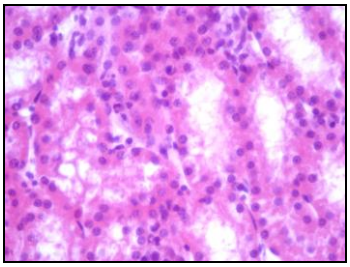
A: 0 min WI 2h CS post-storage



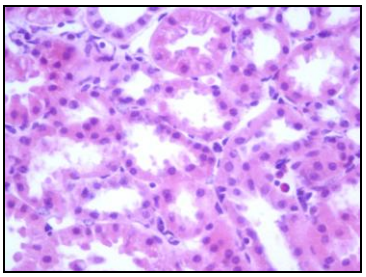
B: 0 min WI 2h CS post-reperfusion



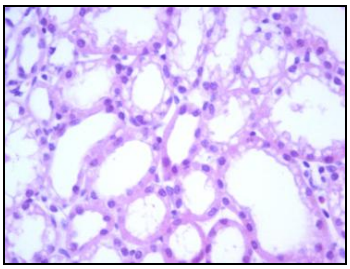
C: 0 min WI 18h CS post-storage



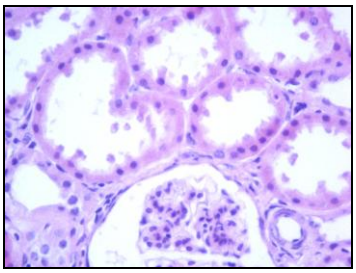
D: 0 min WI 18h CS post-reperfusion



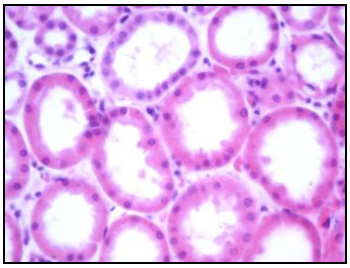
E: 10 min WI 2h CS post-storage



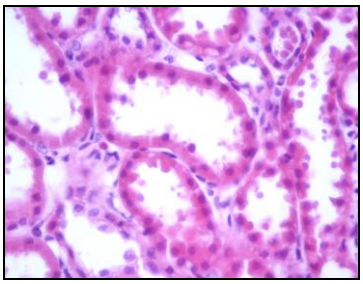
F: 10 min WI 2h CS post-reperfusion



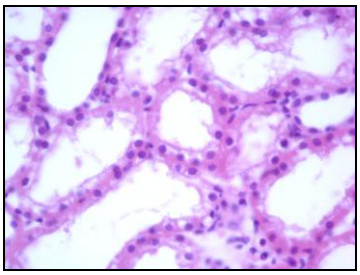
G: 10 min WI 18h CS post-storage



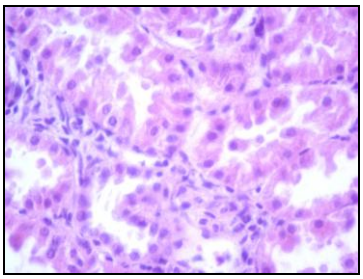
H: 10 min WI 18h CS post-reperfusion



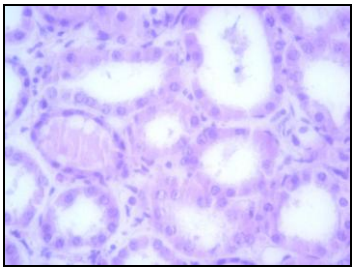
G: 25 min WI 2h CS post-storage



H: 25 min WI 2h CS post-reperfusion



I: 25 min WI 18h CS post-storage



J: 25 min WI 18h CS post-reperfusion

Figure 5.323 a-j Histology. Wedge biopsies were taken post-storage and post-reperfusion and stained with H&E for evaluation using light microscopy after warm ischaemic (WI) periods of 0, 10 and 25 minutes and 2 and 18 hours of cold storage (CS). Sections were scored blinded over ten fields, assessing changes in five morphological variables, tubular dilation, tubular epithelial loss, tubular debris, vacuolation, and glomerular shrinkage. Samples were scored from 0 to 3 according to the level of damage; 0 representing normal, 1 mild, 2 moderate and 3 severe morphological changes.

5.4 Summary

The findings from this study suggest that with a limited period of CS, a WI period of up to 25 minutes does not have a significant impact on renal function compared to kidneys without WI injury. The combination of either 10 or 25 minutes of WI plus 18 hours of CS led to severe impairment of creatinine clearance, renal tubular function and injury, deterioration of the microvasculature and abnormal acid base homeostasis. This suggests that greater attention needs to be given to limiting the cold storage interval or to improve preservation techniques to avoid injury.

The next stage of this research was to assess the effects of reducing the CI time by altering the preservation technique by introducing a period of normothermic perfusion (NP) after hypothermic preservation.

5.5 Discussion

The effects of warm and cold ischaemic injury

This experimental study provides new insights into the effects of renal WI. In the clinical situation a period of cold storage is inevitable and so the effects of WI alone are difficult to determine. The results suggest that large animal kidneys can tolerate a significant period of WI alone without immediate loss of function and that it is the addition of a CI insult that leads to poor function in DCD kidneys.

The results presented are consistent with other experimental and clinical evidence demonstrating that as the duration of CI increases so does the risk of developing early graft dysfunction (253-255). Several experimental models suggest that even after 6 hours of CI time, significant injury occurs (48, 49). Porcine autotransplant models have also shown that prolonging the CI above 24 hours reduces survival (256, 257). The impact of CI has also been demonstrated in a similar bovine model using an acellular normothermic solution (258). Although the emphasis was on repair, there was an initial reduction in metabolism after prolonged CI. The results contradict other studies that suggest that only when the preservation period exceeds 18 hours is the CI time associated with reduced graft survival even in kidneys from ECD and DCD donors (254). However, it is difficult to compare studies due to the number of confounding variables.

The assessment of early kidney graft function is based on serum creatinine levels, reduced urine output and progressive metabolic instability. Close evaluation of the decline in serum creatinine levels is also used as more sensitive measures of graft function (259-261). Although practical and widely used, serum creatinine is not the

most precise marker of kidney injury (262). Levels are dependent on many factors such as age, gender and muscle mass. Furthermore, creatinine secretion through the proximal tubule cells can account for 10-40% of the creatinine clearance value which can mask a decrease in estimated glomerular filtration rate (eGFR) (262).

There has been much emphasis on the development of urinary biomarkers as a means to determine kidney injury at an early stage in order to pre-empt graft dysfunction. An ideal marker should be one that can be obtained non-invasively, is easy to measure and accurately assesses the severity of injury. In this study in addition to the functional outcome, a collective array of urinary biomarkers was used to assess the severity of injury in relation to the defined WI and CI insults; IL-6 and TNFα as markers of inflammation, endothelin-1, a marker of endothelial cell injury and 8-isoprostane as a marker of lipid peroxidation. IL-6 and TNFα are proinflammatory cytokines and important mediators of tissue injury. They are up-regulated in response to ischaemic injury and have been associated with renal dysfunction and acute rejection after transplantation (263-265). Endothelin-1 is a potent vasoconstrictor and known to be up-regulated by ischaemic injury (266). It plays a major role in regulating renal vascular tone and tubular secretion of electrolytes and water. Urinary levels have been found to be increased with chronic kidney disease (267). Lipid peroxidation is a product of the free radical mediated oxidation of arachidonic acid which is associated with membrane damage and is known to instigate pro-inflammatory mediators and stimulate the release of cytokines and chemokines causing tissue injury (268).

This study demonstrated elevated levels of IL-6 and TNFα 3 hours after reperfusion in kidneys with 10 and 25 minutes WI injury after 18 hours CS compared to kidneys without WI and only 2 hours CI injury. Although, the levels were reflective of the functional and metabolic decline, evidence from this and previous studies suggest that these inflammatory markers may not be particularly sensitive to the severity of WI injury (70, 269). Surprisingly, urinary levels of 8-isoprostane did not reflect the degree of ischaemic injury. Elevated levels of lipid peroxidation have been associated with an increased release of free iron during cold ischaemia (270). Proximal tubular cells have also been found to release redox active iron during hypoxia and iron chelators have been successfully used to prevent lipid peroxidation during hypothermic preservation (141, 271). However, other studies have found that the addition of iron chelators during hypothermic storage did not reduce cold ischaemic injury (272).

Levels of ET-1 were only significantly raised after 25 minutes of WI followed by 18 hours cold storage and are therefore, perhaps a more specific indictor of ischaemic injury. The histology in this study was not uniformly representative of the ischaemic injury, possibly due to the short duration of reperfusion and therefore was of limited importance.

Although, the long term outcome was not determined, collectively the decline in renal and tubular cell function, deterioration in metabolic state and increase in intra-renal resistance, in addition to the biomarkers of inflammation and endothelial cell damage reflected the combined degree of warm and cold ischaemic injury.

Conclusion

In this experimental model of DCD and HBD kidneys, WI for periods of 10 and 25 minutes were only detrimental to renal function when kidneys were subsequently preserved in cold storage for 18 hours. Therefore, intensive efforts to limit the duration of the cold storage period or alternative methods of preservation should become a fundamental principle when transplanting kidneys from DCD donors.

Chapter 6

The effects of *ex-vivo* normothermic perfusion

Chapter 6: The effects of *ex-vivo* normothermic perfusion

6.1 Introduction

Since the introduction of hypothermic preservation there has been much debate over whether or not HMP has any benefit over CS techniques in kidney transplantation. Many experimental and clinical studies suggest that HMP is a better method of preservation and is particularly advantageous for kidneys from DCD donors (129, 272, 273). However, there have been some conflicting results (131). The alternative to hypothermic preservation is to maintain the kidney at a relatively normal body temperature. Brasile *et al* found that if kidneys underwent a period of NP with an acellular based solution at 32ºC some of the detrimental effects of CI injury could be reversed (17,258). The aim of this laboratory study was to assess the effects of a short period of normothermic perfusion (NP) after CS, compared to the traditional CS and HMP techniques.

6.12 Experimental Design (Figure 6.12)

Kidneys were retrieved from large white pigs sacrificed under Home Office Scientific act (1986) schedule 1 guidelines. They were subjected to 10 minutes of WI injury, flushed with cold preservation solution then preserved by either CS for 2 hours (minimal ischaemia) or 18 hours, HMP for 18 hours, or CS for 16 hours followed by 2 hours of NP. After preservation kidneys in all groups were reperfused with autologous blood at normal body temperature on the isolated organ perfusion system for 3 hours to assess the outcome (Table 5.24).

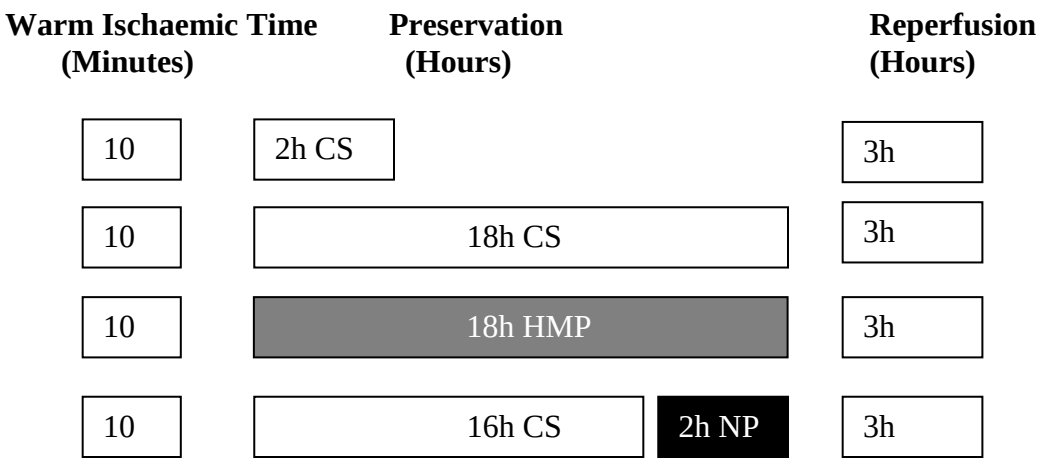


Figure 6.12 Experimental design. The effects of *ex-vivo* normothermic perfusion (NP) (n = 6 kidneys per group) (2h CS, 18h CS, HMP, NP). Static cold storage (CS), Hypothermic machine perfusion (HMP), normothermic perfusion (NP).

6.2 Methods

6.21 Kidney retrieval

All experiments were in accordance with Home Office animals (Scientific Procedures) Act 1986. Kidney retrieval was carried out under schedule 1 sacrificed large white pigs (60-70kg) as previously described in chapter 5.22. Kidneys in all groups were removed from the animal after 10 minutes of WI and flushed with 500ml of HOC at 4ºC. Kidneys undergoing static cold storage (CS) were placed in a sterile bag with approximately 300ml of HOC and stored in a polystyrene organ retrieval box and packed in crushed ice for the duration of the preservation period.

6.22 Hypothermic machine perfusion

The Lifeport Kidney Transporter (Organ Recovery System®, IL. USA) was used for HMP (Figure 6.22). The system was primed with Kidney Perfusion Solution 1 (KPS-1, Organ Recovery System®) and allowed to re-circulate below 8ºC. After the kidneys were retrieved and flushed with 500ml of HOC at 4ºC, the renal artery was cannulated with a straight 8mm arterial cannula (Organ Recovery System®), and secured with a vicryl tie (2-0, Ethicon, Johnson & Johnson, St-Stevens-Woluwe. Belgium). Kidneys were then placed on the perfusion system and perfused at a mean arterial pressure of 30mmHg throughout perfusion and at a mean (SD) temperature of 7.0 ± 0.47 ºC for 18 hours.

Figure 6.22 Lifeport Kidney Transporter (Organ Recovery Systems®)

6.23 Normothermic perfusion

The isolated organ perfusion system was used for reperfusion of the kidneys as described in chapter 5.23 was also used for NP. The kidneys were prepared by cannulating the renal artery, vein and ureter with soft silastic catheters (Pennine, UK) as previously described in chapter 5.24. They were then flushed with 100ml of Ringer's lactate solution at 4ºC immediately before perfusion to remove the preservation solution.

The NP conditions differed from the reperfusion conditions and were designed to provide a protective environment. The circuit was primed with autologous whole blood, which was depleted of leukocytes using a white cell filter (LeukoGuard RS; Pall Medical, Portsmouth, UK) (Table 6.23). The perfusate solution contained Ringer's lactate solution, mannitol and cefuroxime and was supplemented with dexamethasone, a nutrient solution containing sodium bicarbonate, insulin and multivitamins. A vasodilator was also infused into the system to enhance renal blood flow (Table 6.23). Kidneys were perfused at a mean arterial pressure of 65mmHg to reduce the risk of endothelial cell damage.

Components	
Normothermic Mode	
Leukocyte depleted autologous blood	500 ml
Ringer's lactate solution (Baxter Healthcare, Thetford, UK)	500 ml
Mannitol 10% (Sigma-Aldrich, UK)	25 ml
Dexamethasone 8mg (Organon Laboratories, Cambridge, UK)	2 ml
Cefuroxime 750mg (Stragen, Reigate, UK)	5 ml
Sodium bicarbonate 8.4% (Fresenius Kabi, Cheshire, UK)	12 ml
Heparin 1000iu/ml (CP Pharmaceuticals, Wrexham, UK)	2 ml
Supplements	
Nutriflex infusion (B Braun, Sheffield, UK) with the following added;	20 ml/hr
Insulin (Novo Nordisk, Denmark)	100 units
Sodium bicarbonate 8.4% (Fresenius Kabi)	25 ml
Multivitamins (Cerenvit®; Baxter Healthcare)	5 ml
Sodium nitroprusside 25mg (Mayne Pharma, Leamington Spa, UK) Infused over the first hour	25 ml/hr
Glucose 5% (Baxter Healthcare)	7 ml/hr
Ringer's solution to replace urine output ml for ml	

Table 6.23 Components of the perfusate solution and supplements added during normothermic perfusion (NP).

Normothermic measurements

During NP the renal blood flow (RBF) and mean arterial pressure (MAP) were recorded continuously and intra-renal resistance (IRR) calculated (MAP/RBF). Urine output was measured after 60 and 120 minutes. Blood gas analysis was used to record P_aO_2, P_vO_2 and acid-base homeostasis as previously described. Oxygen consumption was calculated as (arterial P_aO_2 – venous P_vO_2) x flow rate/weight.

6.24 *Ex-vivo* Reperfusion

After the set preservation period, kidneys were removed from the HMP and NP systems and flushed with Ringer's lactate solution to remove the preservation solution (HMP) and blood based perfusate (NP).

Kidneys were then reperfused on the isolated organ perfusion system with autologous blood in the reperfusion mode as previously described in chapter 5.23 for 3 hours to assess the renal function and injury.

6.25 Reperfusion outcome measurements

Renal blood flow, intra-renal resistance, creatinine fall, creatinine clearance, fractional excretion of sodium, oxygen consumption, acid base homeostasis and intracellular enzymes were measured as detailed in chapter 5.25. Urine samples were used to measure lipid peroxidation, endothelin-1, IL-6 and TNFα as described in chapters 5.26.

Formalin fixed wedge biopsy samples taken pre-storage, post-storage and post-reperfusion were used to measure the morphology as previously described in chapter 5.28 and to carry out immunohistochemistry staining for myeloperoxidase as a measure of neutrophil infiltration.

Needle core biopsies taken post-storage and post-reperfusion were snap frozen in liquid nitrogen to determine the ATP: ADP ratio.

6.26 Neutrophil Infiltration

As a measure of neutrophil infiltration immunohistochemical staining of Myeloperoxidase (MPO), a marker mainly for neutrophil granulocytes, was undertaken on paraffin sections using a DAKO ChemMate EnVisionTM Detection Kit (DAKO, Glostrup, Denmark). The sections were digested by 40 µg/ml proteinase K for 15 min at 37°C then blocked by peroxidase-blocking reagent. The sections were labelled by an anti-MPO antibody (1:600, DAKO) at 4°C overnight. The antibody binding was revealed by 3'-amino-9-ethylcarbazole. MPO+ cells in the tubular, interstitial and

glomerular were semi-quantitatively scored by counting the number of positive cells in 20 fields at 400× magnification.

6.27 Renal adenosine 3-triphosphate : adenosine 3-diphosphate ratio

The renal adenosine 3-triphosphate (ATP) : adenosine3-diphosphate (ADP) ratio was measured in snap frozen biopsies obtained post-storage and post-reperfusion using an adenylate nucleotide assay kit (Cambrex Bio Science, Rockland, Maine, USA).
The detection is based upon the bioluminescent measurement of ATP. The method utilised an enzyme, luciferase, which catalysed the formation of light from ATP and luciferrin according to the reaction below. The amount of light generated by the reaction was detected using a luminometer.

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{light}$$

Three readings were used to calculate the ADP: ATP ratio. These represent the amount of ATP in the sample (A), a new baseline (B) after conversion of ATP to its reaction products; and the amount of ADP (C). (ADP is measured indirectly following its chemical conversion to ATP). The ADP: ATP ratio was calculated as follows:

$$\text{ADP: ATP} = \frac{C - B}{A}$$

Needle core biopsies were attached to cork blocks using tissue-tek, submersed in liquid nitrogen and transferred to cryotubes for storage at –80^{o}C. For each core biopsy nine sections of 10µM thickness were cut using a cryostat and placed in an eppendorf pre-cooled to –20^{o}C. Nucleotide releasing reagent, 300µl, was added to the sections and the sample was vortexed and stored at 4^{o}C. A 80µl aliquot of this sample was brought to room temperature (the remainder of the sample was kept at 4^{o}C for replicate analysis). 20µl of nucleotide monitoring reagent was added to the aliquot and the sample was placed a luminometer (1250 Bio Orbit). An immediate voltage reading (A) on a chart recorder (2210 LKB Bromma) was noted. A second reading (B) was taken after 10 minutes. The sample was removed, 20µl of ADP converting reagent added and the sample placed back in the luminometer. A final reading (C) was taken after 5 minutes.

6.28 Statistics

There were 6 kidneys in each of the experimental groups. Statistical analysis was performed as described in chapter 5.29. Values are presented as mean ± S.D. $P<0.05$ was taken as statistically significant.

6.3 Results

6.31 Preservation

Hypothermic machine perfusion

Perfusion parameters

The renal perfusate flow increased and intra-renal resistance fell in all kidneys that underwent HMP (Figure 6.31a,b).

A **B**

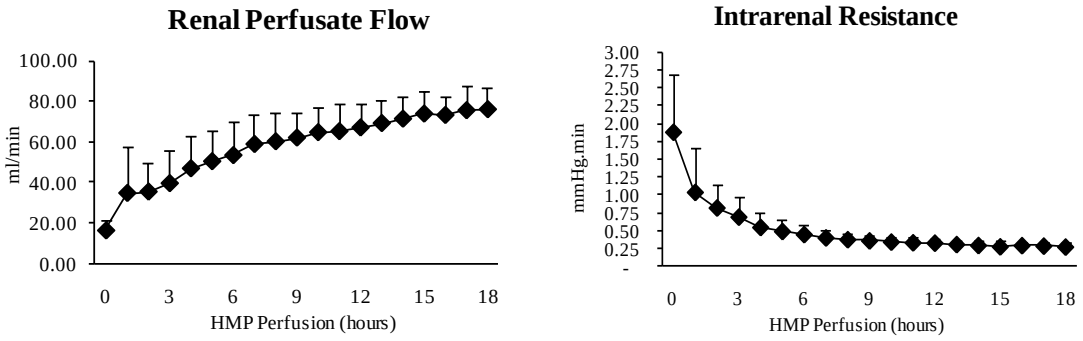


Figure 6.31 Perfusion parameters during 18 hours of hypothermic machine perfusion (HMP) on the Lifeport Kidney Transporter. (A) renal perfusate flow, (B) intra-renal resistance. Values are mean ± SD.

Normothermic perfusion

Perfusion parameters

Renal blood flow peaked during the first hour then declined in the second hour of NP (Figure 6.311a). Intra-renal resistance remained low throughout perfusion in all kidneys (Figure 6.311b). Oxygen consumption reached 40.6 ± 12.5 ml/min/g after 2 hours and the total mean urine output was 406 ± 200 ml.

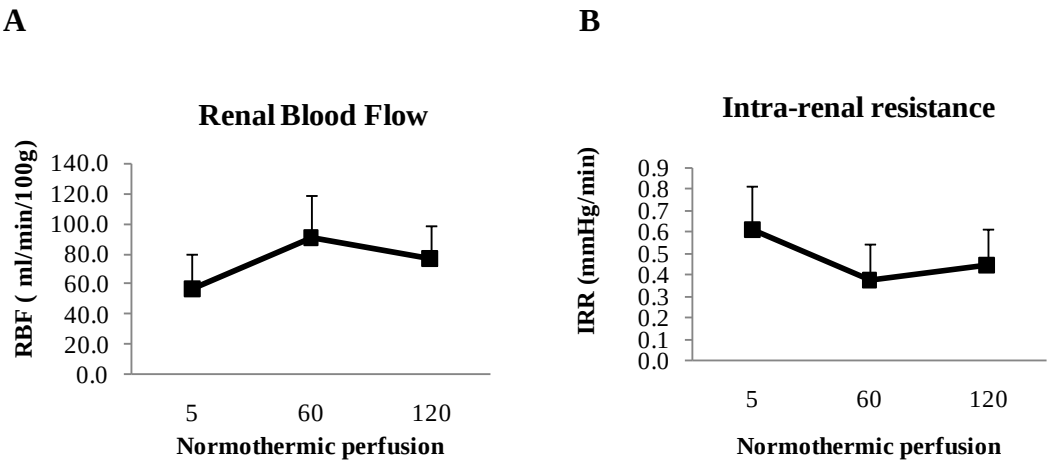


Figure 6.311 (A) renal blood flow and (B) intra-renal resistance during 2 hour of normothermic perfusion (NP). Values are mean ± SD.

6.32 Reperfusion

Haemodynamics

The AUC renal blood flow was reduced in the 18h CS kidneys compared to the other groups, however this did not reach statistical significance (P = 0.083; Figure 6.32a). AUC intra-renal resistance was significantly lower in the NP group compared to the 2 and 18 hour CS kidneys and intra-renal resistance lower for the HMP compared to the 18 hour CS kidneys (P = 0.003; Figure 6.32b).

Renal Function

Serum creatinine levels fell throughout reperfusion in all groups. AUC levels were significantly lower in the 2h CS group kidneys compared to the 18h CS and NP kidneys (P = <0.0001; Figure 6.32c). AUC creatinine clearance was significantly higher in the 2h CS kidneys compared to all other groups (P = 0.007; Figure 6.32d).

Tubular Function

Tubular function was significantly better with a lower level of fractional excretion of sodium in the 2h CS, HMP and NP groups compared to 18h CS kidneys (P = 0.003; Figure 6.32e). Kidneys in the 2h CS group produced significantly more urine than the NP group (P = 0.027; Figure 6.32f).

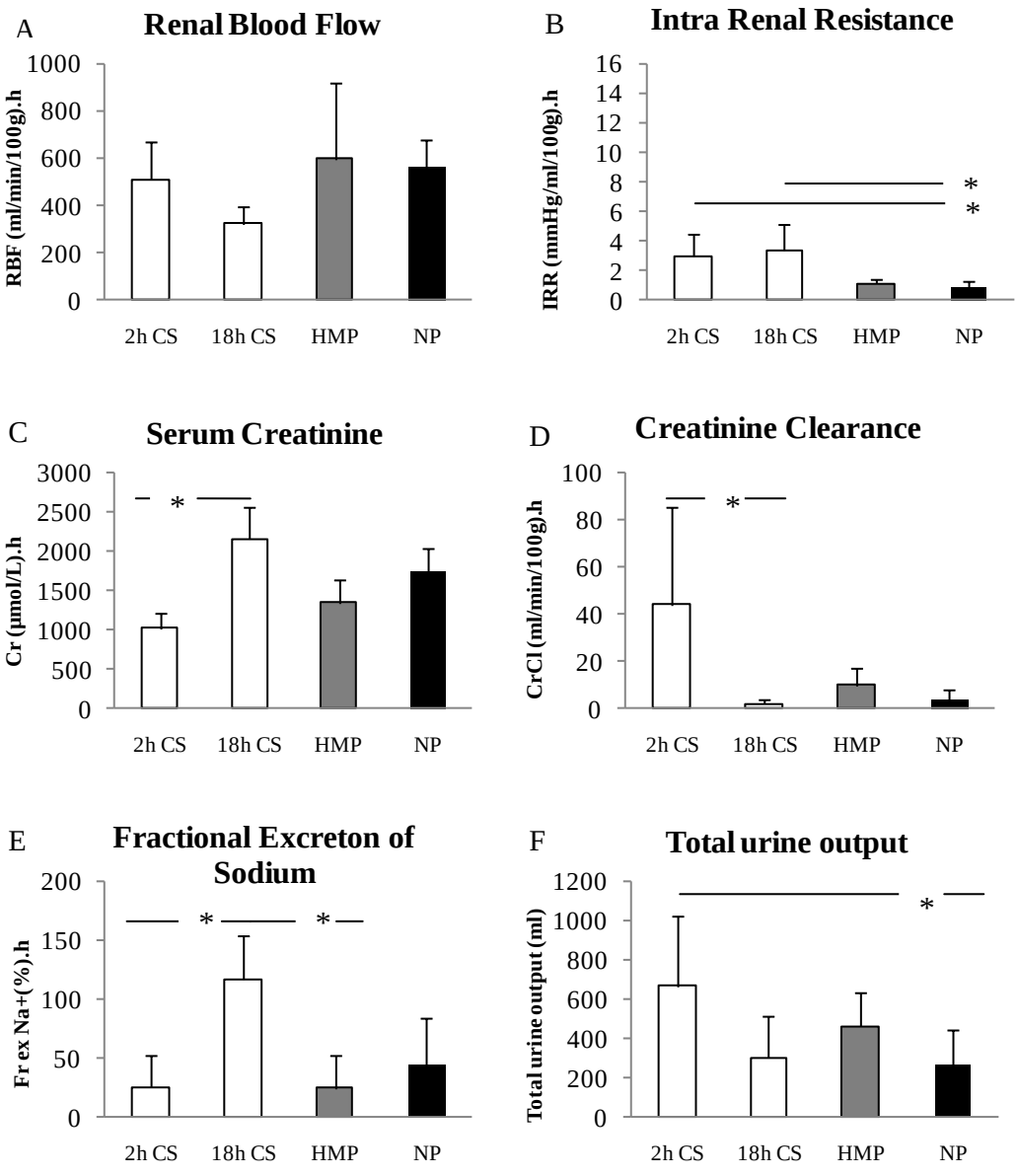


Figure 6.32 Results**.** Haemodynamics, renal and tubular function during 3 hours of *ex-vivo* reperfusion on the isolated organ perfusion system with oxygenated autologous blood. Kidneys were preserved for 2 and 18 hours by cold storage (CS), hypothermic machine perfusion (HMP) or normothermic perfusion (NP). Values are mean ± SD presented as area under the curve (AUC) figures (A-E) and total output (F). (Kruskal Wallis with post test). (B) *P<0.05 NP vs 2 + 18h CS, (C) 2h CS vs 18h CS and NP, (D) 2h CS vs all other groups, (E) 18h CS vs all other groups, (F) 2h CS vs NP.

Metabolism and acid base homeostasis

There were no significant differences in the level of oxygen consumption after 3 hours of reperfusion between the groups (P = 0.140; Table 6.32). The pH after reperfusion was significantly higher in the NP group kidneys compared to the HMP group (P = 0.017; Table 6.32). Levels of bicarbonate were also significantly higher in the NP group compared to the HMP and 18h CS group kidneys (P = 0.004; Table 6.32). Potassium levels were significantly lower in the 2h CS, HMP and NP groups compared to the 18h CS group kidneys (P = 0.029; Table 6.32).

Cellular Injury

Levels of AST after 3 hours of reperfusion were significantly higher in the NP group compared to kidneys in the 2h CS, 18h CS and HMP groups (P = 0.001; Table 6.32). There were no significant differences in levels of LDH (P = 0.069; Table 6.32).

Parameters after 3 hours of reperfusion in each of the experimental groups

Parameters at 3h reperfusion	2h CS	18h CS	HMP	NP	P value
O_2 (ml/min/g)	44.5 ± 19.8	23.7 ± 11.9	35.1 ± 18.9	39.4 ± 9.7	0.140
pH	7.38 ± 0.1	7.37 ± 0.1*	7.33 ± 0.1	7.51 ± 0.1**	0.017
Bicarbonate (mmol/L)	25.8 ± 3.9	21.6 ± 3.7	22.0 ± 6.9	32.8 ± 3.8***	0.004
Potassium (mmol/L)	6.5 ± 3.1	10.0 ± 0.04	5.6 ± 1.2¶	6.3 ± 2.6¶¶	0.029
AST (iu/L)	54.7 ± 8.1	125.3 ± 40.1	96.2 ± 32.2¥	333.0 ± 109.	0.001
LDH (iu/L)	399.2 ± 94.9	381.5 ± 28.2	357.8 ± 83.9	489.2 ± 128.2	0.069

Table 6.32 Parameters after 3 hours of *ex-vivo* reperfusion with oxygenation autologous blood on the isolated organ perfusion system after 2 and 18 hours of preservation by either cold storage (CS), hypothermic machine perfusion (HMP) or normothermic perfusion (NP). Oxygen consumption (O_2), pH, bicarbonate, serum potassium, asparatate transaminase (AST) and lactate dehydrogenase (LDH) levels. *P = <0.05, CS vs NP, ** P <0.05, NP vs HMP, *** P < 0.05, NP vs 18h CS & HMP, ¶ P < 0.05, HMP vs 18h CS, ¶¶ P < 0.05, NP vs 18h CS, ¥ P <0.05, HMP vs NP. Values are mean ± SD (Kruskal Wallis with post test).

6.33 Injury markers

Oxidative damage/Inflammation

Urine levels of 8-isoprostane after 3 hours of reperfusion were significantly higher in the HMP kidneys compared all the other groups suggesting a higher level of lipid peroxidation induced by this method of preservation (P = 0.0007; Figure 6.33a). However, the preservation techniques did not cause any apparent endothelial cell injury with no significant differences in the levels of ET-1 between the groups (P = 0.213; Figure 6.33b).

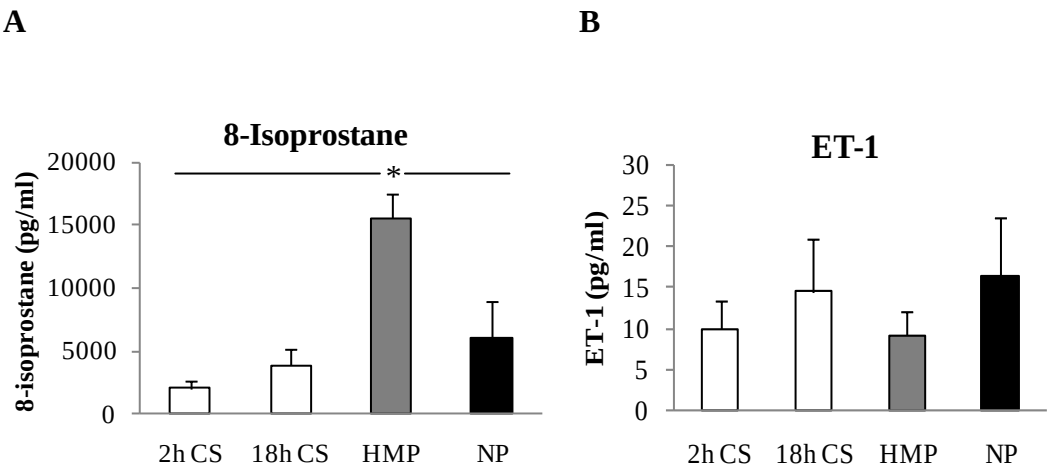


Figure 6.33 Urine levels of (A) 8- isoprostane, (B) endothelin1 (ET-1) after 3 hours of *ex-vivo* reperfusion in the 2h CS, 18 hour CS, HMP and NP groups. (A) **P = 0.0007, HMP vs 2h CS, 18h CS and NP groups. Values are mean ± SD. (Kruskal Wallis with post test).

The inflammatory marker TNFα measured in the urine after 3 hours of reperfusion was significantly increased after 18h CS (P = 0.028; Figure 6.331a). However, levels of IL-6 were significantly higher after NP in addition to the 18h CS kidneys (P = 0.001; Figure 6.331b)

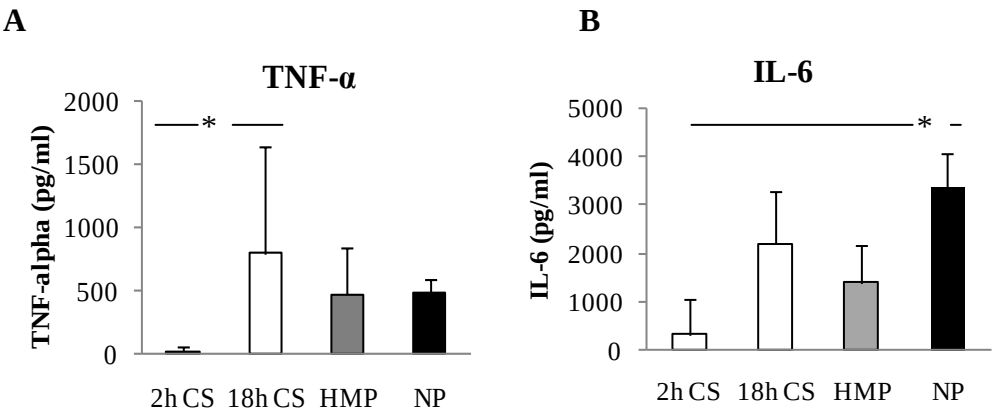


Figure 6.331 Urine levels of (A) tumour necrosis factor alpha (TNFα) and (B) interleukin 6 (IL-6), after 3 hours of reperfusion in the 2h CS, 18 hour CS, hypothermic machine perfusion (HMP) and normothermic perfusion (NP) groups. Values are mean ± SD. (Kruskal Wallis with post test). (A) *P = 0.028, 18h CS vs 2h CS. (B) *P = 0.0001, 18hCS and NP vs 2h CS.

Renal adenosine 3'-triphosphate : adenosine 3'-diphosphate ratio

Two hours of NP increased the ATP : ADP ratio to a significantly higher level than the post-storage values in the 2h CS, 18h CS and HMP group kidneys (P = 0.046; Figure 6.332a). Reperfusion increased the ATP : ADP ratio in all groups (P = 0.088; Figure 6.332b).

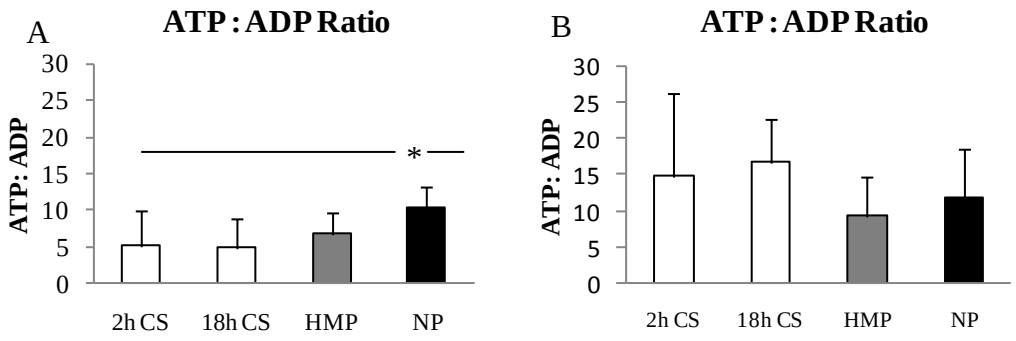


Figure 6.332 ATP : ADP ratio in the tissue (A) post storage (B) after reperfusion in the 2h cold storage (CS), 18h CS, hypothermic machine perfusion (HMP) and normothermic perfusion (NP) groups. Values are mean ± SD. (Kruskal Wallis with post test). (A) ** P = 0.046, NP vs 2h CS and 18h CS post storage.

Neutrophil infiltration (Myeloperoxidase)

The number of positive cells was extremely low in all of the biopsies taken pre-storage and post-storage. However, there was a significantly higher number of positive cells in the HMP group after preservation compared to the other storage groups (P = 0.009; Figure 6.333). After 3 hours of reperfusion the number of positive cells was significantly increased in all of the groups with increased infiltration in the HMP and NP groups compared to the 2h and 18h CS groups (P = 0.003; Figure 6.333)

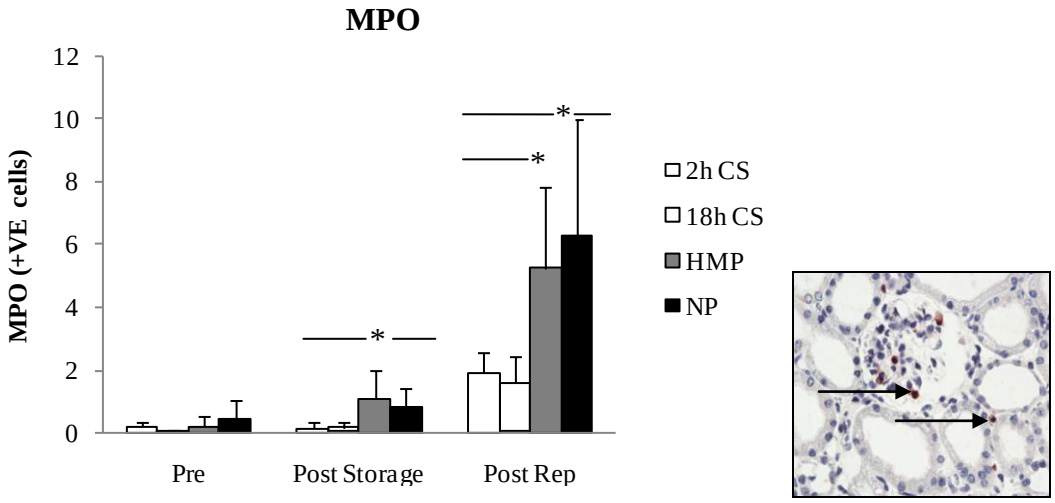
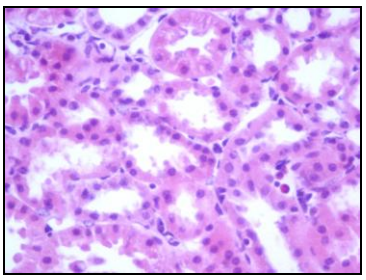


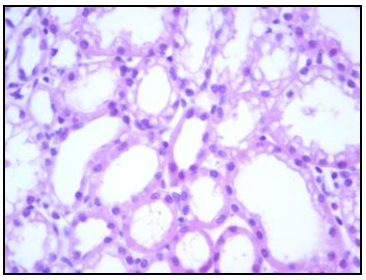
Figure 6.333 Immunohistochemical staining of myeloperoxidase (MPO). MPO+ cells in the tubular, interstitial and glomerular were semi-quantitatively scored by counting the number of positive cells in 20 fields at 400× magnification. Pre-storage, post-storage and post-reperfusion in the 2h CS, 18h CS, HMP and NP groups. Values are mean ± SD (Kruskal Wallis with post test). (Post Storage) ** P =0.009 HMP vs 2h CS, 18h CS and NP. (Post reperfusion) **P = 0.003, HMP and NP vs 2h CS and 18h CS. The photograph shows the tubular cells and glomerular with the arrows pointing to the positive MPO cells.

Histology

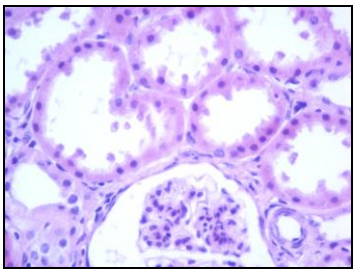
There was a significant amount of morphological change in the post-storage biopsies with an increase in the level of vacuolation in the NP kidneys compared to the other groups and glomerular shrinkage in the NP and HMP kidneys compared to the 2h CS group ($P = 0.038, 0.003$ respectively; Figure 6.334a-h; Values are detailed in Appendix 10.3; Table 10.3a). There was a significant increase in the amount of vacuolation in the NP reperfusion biopsies compared to the 18h CS kidneys and tubular dilatation in the 2h CS kidneys compared 18h CS ($P = 0.032, 0.014$; Figure 6.334). Values are detailed in Appendix 10.3; Table 10.3b).



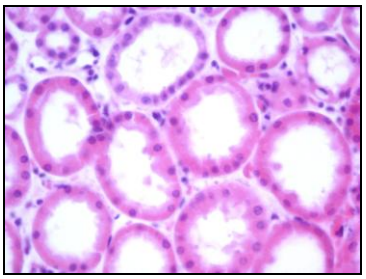
A: 2h CS post-storage



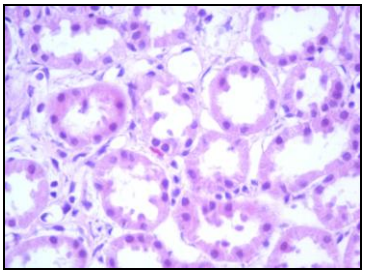
B: 2h CS post-reperfusion



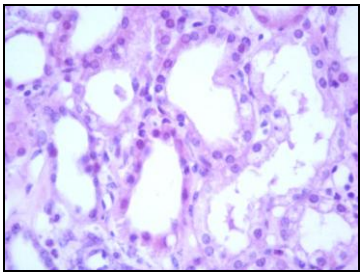
C: 18h CS post-storage



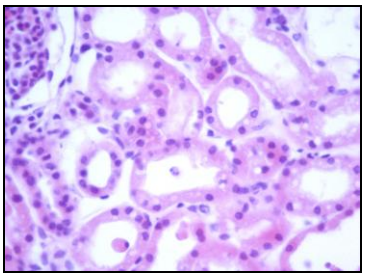
D: 18h CS post-reperfusion



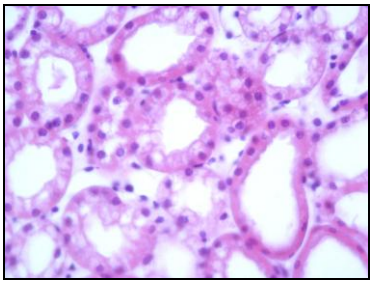
E: HMP post-storage



F: HMP post-reperfusion



G: NP post-storage



H: NP post-reperfusion

Figure 6.334 a-h Histology. Wedge biopsies in the 2h CS, 18h CS, HMP and NP groups were taken post-storage and post-reperfusion and stained with H&E for evaluation using light microscopy. Sections were scored blinded over ten fields, assessing changes in five morphological variables, tubular dilation, tubular epithelial loss, tubular debris, vacuolation, and glomerular shrinkage. Samples were scored from 0 to 3 according to the level of damage; 0 representing normal, 1 mild, 2 moderate and 3 severe morphological changes.

6.4 Summary

This study demonstrated that NP led to the replenishment of ATP during preservation and was able to reverse some of the detrimental effects of CI kidneys. Kidneys undergoing NP had significantly improved tubular function and metabolic stability during reperfusion compared to kidneys with 18 hours CS. HMP kidneys showed improved renal function and reduced tubular cell injury compared to CS. NP was at least equivalent to HMP from the variables assessed and was perhaps superior in terms of oxygen consumption and acid base homeostasis.

The next set of experiments where designed to assess the feasibility of a short period of NP after a longer WI insult and preservation period in a porcine auto-transplant model.

6.5 Discussion

The effects of *ex-vivo* normothermic perfusion

The detrimental combination of WI and CI damage represents a key obstacle to expanding the DCD organ donor pool. Hypothermic techniques have been the traditional method of organ preservation for many years, based on the principle that cooling overcomes the detrimental effects of ischaemic hypoxia by reducing the oxygen demand and overall metabolic rate of the organ. Nonetheless, the previous study demonstrated that prolonged CI after a period of WI exacerbated the severity of the injury after reperfusion.

In the clinical setting, the logistics of organ retrieval, transfer and cross-matching necessitate a substantial period of hypothermic preservation and therefore a brief period of NP is an attractive concept. This research has shown that 2 hours of NP with diluted, leukocyte depleted autologous blood led to an improvement in the preservation of porcine kidneys after 16 hours of CS. Kidneys that underwent NP, demonstrated reperfusion haemodynamics equivalent to organs undergoing only 2 hours of CS. Renal tubular function was also improved and was at least equivalent to HMP from the variables assessed. It was perhaps superior in terms of oxygen consumption and acid base homeostasis, although there appeared to be more cellular injury in terms of levels of AST and more inflammation with higher levels of IL-6. This might suggest a degree of tubular damage, but may also have been related to the kidney being reperfused twice. Nonetheless, higher levels of IL-6 and neutrophil infiltration may also suggest a beneficial regenerative response. Cytokines have both pro and anti inflammatory

properties and can contribute to the generation of signals that promote infiltration to instigate regeneration and repair mechanisms (53). Therefore, it may be through these mechanisms and the replenishment of ATP that NP reversed some of the detrimental effects of CS. The inhibition of cellular metabolism during hypothermic preservation eliminates the possibility of any substantial repair process. However, it is noteworthy that kidneys undergoing HMP also had a higher degree of neutrophil infiltration and higher levels of ATP after preservation compared to CS kidneys. This may also be linked to regeneration. Higher levels of ATP during HMP have been reported by La Manna *et al* who concluded that it was by this mechanism that HMP limited cellular impairment during preservation compared to CS (273).

The histology in the HMP and NP kidneys both showed an increase in tubular cell dilation after perfusion and reperfusion. The increase in cytoplasmic vacuolation during NP was also of concern, but must be interpreted in the face of good renal functional parameters suggesting that this was not deleterious, at least in the acute stage.

Another benefit of NP may include maintaining better membrane stability compared to hypothermic techniques. Mayfield *et al* (274) demonstrated that kidneys perfused with normothermic blood had an improved capacity to limit tissue oedema and ion permeability compared with those perfused hypothermically. Brasile *et al* (198) showed that warm reperfusion using an acellular solution not only prevented further injury but also reverse injury caused during WI. Porcine kidneys subjected to 120 min of WI did not function when transplanted immediately without preservation or after

cold preservation but did so after normothermic reperfusion. Schon *et al* (275) demonstrated that normothermic oxygenated perfusion could restore the metabolic process in *ex vivo* perfused pig livers.

Other studies in kidney and liver transplantation have also demonstrated the value of normothermic recirculation applied before organ retrieval in recovering adenine nucleotide levels after WI (212,276), restoring reduced glutathione levels (276) and improving post-transplant viability (212,277,278). Net *et al* (279) concluded that normothermic recirculation appeared to have a similar effect to ischaemic preconditioning; the mechanisms involved adenosine and a reduction in hepatic xanthine levels (279,280).

Clinically, HMP is increasingly being used for DCD and ECD kidneys as an alternative to CS. Although there is increasing evidence to suggest its superiority, the results are not conclusive. A recent European multicentre clinical trial of CS versus HMP in DBD kidneys found that HMP reduced the risk of DGF (adjusted odds ratio, 0.57; P=0.01) and improved 1 year graft survival compared to CS (94% vs 90%) (130). In a sub-analysis of 82 pairs of DCD kidneys the DGF rate in the HMP group was 53.7% compared to 69.5% in kidneys that were statically stored (281). However, there was no significant difference in graft survival at 1 year (94% vs 95%). Sub-analysis of ECD donors in this trial, found that HMP reduced rates of DGF from 29.7% to 22% and also improved 1 year graft survival (282). In contrast to this support for HMP, a multicentre UK trial found no beneficial effects of HMP. 45 pairs of controlled DCD kidneys were randomized to HMP or CS (131). The DGF rates were 58% vs 56% in the HMP and

CS groups respectively. These findings have questioned the benefits of HMP however there has been criticism of the UK trial for the sequential design and the small number of patients (283).

In the present research, HMP kidneys demonstrated reduced preservation injury compared to static storage with lower levels of intra-renal resistance, improved glomerular and tubular cell function during reperfusion. Serum potassium levels were relatively stable, indicating better renal function, although there was no improvement in the acid base homeostasis compared to the static techniques. Contrary to these beneficial findings of HMP, levels lipid peroxidation were elevated compared the NP and static cold stored kidneys. However, this marked elevation did not appear to affect renal or tubular cell function during this short reperfusion phase. Increased levels of malondialdehyde, another product of lipid peroxidation, have also been found with the use of HMP in a porcine autotransplant model (153). The authors concluded that these increased levels may be an indicator of vascular and endoplasmic alteration and deterioration of tissue integrity after reperfusion (152). In the previous study, levels of 8-isoprostane did not reflect the severity of ischaemic injury and therefore it appears that lipid peroxidation may be a consequence of the preservation technique. In a study of DCD kidneys de Vries *et al* found that redox active iron was released during HMP as a result of WI injury and that this could be used as a measure of viability (141). Redox active iron is associated with lipid peroxidation and therefore increased levels after HMP are a plausible explanation for the elevated levels during reperfusion in this present study.

Conclusion

A short period of NP after static storage lead to the replenishment of cellular ATP and appeared to improve the condition of kidney. Some of the detrimental effects of warm and cold ischaemic injury were reversed and kidneys had improved function compared to kidneys undergoing static cold storage and better metabolic stability compared to HMP.

Chapter 7

A pilot study assessing the feasibility of a short period of normothermic perfusion in a porcine renal autotransplant model

Chapter 7: A pilot study assessing the feasibility of a short period of normothermic perfusion in a porcine renal autotransplant model

7.1 Introduction

Introducing a short period of normothermic perfusion (NP) at the end of the ischaemic period is a practical approach to kidney preservation in the clinical setting. The preliminary *ex-vivo* studies using clinical grade equipment and 2 hours of NP with leukocyte depleted autologous blood demonstrated that it was possible to resuscitate ATP levels and reverse some of the deleterious effects of cold storage in porcine kidneys in a model of the controlled DCD donor. The aim of this study was to assess the feasibility of a short period of NP with autologous blood in a porcine autotransplant model of the uncontrolled DCD donor.

7.12 Experimental design (Figure 7.12)

A porcine autotransplant model was used to assess the feasibility of a short period of NP. Kidneys were subjected to 30 minutes of WI injury then randomised into two groups, 22 hours HMP or 20 hours of HMP followed by 2 hours of NP. After preservation a right nephrectomy was performed and the preserved kidney autotransplanted into the vacant right renal bed. Animals were recovered for a period of 10 days after transplantation to assess renal function, graft survival and injury parameters (Figure 7.12).

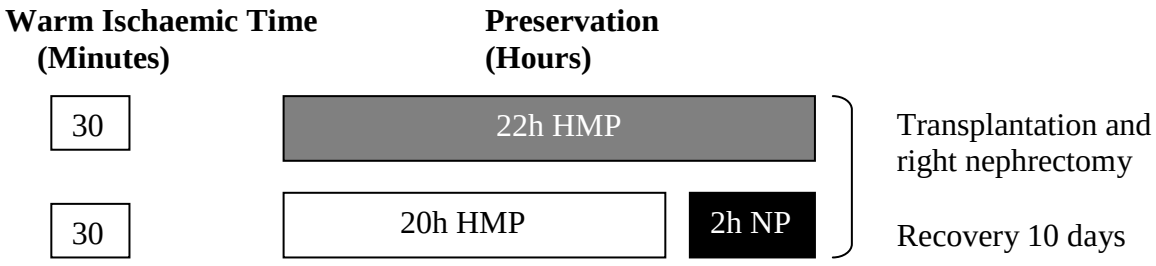


Figure 7.12 Experimental design. A pilot study assessing the feasibility of a short period of NP (n = 6 animals per group). Hypothermic machine perfusion (HMP), normothermic perfusion (NP).

7.2 Methods

7.21 Autotransplant model

The autotransplant experimental model was performed at the Department of Surgery, University of Maastricht, Maastricht, The Netherlands.

Under Dutch animal welfare authority male Landrace pigs weighing approximately 40kg (range 37- 43.8kg) were used in this study. The anaesthetic protocol was identical for the nephrectomy and transplant procedure. The animals were pre-medicated with azaperone (Janssen Animal Health, Beerse, Belgium) 4mg/kg and ketamine 15mg/kg. General anaesthesia was induced by thiopental 6mg/kg IV, and following intubation, was maintained with 1% isoflurane, fentanyl IV 8µg/kg/h and atropine 10µg/kg IV. Ringer's solution 40ml/kg and gelofusine® (B.Braun, Sheffield UK) 20ml/kg were administered IV during both procedures.

7.22 Surgical technique

Left (donor) nephrectomy: The abdomen was opened through a midline incision. The left kidney was mobilized from its bed and its artery and vein were ligated at the aorta and the junction with the inferior vena cava respectively. The ureter was also ligated and divided. The kidney was left free in the abdominal cavity and the abdomen was then partially closed using a loop nylon mass closure technique. A double lumen cuffed silicone vascular access catheter (Tyco Healthcare, Rugby UK) was placed in the right

external jugular vein by surgical cut-down. The catheter was tunnelled to appear behind the right ear. A sterile blood transfusion bag containing CPDA (Baxter Healthcare) was attached to one of the lumens of the jugular central line and approximately 250 ml of blood was collected from each animal in both groups and stored at 4°C. The lumens of the central line were then locked with heparin (Multiparin®; CP Pharmaceuticals, Wrexham UK) 1000 IU/ml. After a period of approximately 30 minutes the left kidney was removed from the abdomen, weighed and then flushed with 300 ml Custodiol® HTK solution (Pharmapal LTD, Athens, Greece) cooled to 4^{o}C and infused at a pressure of 100 cm H_2O. The abdominal muscle closure was completed and the skin closed with a continuous absorbable suture. Animals were then recovered and given water *ad libitum* overnight. The left kidney was randomised using a computerised selected sequence and sealed envelope system to either HMP for 22 hours or HMP for 20 hours followed by 2 hours of NP.

Right nephrectomy and renal autotransplantation: The midline wound was re-opened and a right nephrectomy was performed. The right renal artery and vein were clamped at the aorta and vena cava respectively and divided close to the renal hilum. The ureter was also divided near the renal hilum. The left kidney was then autotransplanted into the right renal bed. The renal artery and vein were anastomosed end to end using continuous 6-0 polypropylene and the ureter was anastomosed end to end using continuous 4-0 PDS over a 4.8 Fr double J stent. The abdomen was closed using mass loop nylon and a continuous absorbable skin suture.

7.23 Hypothermic machine perfusion

After the initial flush, kidneys were re-weighed and then cold perfused using the Lifeport ^{TM} preservation machine (Organ Recovery Systems, Des Plaines, Illinois, USA). This delivered kidney perfusion solution (1L KPS-1$^{®}$; Organ recovery systems) at a mean arterial pressure of 30mmHg and a temperature below 8ºC. After the preservation period kidneys were removed from the Lifeport system, flushed with a further 200ml of HTK solution and then re-weighed before transplantation.

The renal perfusate flow, mean arterial pressure (MAP) and intra renal resistance (IRR) were recorded continuously during HMP.

7.24 Normothermic perfusion

After approximately 20 hours of HMP as described above, kidneys were removed from the Lifeport system and then transferred to the isolated organ preservation system for NP under sterile conditions. Kidneys were perfused for 2 hours with a leukocyte depleted autologous blood based solution (blood collected during the nephrectomy) as previously described in chapter 6.23. However, the circulating volume totalled 500ml rather than 1 litre in the *ex-vivo* experiments (250ml of leukocyte depleted blood and 250ml Ringer's lactate solution). 1500μmol/L of creatinine was also added to determine renal function.

The renal blood flow (RBF) and mean arterial pressure (MAP) were recorded continuously and intra-renal resistance (IRR) calculated (MAP/RBF). Urine output was measured after 60 and 120 minutes. Serum and urine creatinine levels were measured at 60 and 120 minutes and creatinine clearance (CrCL) (urinary creatinine x urinary volume / plasma creatinine) calculated.

After 2 hours (Range 1.55 – 2.0 hours) of NP, kidneys were removed from the perfusion system, flushed with 200ml of HTK and weighed immediately before transplantation.

7.25 Post-operative care

Buprenorphine 30µg/kg IV (Schering-Plough, UK) was administered 8-12 hourly up to 48 hours post-op for postoperative analgesia. Ringer's lactate solution (40ml/kg/24h) and augmentin 500mg (GlaxoSmithkline, Hartfordshire, UK) were administered IV for up to 48 hours post operatively. Animals were allowed free access to water immediately and food was introduced on the first post-operative day.

Animals with severe signs of distress or lethargy caused by renal failure were euthanized by lethal injection of IV pentobarbitone. The surviving animals were euthanized 10 days postoperatively by similar lethal injection and the transplanted kidney explanted.

7.26 Outcome measures

Primary

- Graft survival (functioning kidney)
- Kidney function (serum creatinine)

Secondary (Summary; Appendix 10.1).

- Histopathological change
- Oxidative stress
- Inflammatory injury
- Incidence of trauma to kidney after preservation (vessels and ureter)

Two needle core biopsies were taken pre-warm ischaemia (Baseline), 30 minutes after reperfusion and on termination (End). Samples were either fixed in 10% formal saline or snap frozen in liquid nitrogen (Summary; Appendix 10.1).

Formalin fixed sections were stained with H&E to determine the morphological injury as described in chapters 5.27 and 5.28 or underwent immunohistochemistry staining for myeloperoxidase as described in chapter 6.26.

Venous blood samples were taken from the jugular permcath, collected into heparin tubes, pre-transplant, 60 minutes, 6 hours, 24 hours then daily post-transplant. Samples were centrifuged at 1000g for 15 minutes then were either sent for biochemical analysis of creatinine and urea levels or stored at -80ºC until analysed for levels of 8-isoprostane, IL-6, TNFα and protein carbonyl (Summary; Appendix 10.1).

7.27 Injury markers

Lipid peroxidation (8-isoprostane)

Plasma levels of 8-isoprostane were determined by ELISA (Cayman Chemical Co, MI, USA). Samples were centrifuged at 10,000g for 2 minutes and the supernatant taken for analysis. Plasma samples were diluted 5 fold prior to analysis. The sample and standards were added in duplicate to the ELISA plate together with an 8-isoprostane-acetylcholinesterase (AChE) conjugate and incubated for 18 hours at 4ºC. During incubation any 8-isoprostane present in the sample competed with the 8-isoprostane AChE conjugate for the 8-isoprostane rabbit antiserum binding sites on the pre-coated plate. The plate was then washed and developed by the addition of the substrate to AChE. The plate was read at 405nm after colour development for 90 minutes.

Inflammation (Interleukin-6 and Tumour Necrosis Factor alpha)

Plasma levels of Interleukin 6 (IL-6) and tumour necrosis factor alpha (TNFα) were determined by the quantitative sandwich enzyme immunoassay technique (R&D Systems. Minneapolis, MN, USA). The samples and standards were added in duplicate to the pre coated ELISA plate and incubated for 2 hours (IL-6; polyclonal antibody specific for porcine IL-6 or TNFα polyclonal antibody specific for porcine TNFα). After washing an enzyme-linked polyclonal antibody specific to IL-6 or TNFα was added. After a further incubation period of 2 hours the plate was washed and developed with the addition of a substrate solution. A stop solution was then added and the plate read at 450nm.

Protein damage (Protein carbonyl)

Plasma levels of protein carbonyls were determined by ELISA (Biocell; AXXORA, Nottingham, UK). Standards and samples were reacted with dinitrophenylhydrazine (DNP) for 45 minutes before addition to the plate in duplicate and incubated for 2 hours at 37ºC. The unconjugated DNP and non-protein constituents were washed and the absorbed protein incubated with biotinylated anti-DNP antibody for 1 hour at 37ºC. The plate was then washed and incubated with streptavidin-linked horseradish peroxidase for 1 hour at room temperature. After the final wash the chromatin reagent was applied. The reaction was stopped after 7 minutes and the absorbance determined at 450nm.

7.28 Statistics

There were 6 kidneys in each of the experimental groups. Statistical analysis was performed as described in chapter 5.29. Fisher's exact test was used for categorical data. Values are presented as mean ± S.D. $P<0.05$ was taken as statistically significant.

7.3 Results

7.31 Perfusion parameters

Hypothermic machine perfusion

Intra-renal resistance fell throughout HMP in both groups with no significant difference in the final measurement (HMP; Start 1.32 ± 0.64, End 0.46 ± 0.1 NP; Start 1.10 ± 0.64, End 0.45 ± 0.19mmHg/ml/min: P = 0.400). Renal perfusate flow increased during HMP with no significant difference between the groups (HMP; Start 20.67 ± 13.89, End 55.33 ± 14.94: NP; Start 25.33 ± 16.66, End 55.33 ± 14.94 ml/min: P = 0.818).

Normothermic perfusion

Intra-renal resistance remained high and renal blood flow remained at a constant level in all kidneys during NP (Figure 7.31a,b). Oxygen consumption levels were also consistent after 60 and 120 minutes of NP (Table 7.31).

Numerically, kidneys in the NP group gained more weight during preservation compared to HMP, however this did not reach statistical significance (NP 22 ± 10% increase in weight vs 13 ± 7% in the HMP group; P = 0.180).

All kidneys were transplanted successfully after preservation with no trauma to the kidney vessels or ureter after either of the preservation techniques (P = 1.30, Fisher's exact test)

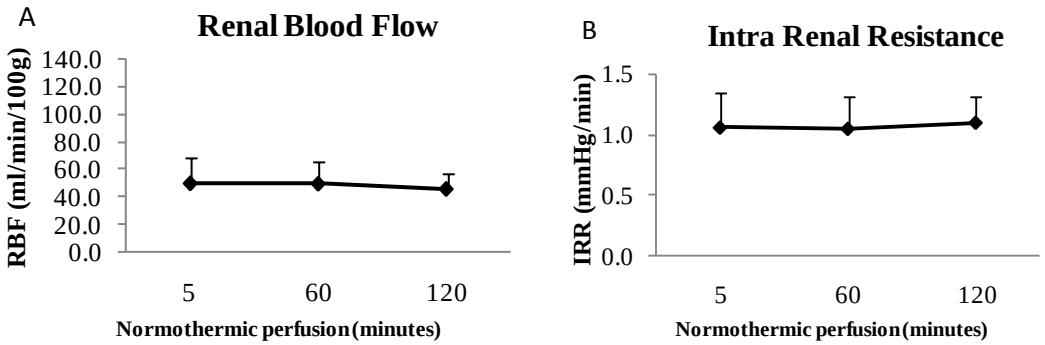


Figure 7.31 Mean renal blood flow (A) and intra-renal resistance (B) during 2 hours of normothermic perfusion (NP). Values are mean ± SD.

	60 minute	120 minute
Urine output (ml)	124 ± 95.1	93 ± 35.0
Creatinine fall (%)	40.3 ± 13.0	51.5 ± 14.2
CrCl (ml/min/100g)	2.2 ± 2.4	2.3 ± 1.9
O_2 consumption (ml/min/g)	31.8 ± 12.0	28.3 ± 6.8

Table 7.31 Functional parameters at 60 and 120 minutes of normothermic perfusion (NP). Creatinine clearance (CrCl), oxygen consumption (O_2 consumption). Values are mean ± SD.

7.32 Animal survival and renal function

There was no significant difference in the WI time (HMP 31.5 ± 3.8 vs NP 33.0 ± 3.2 minutes; P = 0.512), total storage time (HMP 22 ± 0.89 vs NP 22 ± 1.0 minutes; P = 0.520), or anastomosis time (HMP 24.5 ± 2.7 vs NP 24.2 ± 2.9 minutes; P = 0.936) between the groups.

Five out of 6 animals in the HMP and 4 out of 6 animals in the NP group survived to day 10 post transplant (P=1.00, Fisher's exact test). The deaths in each group were caused by renal failure and the decision to euthanize these animals was made on a clinical basis with the animals presenting with increased lethargy, uraemia, loss of appetite and rising serum creatinine and urea levels (Table 7.32).

Comparison of renal function in the HMP and NP groups showed no significant differences in the mean peak creatinine or urea levels, duration to reach the peak levels and time to return to a critical level defined as a serum level of less than 250μmol/L and urea less than 10mmol/L (Figure 7.32a-c, Table 7.32). However, in the HMP group only 2 animals recovered renal function with serum creatinine levels below 250μmol/L compared to all 4 of the survivors in the NP group by day 10 (Table 7.32). There were also no significant differences in the mean and mean daily AUC creatinine and AUC urea between the groups (Table 7.32).

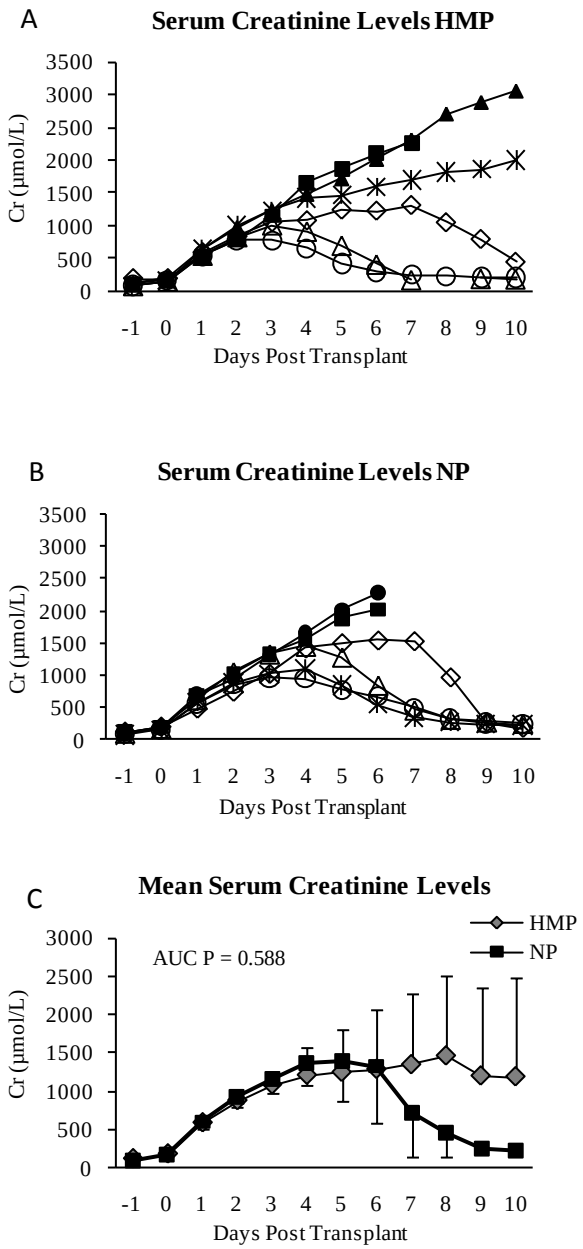


Figure 7.32 Serum creatinine levels from pre-nephrectomy (Day-1) to day 10 post-transplant. (A) serum creatinine levels in the hypothermic machine perfusion (HMP) group, (B) normothermic perfusion (NP) group and (C) serum creatinine in the HMP and NP groups (AUC P = 0.699). Values in A and B are individual animals and in C the mean ± SD (Kruskal Wallis with post test).

Survival and renal function results

	HMP	NP	P value
Survival	5/6	4/6	1.000
Peak Cr (µmol/L)	1736 ± 866	1553 ± 516	0.999
T_{max} Cr (days)	6.2 ± 3.6	4.8 ± 1.3	0.630
T_{crit} Cr (days) <250µmol/L	5.5 ± 0.7	9.3 ± 0.5	0.629
AUC Cr (µmol/L.day)	10085 ± 5089	7594 ± 1377	0.588
AUC Cr (daily mean)	1076 ± 523	934 ± 320	0.699
Peak Urea (mmol/L)	56.9 ± 25.9	47.6 ± 24.2	0.818
T_{max} Urea (days)	6.5 ± 3.4	4.5 ± 1.64	0.260
T_{crit} Urea <10mmol/L	7.0 ± 0.0	7.3 ± 0.5	0.500
AUC Urea (mmol/L.day)	289.4 ± 144.5	225.3 ± 84.6	0.484
AUC Urea (daily mean)	30.7 ± 14.5	27.7 ± 11.95	0.937

Table 7.32 Results. Survival, mean serum creatinine and blood urea, peak level, time to maximum level, time to critical level and AUC. Values are mean ± SD (Kruskal Wallis with post test) hypothermic machine perfusion (HMP), normothermic preservation (NP), creatinine (Cr), area under the curve (AUC), time to maximum level (T_{max}), time to a critical level (creatinine less than 250 µmol/L and urea less than 10mmol/L) (T_{crit}).

7.33 Injury markers

Oxidative damage and inflammatory markers

Levels of 8-isoprostane in the plasma post-transplant remained stable in the NP group. The level was significantly lower in the NP group 60 minutes post-transplant compared to the HMP group (P = 0.026; Figure 7.33a) There was a significant fall in the level at 6 hours in the HMP group (P = 0.015; Figure 7.33a). Levels of protein carbonyl fell significantly after 24 hours post transplant in both groups (HMP P = 0.002, NP P = 0.01; Figure 7.33b). There was no significant difference in the protein carbonyl levels between the two groups at any of the time points (P >0.05).

Levels of IL-6 increased significantly after 6 hours post transplant before falling to the pre-transplant level at 24 hours in the HMP group (P = 0.009; Figure 7.33c). Numerically, levels also increased in the NP group at 6 hours then fell at 24 hours post-transplant, however there was no significant difference between the time points or between the groups (P >0.05; Figure 7.33c). Levels of TNFα remained constant at all time points with no significant difference between the groups (P >0.05; Figure 7.33d).

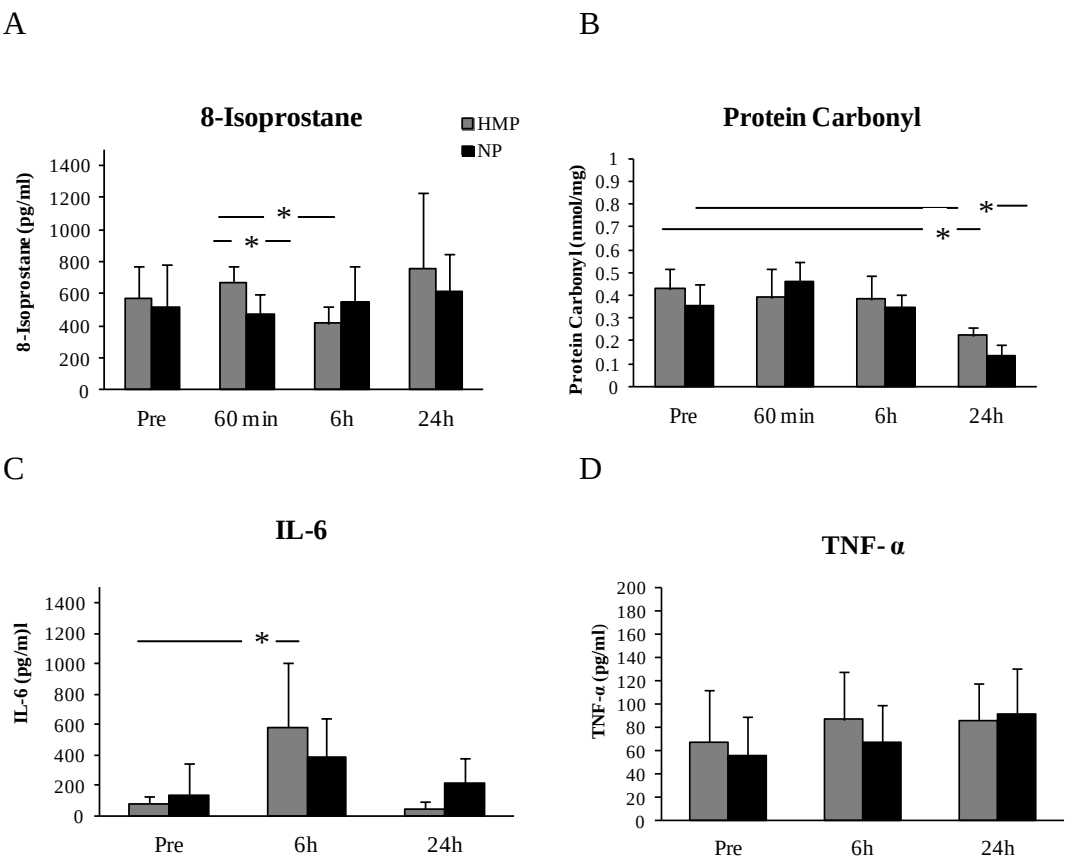


Figure 7.33 (A) Plasma levels of 8-isoprostane, pre-transplant, 60 minutes, 6h and 24h post-transplant. ** P = 0.026 between groups at 60 minutes post-transplant. * P = 0.0015 between 60 minutes and 6 h post-transplant in the HMP group. (B) Plasma levels of protein carbonyl, pre-transplant, 60 minutes, 6h and 24h post-transplant. ** P = 0.002 and 0.010 in the HMP and NP group 24h post-transplant compared to the pre-transplant level. (C) Plasma levels of IL-6, pre-transplant, 6hr and 24h post-transplant. ** P = 0.009 in the HMP group 6h post-transplant compared to the pre-transplant level. (D) Plasma levels of TNFα, pre-transplant, 6h and 24h post-transplant. No significant difference between time points or groups. Values are mean ± SD (Kruskal Wallis with post test).

Neutrophil Infiltration: Myeloperoxidase

The number of positive cells was extremely low in the pre (baseline) biopsies in both groups (P = 0.323). Neutrophil infiltration increased significantly after reperfusion but there were no differences between the two groups (P = 0. 233; Figure 7.331). On termination there was significantly more positive cells in both groups but again with no difference between the HMP and NP groups (P = 0.381; Figure 7.331).

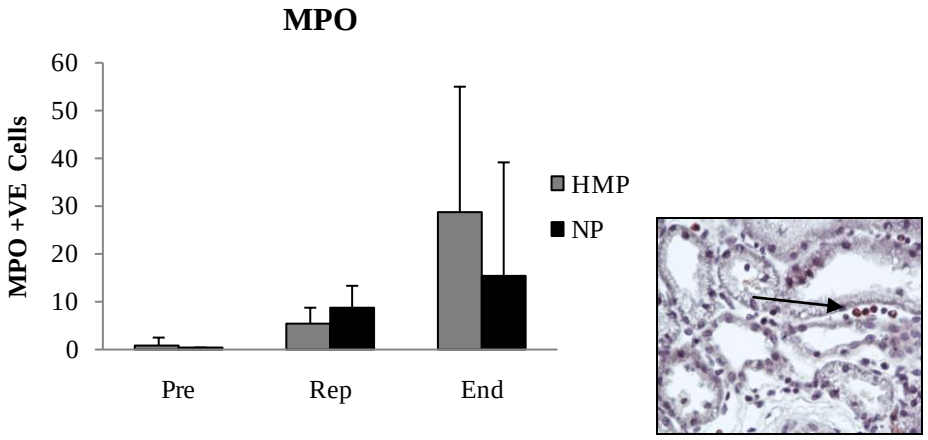
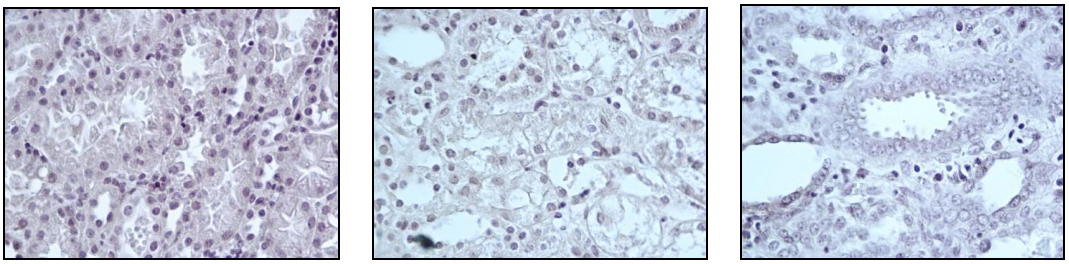


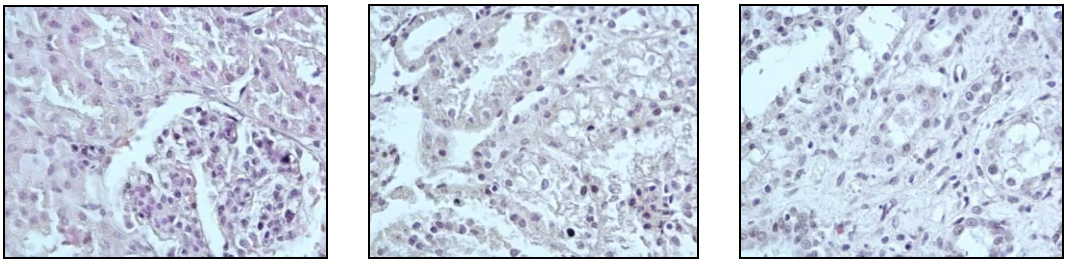
Figure 7.331 Immunohistochemical staining of myeloperoxidase (MPO). MPO+ cells in the tubular, interstitial and glomerular were semi-quantitatively scored by counting the number of positive cells in 20 fields at 400× magnification. MPO positive cells in the HMP and NP groups pre-transplant, 60 minutes post-reperfusion and on termination. Values are mean ± SD (Kruskal Wallis with post test). The photograph shows the tubular cells with the arrow indicating the positive MPO cells in the interstitium.

Histology

There was a significant amount of morphological change with an increase in the level of dilated tubules and tubular debris in the HMP group, and dilated tubules and vacuolation in the NP group in the reperfusion biopsies compared to the baseline samples in both groups ($P < 0.05$). There was also a significant increase in the amount of vacuolation in the NP reperfusion biopsies compared to the HMP (Figure 7.332); values are detailed in appendix 10.4; Table 10.4). In the end biopsies, there was a significant amount change in both groups (Appendix 10.4; Table 10.4).



A: HMP Pre B: HMP Post-reperfusion C: HMP Day 7 (end)



D: NP Pre E: NP Post-reperfusion F: NP Day 7 (end)

Figure 7.332a-f Histology. Needle core biopsies were taken after at baseline (Pre) 30 minutes after reperfusion and at 7 days in the HMP and NP groups. Sections were stained with H&E for evaluation using light microscopy Sections were scored blinded over ten fields, assessing changes in five morphological variables, tubular dilatation, tubular epithelial loss, tubular debris, vacuolation, and glomerular shrinkage. Samples were scored from 0 to 3 according to the level of damage; 0 representing normal, 1 mild, 2 moderate and 3 severe morphological changes. 400× magnification.

7.4 Summary

This study showed that a short period of NP with oxygenated leukocyte depleted autologous blood, staged at the end of the main cold preservation period, was a safe and technically feasible method of kidney preservation. The isolated organ perfusion system used clinical grade paediatric cardiopulmonary bypass technology and therefore could be translated into clinical practice.

7.5 Discussion

A pilot study assessing the feasibility of a short period of normothermic perfusion in a porcine renal auto-transplant model

This study showed that a short period of NP with oxygenated autologous blood, staged at the end of the main cold preservation period, was a technically feasible method of kidney preservation. The ischaemic injury (a combination of 30 minutes warm time and 22 hours cold storage) was quite severe and was intended to mirror the situation of an uncontrolled DCD donor (16). The severity of the chosen injury was confirmed by the fact that several animals in each group demonstrated a progressive and irreversible rise in serum creatinine in the 10 day post transplant period. These animals did not produce urine and their transplanted kidneys did not recover function during this time period. Human kidneys subjected to a similar combination of WI and CI injury demonstrate initial function, DGF and PNF in approximately 10%, 80% and 10% of cases respectively (284). The porcine autotransplant model has important differences compared to human transplantation. The use of healthy, young donor animals and absence of both an allo-immune response and nephrotoxic immunosuppressive drugs may have accounted for the fact that 58% of the autotransplants in this study had initial graft function.

HMP was chosen over CS as the control group in this study because of the increasing use of this technology for the preservation of human DCD and ECD kidneys and growing evidence of its benefits in reducing rates of DGF (130,281,282,285-287).

The short period of NP did not result in any survival or functional advantage in this pilot study and larger experimental numbers may be needed to examine the benefits of NP in DCD kidney transplantation compared to other preservation techniques. However, there was some evidence to suggest some underlying advantage. The generation of oxygen free radicals and up-regulation of pro-inflammatory cytokines are central mediators of I/R injury that cause tissue damage and reduce graft function (70,71). Systemic levels of 8-isoprostane, a well recognised marker of lipid peroxidation (81) were lower in the NP group compared to the HMP group after 60 minutes of reperfusion. This confirms the findings of the previous *ex-vivo* study that HMP appears to cause additional oxidative injury. Although, NP demonstrated no further reduction in the level of oxidative damage or in the expression of pro-inflammatory cytokines, nor any evidence of repair with an increased cytokine response, these findings suggest that NP may have some beneficial effects against I/R injury and this warrants further investigation.

The morphology after reperfusion did reveal a slight increase in the level of ischaemic change with increased vacuolation in the NP group compared to the HMP group. This observation has been noted previously in the *ex-vivo* model and may be attributed to the action of additional warming. Nonetheless, there was no apparent effect on renal function and no further increase in the severity compared to the HMP group, which suggests that this was a transient, reversible process.

Restoring renal metabolism and function prior to transplantation may also allow a comprehensive assessment of graft viability. The small number of animals and

predetermined level of ischaemic injury in this study did not allow us to directly address organ viability. However the perfusion parameters measured during NP clearly demonstrated the extent of the WI and CI injury and where consistent with similar findings in the *ex-vivo* experiments. Kidneys had a low level of creatinine clearance, low renal blood flow and constant high level of intra-renal resistance. Previous *ex-vivo* studies using the isolated organ perfusion system have demonstrated that functional parameters measured during NP significantly correlated with the level of renal function during reperfusion (209). Therefore, in the future, NP may have an important role in the development of accurate biochemical indices to screen organs before transplantation.

The period of *ex-vivo* NP was deliberately limited to 2 hours. Previous studies suggest that re-establishing metabolism during NP enhances cell membrane integrity and function and this should allow sufficient time to deliver relatively simple therapies against I/R injury (209,288). Much longer isolated haemoperfusion times have been used successfully and further work could explore extending the perfusion time in porcine kidneys. However, shorter periods of NP may also be just as beneficial. In the clinical setting a period of 1- 2 hours of NP would be convenient as this could be started as soon as the transplant recipient was ready. The time taken to transfer the patient to the operating theatre, the induction of anaesthesia and the preparation of the iliac vessels for graft implantation would provide sufficient time to complete NP without prolonging the total ischaemic period. It would also avoid the logistical difficulties of more prolonged NP during organ transportation

Conclusion

This pilot study demonstrated that the addition of a period of NP after hypothermic preservation in DCD kidneys is a safe and feasible method of kidney preservation. Graft survival and kidney function was equivalent to HMP however there appeared to be some reduction in the level of oxidative damage. Larger numbers may be required to examine the benefits of NP in DCD kidney transplantation.

Chapter 8

A clinical study of normothermic perfusion in marginal donor kidney transplantation

Chapter 8: A clinical study of normothermic perfusion in marginal donor kidney transplantation

8.1 Introduction

In clinical practice preservation techniques have been dominated by hypothermic conditions. These are a practical and simple approach that have served kidney transplantation well for over 50 years. Nonetheless, the evidence from this research and from the literature, suggests that limiting the CI time is essential in preventing injury in marginal kidneys. The *ex-vivo* and autotransplant models used in this research has shown that a short period of normothermic perfusion (NP) before transplantation may be beneficial in reducing I/R injury and improving early graft function. Although, not routinely used, *ex-vivo* NP has been introduced clinically in heart (234,235) and lung transplantation (240,242,289,290). The early results from these initial series are extremely encouraging. The study described here is the first report of the application of NP in marginal donor clinical kidney transplantation.

8.12 Experimental Design (Figure 8.12)

15 patients undergoing a kidney transplant from a marginal donor were entered into the study. The donor kidneys were stored under CS conditions for transportation as normal practice in the UK. On arrival and once identified as suitable for NP, kidneys were prepared and the renal artery and vein cannulated ready for perfusion in the operating theatre. This was carried out whilst the patient was being prepared for surgery (Figure 8.12). In four cases both kidneys from the same donor were accepted. One remained in CS until transplanted and the other underwent NP, thus allowing a direct comparsion.

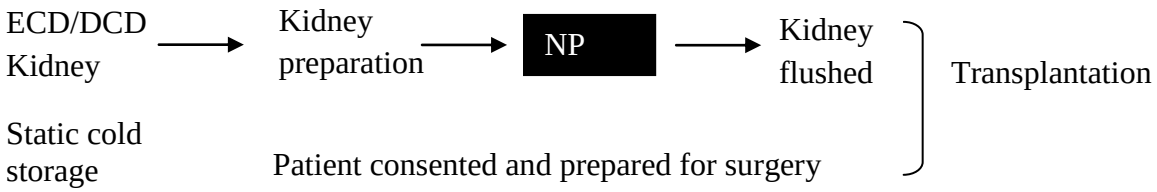


Figure 8.12 Experimental design: Normothermic perfusion (NP) for kidneys from marginal donors.

8.2 Methods

8.21Ethical Approval

Approval was sought from The University Hospitals of Leicester (UHL) Clinical Ethical committee and from the Hospitals New Interventional Procedures Advisory Group (NIPAG) to implement this technique into clinical practice. This committee is an advisory board appointed by the Hospital Trust to review new clinical procedures. After a formal application and series of committee meetings, approval was granted in November 2010. The committee recommended strict guidelines to ensure patient safety, leaving 1 month between each case and detailed audit for the first 5 patients. Approval was also sought from the infection control team, hospital managers and Haematology Department and they granted their support in August 2010. The Research & Development Office also sanctioned the technique as a new procedure rather than a research study at this early stage. Approval was also granted from the Chairman of the Kidney Advisory Group, NHS Blood and Transplant (NHSBT) in August 2010.

Written consent was gained from the recipient after the procedure was explained in detail and the patient was provided with an information sheet prior to the transplant procedure.

8.22 Indications

Preservation of kidneys from brain dead donors for transplantation especially those from marginal donors or with long hypothermic storage periods.

Inclusion criteria

Patients were eligible if all of the following criteria were met:

1: Age ≥ 18 years

2: Undergoing a renal transplant from a deceased donor.

3: Written, signed informed consent to the procedure.

Exclusion criteria

Patients were not eligible if any of the following criteria applied:

1: Anatomy of the kidney (complex arterial anatomy and multiple vessels).

2: Any condition which, in the opinion of the investigator, made the procedure unsuitable for the patient.

8.23 Isolated Perfusion System

The perfusion system was designed using clinical grade paediatric cardio-pulmonary by-pass technology (Medtronic, Watford, UK). The hardware consisted of a centrifugal blood pump (Bio-pump 560), a heat exchanger (Chalice Medical), a speed controller (Bisconsole), a TX50P flow transducer, pressure transducer, a temperature probe (Cole-Parmer, London, UK) and two Alaris infusion pumps (Carefusion, Basingstoke, UK). The stainless steel kidney chamber was custom made and sterilized (Yorklabs, York, UK).

The disposable components were custom designed and sterilized (Medtronic) and consisted of a 5 litre venous reservoir (Medtronic), ¼ inch PVC tubing, a Minimax Plus® membrane oxygenator and bio-head pump (Medtronic) (Figure 8.23).

The circuit was primed with the perfusate solution (Ringer's solution, Baxter Healthcare), 1 unit of compatible cross matched packed red blood cells and some supplements (Table 8.23). Supplements were also infused into the venous reservoir and arterial arm of the circuit to maintain normal homeostatic conditions during perfusion (Table 8.23). Kidneys were perfused at a mean arterial pressure of 65mmHg.

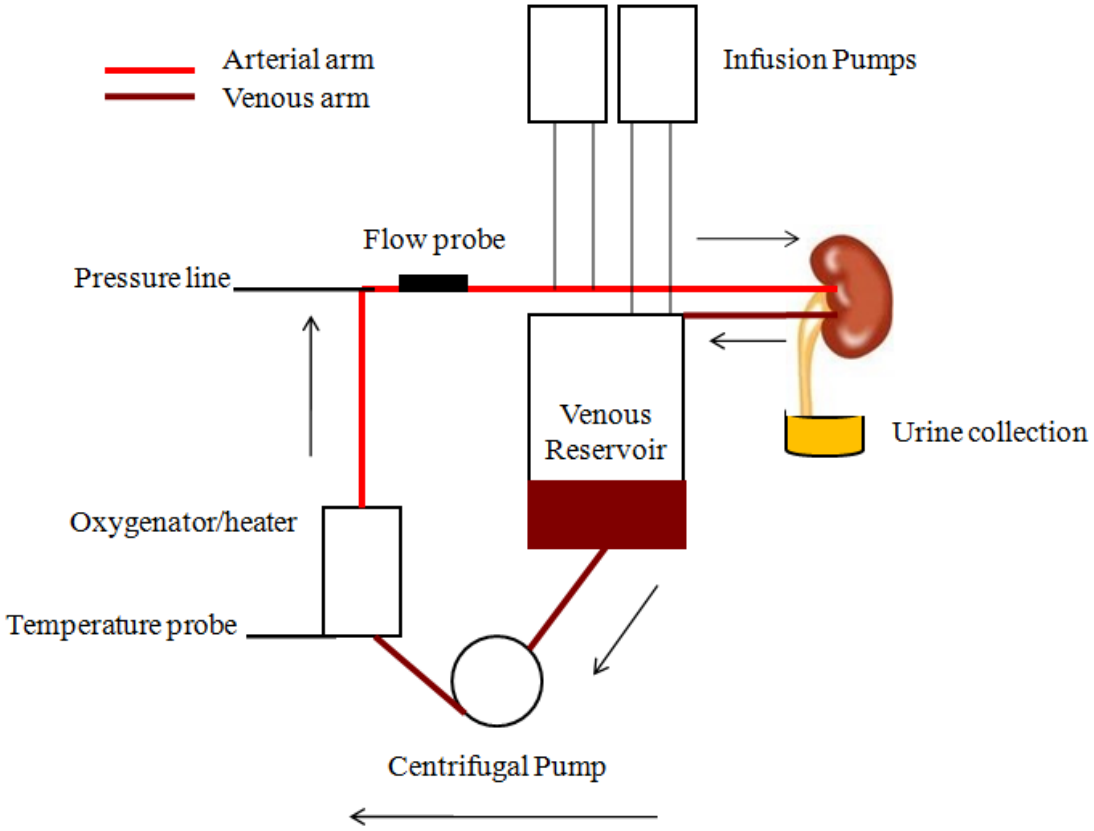


Figure 8.23 Schematic diagram of the clinical *ex-vivo* normothermic perfusion system. The arrows show the direction of blood flow. The system hardware is based on paediatric cardiopulmonary bypass technology (Bioconsole 560). Under sterile conditions the renal artery, vein and ureter were cannulated and the kidney placed in a custom made stainless steel perfusion chamber on the perfusion trolley. The perfusate was pumped from the venous reservoir via the centrifugal pump into the membrane oxygenator and heat exchanger before entering the arterial arm of the circuit. The oxygenated and warmed blood entered the kidney via the renal artery and then allowed to drain from the vein back into the reservoir were it was re-circulated. The arterial pressure was fixed allowing the kidney to autoregulate its own blood flow. The urine was collected into a sterile container. Nutrients, fluids and glucose were continually infused into the circuit.

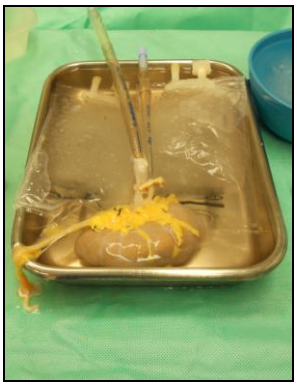
Components	Volume
Perfusate	
Compatible crossed matched packed red blood cells	1 unit
Ringer's solution (Baxter Healthcare)	400ml
Mannitol 10% (Baxter Healthcare)	25ml
Dexamethasone 8mg (Organon Laboratories, Cambridge, UK)	2ml
Sodium bicarbonate 8.4% (Fresenius Kabi, Cheshire, UK)	25ml
Heparin 1000iu/ml (CP Pharmaceuticals, Wrexham, UK)	2ml
Supplements	
Nutriflex infusion (B.Braun) with the following added	20ml/hr
Insulin (Novo Nordisk, Denmark)	100 units
Sodium bicarbonate 8.4% (Fresenius Kabi)	25 ml
Prostacyclin 0.5 mg (Folan, Glaxo-Wellcome, Middlesex, UK)	4ml/hr
Glucose 5% Baxter Healthcare)	7ml/hr
Ringer's solution to replace urine output ml for ml	

Table 8.23 Components of the perfusate solution and supplements added during normothermic perfusion.

8.24 Kidney Preparation

The renal artery and vein were cannulated with cardiac arterial and venous cannulas (Medtronic) secured in place with a vicyrl ties (2-0, Ethicon) (Figure 8.24a-e).

A



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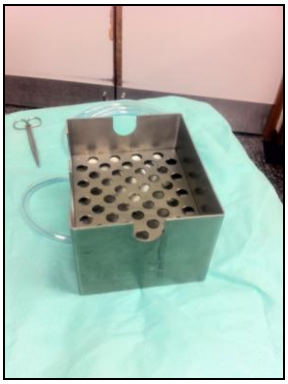

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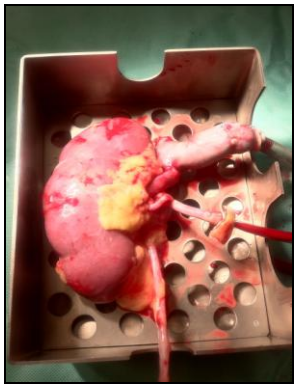

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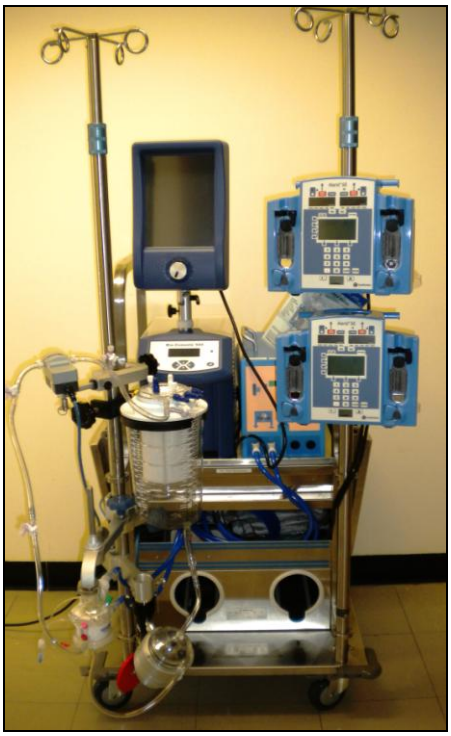

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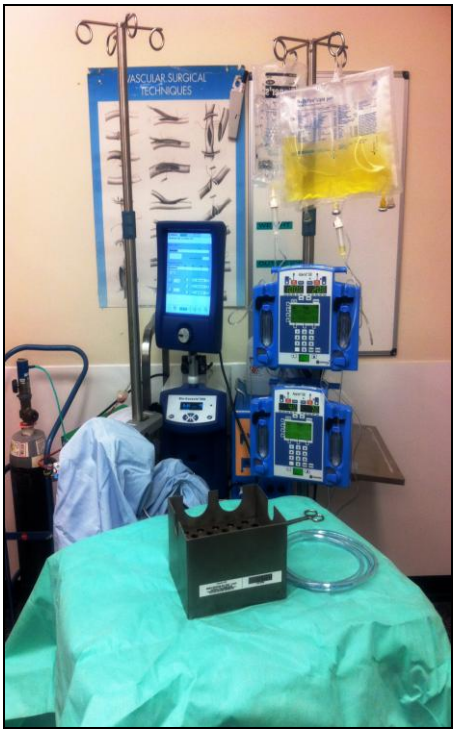


Figure 8.24 Normothermic perfusion system.(A) kidney preparation with the renal artery and vein cannulated, (B) kidney perfusion chamber, (C) kidney in the perfusion chamber during normothermic perfusion, (D) kidney perfusion system, (E) kidney perfusion system in the operating theatre.

8.25 Normothermic perfusion

The renal blood flow (RBF) and mean arterial pressure (MAP) were recorded continuously and intra-renal resistance (IRR) calculated (MAP/RBF). Urine was collected into a sterile container and output and was measured throughout perfusion. Blood gas analysis of arterial blood was used to record and maintain acid-base homeostasis.

After perfusion, the kidney was flushed with approximately 200mL of cold (4°C) Hyperosmolar Citrate (HOC, Baxter Healthcare, UK) to remove the perfusate.

The arterial Carrel patch was excised along with a short segment of vein, where the cannula ligatures had been and the kidney immediately transplanted.

8.26 Transplantation

The normothermically perfused kidneys were transplanted into the right iliac fossa with anastomosis of the artery and vein to either the external or internal iliac vessels and the ureter to the bladder as an extravesical onlay over a double J stent.

8.27 Immunosuppression

Patients were immunosuppressed with the standard unit regimen of basiliximab (20 mg on days 0 and 4), tacrolimus (0.1 mg/kg/day to maintain trough levels of 6–10 ng/mL), mycophenolate mofetil (500 mg twice daily) and prednisolone (20 mg daily) reducing to 5mg at 6 weeks post-transplant.

8.28 Patient assessment and outcome measures

- Daily serum creatinine levels to measure creatinine fall over the first 7 days then at 1 and 3, 6 and 12 months after transplantation
- Rates of delayed graft function (DGF); defined as the need for dialysis within the first 7 days after transplantation
- Rates of primary non function (PNF); defined as the graft never functioning
- Slow graft function rate (SGF); defined as a less than 10% reduction in serum creatinine levels over 3 consecutive days within the first week after transplantation
- Episodes of acute rejection within the first 3 months. Categorised into cell mediated and antibody mediated rejection
- Adverse reactions eg infection, thrombosis of the graft
- Length of hospital stay (days)
- Graft loss

8.3 Results

8.31 Donor demographics

From December 2010 to November 2011 15 patients received marginal donors kidneys that underwent NP. 13 kidneys fell into the criteria for ECD, 1 was from a DCD donor who suffered a cardiac arrest for a period of 17 minutes and the other a 37 year old who suffered hypoxic brain death and cardiac arrest. The average donor age was 59 ± 9.9 years (range 37 – 73 years) and in the majority of cases death was caused by an intracranial haemorrhage. There were 4 male and 11 female donors. The average cold storage period was 12.8 ± 4.8 hours (range 6 – 21 hours). The demographics are detailed in table 8.31.

Donor demographics

Donor	Age (Years)	Gender	Cause of Death	WI minutes	CI hours/minutes
1	62	F	Intracranial haemorrhage + Cardiac arrest	30	10.37
2*	74	M	Trauma/ cardiac arrest	17	20.10
3	48	F	Hypoxic brain death		12.02
4	68	F	Intracranial haemorrhage		9.20
5	52	M	Intracranial haemorrhage		8.20
6	54	F	Intracranial haemorrhage		4.31
7	63	F	Hypoxic brain death		9.32
8	58	F	Intracranial haemorrhage		6.20
9	37	F	Hypoxic brain death + Cardiac arrest		16.14
10	63	F	Intracranial haemorrhage		16.51
11	69	F	Intracranial haemorrhage		15.31
12	55		Intracranial haemorrhage		6.53
13	52	M	Intracranial haemorrhage+ Cardiac arrest		18.55
14	54	F	Intracranial haemorrhage		13.22
15	73	F	Intracranial haemorrhage		12.28

Table 8.31 Donor demographics. Donor age, gender, cause of death, warm ischaemic (WI) time and cold ischaemic (CI) time. * DCD.

8.32 Normothermic perfusion

All kidneys underwent NP using one unit of compatible cross matched packed red blood cells supplemented with a priming solution, nutrients, multivitamins and a vasodilator. The mean haematocrit level pre-perfusion was 22.3 ± 3.4% and fell to 19.9 ± 5.1% after perfusion. The perfusion conditions remained stable throughout perfusion and the average duration of perfusion was 66 ± 15.5 minutes (Range 35 – 100 minutes) (Table 8.32).

Acid base balance

	pH	PO_2 (kPa)	PCO_2 (kPa)
Pre	7.39 ± 0.1	68.9 ± 3.4	5.69 ± 1.2
Post	7.39 ± 0.1	67.1 ± 7.5	5.26 ± 0.73

Table 8.32 Perfusion paramters Mean ± SD pH, partial pressure of oxygen (PO_2) and carbon dioxide (PCO_2) pre and post normothermic perfusion.

The perfusion was carried out in the operating theatre whilst the patient was anaesthetised and prepared for surgery to avoid delaying the transplant. Once the transplant bed was prepared ready for transplantation the kidney was removed from the perfusion system, flushed with cold preservation solution to remove the blood and immediately transplanted. This was case in all kidneys except in patient number 10. Here the initial recipient had a severe anaphylactic reaction to the prophylactic antibiotics given after the induction of anaesthesia whilst the kidney was undergoing

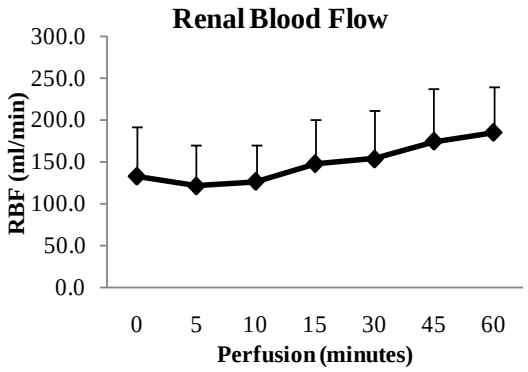
NP. After successful treatment the decision was made to not proceed with the transplant and the patient went to the high dependence ward. Perfusion was stopped after 60 minutes, the kidney was flushed with HOC and then stored on ice until transplanted into another suitable recipient 5 hours later.

Renal blood flow increased and intra-renal resistance fell throughout perfusion in all kidneys although blood flow was extremely variable (Figure 8.32). The amount of urine produced also varied significantly, ranging from 50 to 450ml in total (Table 8.321).

All of the kidneys except one had a single renal artery. In the kidney with two arteries the circuit was adapted and both arteries were cannulated and the kidney perfused successfully. However, this did appear to alter the perfusion conditions and the kidney had a relatively low renal blood flow but appeared well perfused.

There were no complications during perfusion and all kidneys were transplanted successfully. The average anastomosis time was 27 ± 6 minutes and total ischaemic time including NP was 13 ± 4.7 hours.

A



B

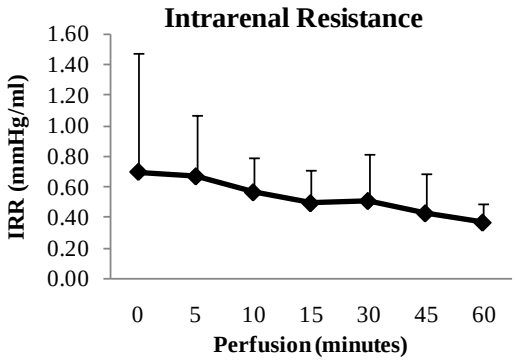


Figure 8.32 (A) Renal blood flow and (B) intra-renal resistance throughout 60 minutes of normothermic perfusion (NP). Values are mean ± SD.

Normothermic perfusion parameters

Patient	Perfusion time (minutes)	RBF (ml/min)	IRR (mmHg/min)	Urine (ml/min)	Temp (ºC)
1	35	58 ± 11	1.26 ± 0.30	1.4	33.9 ± 0.6
2	60	106 ± 10	0.65 ± 0.07	2.5	35.5 ± 0.5
3	65	167 ± 48	0.39 ± 0.09	3.8	33.3 ± 0.8
4	82	136 ± 30	0.49 ± 0.16	3.7	34.1 ± 0.8
5	65	190 ± 26	0.32 ± 0.05	3.7	34.7 ± 0.3
6	100	211 ± 59	0.34 ± 0.14	2.5	34.2 ± 0.8
7	60	230 ± 42	0.23 ± 0.06	3.8	33.7 ± 0.9
8	77	164 ± 52	0.43 ± 0.24	3.4	34.5 ± 0.5
9	70	186 ± 55	0.35 ± 0.12	2.3	34.6 ± 0.6
10	60	187 ± 27	0.32 ± 0.10	1.0	35.0 ± 0.3
11	75	146 ± 21	0.45 ± 0.06	5.3	31.5 ± 0.9
12	70	125 ± 33	0.57 ± 0.25	6.4	36.0 ± 0.1
13	72	145 ± 27	0.44 ± 0.1	2.8	36.0 ± 2.4
14	60	110 ± 8.9	0.61 ± 0.11	4.1	34.9 ± 0.3
15*	42	52 ± 13	1.40 ± 0.98	1.2	34.6 ± 0.7
Mean	**66.2 ± 15.5**	**148 ± 52.4**	**0.55 ± 0.3**	**3.2**	**34.4 ± 1.1**

Table 8.321 Parameters during normothermic perfusion (NP) for each kidney. Perfusion time, mean renal blood flow (RBF), mean intra-renal resistance (IRR), urine output and mean temperature throughout perfusion (*Patient 15, the kidney had 2 arteries).

8.33 Recipient demographics

The average age of the recipient was 57 ± 10.5 years (range 41-74 years). Ten were male and 5 were female (Table 8.33). Three of the recipients were pre-dialysis, five on continuous ambulatory peritoneal dialysis (CAPD) and 7 on haemodialysis (HD) (Table 8.33).

Recipient	Age (Years)	Gender	Dialysis	HLA Mismatch
1	55	F	CAPD	1-0-1
2	59	M	Pre-dialysis	1-1-1
3	48	M	CAPD	2-1-1
4	47	M	HD	1-1-0
5	68	F	CAPD	0-1-1
6	72	M	HD	0-1-1
7	74	F	HD	1-1-1
8	58	M	HD	0-1-1
9	51	M	HD	1-0-0
10	54	F	Pre-dialysis	2-1-2
11	43	M	CAPD	0-0-0
12	69	M	HD	2-2-2
13	41	M	HD	1-2-1
14	51	M	Pre-dialysis	2-2-2
15	64	F	CAPD	1-1-2

Table 8.33 Recipient demographics, age, gender, type of dialysis, haemodialysis (HD) and HLA mismatch.

8.34 Outcome

One patient had delayed graft function requiring two episodes of dialysis within the first week of transplantation. Two patients had slow graft function (SGF) defined as a less than 10% fall in serum creatinine levels for three consecutive days during the first week. Four patients had an episode of acute rejection, (2 cell mediated, 1 antibody mediated, 1 borderline) within the first month and were treated successfully with methylprednisalone. 1 patient had an episode of rejection 3 months after transplantation and was treated successfully. The median length of hospital stay was 7 ± 7.1days (Appendix 10.5; Table 10.5).

Renal function

The average pre-serum creatinine levels were 702 ± 222µmol/L and fell to 229 ± 172µmol/L after 7 days (Figure 8.341). Serum creatinine levels fell in all patients after 1, 3 and 6 months (Figure 8.342). Graft and patient survival at 1, 3 and 6 months was 100% (Appendix 10.5; Table 10.5).

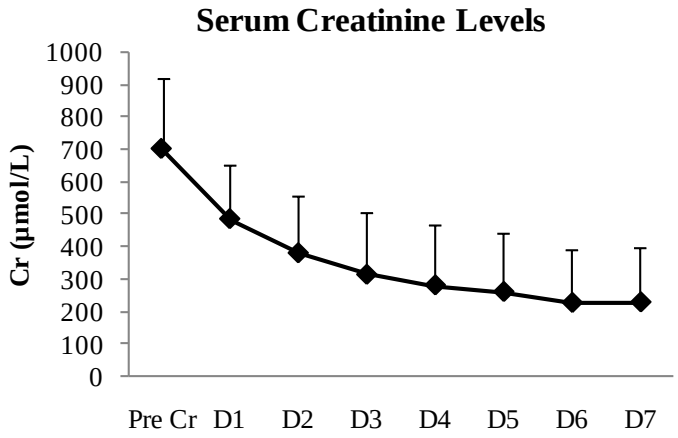


Figure 8.341 Mean serum creatinine levels of patients receiving a kidney that underwent normothermic perfusion (NP) from pre-transplant to 7 days post-transplant.

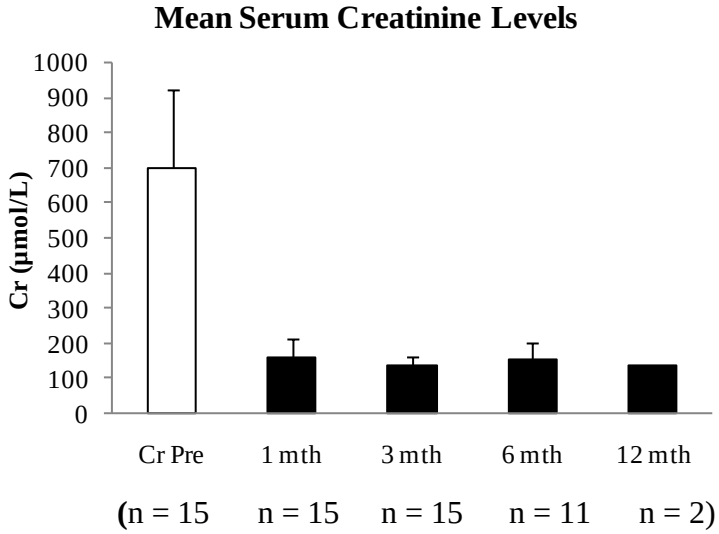


Figure 8.342 Mean serum creatinine levels all patients pre-transplant, in 15 at 1 month, 15 at 3 months ,11 at 6 months and 2 patients at 12 months post-transplant.

8.35 Paired kidneys

Four of the kidneys that underwent NP were of a pair excepted by the the transplant department at Leicester General Hospital. The following results report the outcome of the normothermic perfused and paired cold stored kidney.

8.351 Pair 1 (Table 8.35, 8.351; Figure 8.35a)

Donor details

The 62-year-old female donor was an ECD after brain death. There was a history of hypertension and death resulted from an intracranial haemorrhage. There was also a 30 minute period of cardiac arrest, with resuscitation for a total of 60 minutes, 24 hours before death.

***Ex-vivo* normothermic perfusion**

Both kidneys had single vessels and the left kidney was chosen for NP. The kidney was perfused for a total of 35 min at a mean temperature of 33.9 ± 0.6°C. The renal blood flow reached a maximum of 70mL/min and the average intra-renal resistance was 1.26mmHg/ml/min. The kidney produced a total of 50 ml of urine during perfusion. This volume of urine was replaced with a crystalloid solution. The kidney seemed well perfused throughout with no complications.

Recipients

The normothermically perfused kidney was transplanted into a 55-year-old woman with adult polycystic kidney disease (patient 1). She also had a history of osteogenic sarcoma with lung metastasis, which had been successfully treated by above knee amputation and right upper lung lobectomy in 1968. The patient had been on peritoneal dialysis for 2 years and was under treatment for hypertension and hyperlipidemia. The anastomosis time was 24 minutes and the cold ischaemic time 10 hours 52 minutes. On clamp release, the kidney was well perfused in all areas and urine production was noted. The human leukocyte antigen (HLA) mismatch for this kidney recipient was 1:0:1. The immunological cross match was negative. The kidney demonstrated slow initial graft function, but the patient was dialysis independent. At 7 days after transplant, a surveillance biopsy showed acute tubular necrosis with Banff 1a acute rejection. This was treated initially with three 0.5 g boluses of methylprednisolone but as the serum creatinine remained elevated at 450μmol/L a 10 day course of antithymocyte globulin (ATG; thymoglobulin) was administered; this consisted of five administrations of 1.5mg/kg. Repeat transplant biopsy at the end of the course of ATG showed minimal resolving acute tubular necrosis but no rejection. At 1, 3 and 6 months posttransplant, the patient was well with serum creatinine levels of 189, 147, 118μmol/L respectively.

The CS kidney from the same donor was transplanted into a 52-year-old man with IgA nephropathy, who had been on haemodialysis for 4 years. He was also being treated for

hypertension and hyperlipidemia. The anastomosis time was 25 min and the cold ischaemic time was 14 hours 9 minutes. On clamp release, this kidney showed rather patchy perfusion throughout and no urine was seen on table.

The HLA mismatch for this recipient was 1:1:1. The immunological cross match was negative. Postoperatively, there was little urine production and the patient remained dialysis dependent. A surveillance needle core biopsy performed on the 7th postoperative day showed severe acute tubular necrosis and Banff 2a acute rejection. This was treated with five doses of ATG (1.5 mg/kg) over a 10-day period. The repeat biopsy at the end of the course demonstrated that the rejection had resolved, but there were still moderate changes of acute tubular necrosis. Urine output improved gradually and the period of DGF lasted 26 days. At 1, 3 and 6 months post transplant, the patient's serum creatinine was 673, 236 and 216μmol/L respectively.

8.352 Pair 2 (Table 8.35, 8.351; Figure 8.35b)

Donor details

68y old female extended criteria donor who died as a result of an intracranial haemorrhage. She had a past medical history of hypertension. Her viral status was CMV positive.

***Ex-vivo* normothermic perfusion**

Both kidneys had single vessels and the left kidney was chosen for NP. The kidney was perfused for a total of 82 minutes at a mean temperature of 34.1± 0.78°C. The renal blood flow reached a maximum of 160mL/min and the average intra-renal resistance was 0.49 mmHg/mL/min. The kidney produced a total of 300 mL of urine during perfusion. The kidney was well perfused throughout with no complications.

Recipients details

The normothermically perfused kidney was transplanted into a 46yr old man with end stage renal failure for 4 years due to polycystic kidney disease (patient 4). The patient had been on peritoneal dialysis until 2008 then haemodialysis. The anastomosis time was 20 min and the cold ischaemic time 9 hours 20 minutes. On clamp release, the kidney was well perfused in all areas. The HLA mismatch for this kidney recipient was 1:1:0. The immunological cross match was negative. Post-operatively the patient was hypotensive and the kidney demonstrated slow initial graft function, but the patient was dialysis independent. At 7 days after transplant, a surveillance biopsy showed acute

tubular necrosis with Banff 1a acute rejection and suspected T cell mediated rejection. This was treated initially with three 0.5 g boluses of methylprednisolone however the serum creatinine remained elevated, but no further evidence of rejection was not found on a second biopsy. Immunofluorescence for C4d was negative. The antibody mediated rejection was treated by ultrafiltration. His immunosuppression was changed from tacrolimus to rapamycin two months after transplantation due to a rise in his serum creatinine levels. His serum creatinine levels at 1, 3 and 6 months were 262, 220 and 157μmol/L respectively.

The static cold stored kidney was transplanted into a 60 year old man with ESRF due to polycystic kidney disease. He also suffered from hypertension. The cold ischaemic time was 16 hours 10 minutes and anastomosis time 38 minutes. The HLA mismatch for this kidney recipient was 0-1-0. The patient had initial graft function but creatinine levels were slow to fall. A surveillance biopsy on day 8 post transplant showed borderline rejection and he was treated with three 0.5 g boluses of methylprednisolone. His serum creatinine levels at 1, 3 and 6 months were 201, 204 and 204μmol/L respectively.

8.353 Pair 3 (Table 8.35, 8.351; Figure 8.35c)

Donor details

The donor was a 63 year old lady that suffered an intracranial haemorrhage. Her viral status was CMV positive.

***Ex-vivo* normothermic perfusion**

Both kidneys had a single artery and the right kidney was selected for NP. It was perfused for 60 minutes at a mean arterial pressure of 58 ± 3.5mmHg and temperature of 35 ± 0.3ºC. The renal blood flow increased throughout perfusion and averaged 187ml per minute. The average intra renal resistance was 0.32 ± 0.1mmHg/ml/min. During perfusion the total urine output was 60ml. There were no complications and the kidney appeared well perfused throughout.

Recipients details

The initial recipient of the right kidney was a 56 year-old man who suffered a severe anaphylactic reaction and cardiac arrest at induction of anaesthesia whilst the kidney was being normothermically perfused. He was resuscitated and transferred to the intensive care unit. After 60 minutes of NP the kidney was flushed with 500ml of HOC at 4ºC and placed back on ice until transplanted. The kidney was stored on ice for 11

hours 31 minutes before NP and 5 hours 21 minutes after until transplanted. The total cold ischaemic time was 16 hours 52 minutes.

The kidney was transplanted into a 54 year-old lady with ESRF and secondary hypertension (Patient 10). She had a BMI of 24 and was pre-dialysis with a GFR of 12. The anastomosis time was 30 minutes. The HLA mismatch for this recipient was 1-1-2 and viral status CMV positive.

The patient had immediate graft function with no complications. Serum creatinine levels fell immediately after transplantation. Her serum creatinine levels at 1 and 3 months were 131 and 122μmol/L.

The recipient of left kidney that underwent CS was a 54 year old man with a past medical history of ESRF due to diabetic nephropathy. He had been on haemodialysis since 2005. The HLA mismatch for this recipient was 1-1-1 and viral status CMV negative. The cold ischaemic time was 10 hours 38 minutes and anastomosis time 42 minutes. The patient was dialysed post-operatively for hyperkalaemia but had immediate graft function thereafter. His serum creatinine levels at 1 and 3 months were 189 and 166μmol/L respectively.

8.354 Pair 4 (Table 8.35, 8.351; Figure 8.35d)

Donor details

The donor was a 54 year old female with an intracranial haemorrhage and past medical history of hypertension and chronic obstructive pulmonary disease (COPD). Her viral status was negative.

***Ex-vivo* normothermic perfusion**

The right kidney had a single artery and was selected for NP. It was perfused for 60 minutes and the mean renal blood flow was 110 ± 8.9ml/min. The average temperature was 34.9ºC and the kidney produced a total of 245ml of urine. It was evenly perfused throughout with no complications.

Recipient details

The normothermically perfused kidney was transplanted into a 51 year old male with diabetes and ESRF but pre-dialysis with an eGFR of 8 (Patient 14). The HLA mismatch was 2-2-2 and viral status CMV positive. The cold ischaemic time was 13 hours 22 minutes and anatomosis time 30 minutes. The kidney was transplanted without any complicatons. However, on reperfusion the kidney initially appeared poorly perfused. Nonetheless, the kidney had initial graft function with a steady fall in serum creatinine levels. The patient was discharged from hospital after 7 days without any complications. His creatinine at 1 month was 146µmol/L.

The recipient of the static cold stored left kidney was a 28 year man with ESRF due to chronic pyelonephritis. He was on haemodialysis and had past medical history of hypertension and schizophrenia. The HLA mismatch was 2-1-0 and viral status CMV positive. The kidney had two renal arteries that were widely displaced and therefore on the bench the patch was divided and the arteries closely approximated. The kidney was well perfused after transplantation and a small amount of urine was produced immediately. The was treated for sepsis and a raised creatinine 7 days after transplantation A needle core biopsy showed evidence of acute kidney injury but no rejection . His creatinine at 1 month was 136µmol/L.

	Age (Yr)	CI (h m)	Anastomosis (m)	Mismatch	Graft function	Rejection
Pair 1						
CS	52	14h 9m	25	1-1-1	DGF	Yes
NP	55	10h 52m	24	1-0-1	SGF	Yes
Pair 2						
CS	60	16h 10m	38	0-1-0	SGF	Yes
NP	46	9h 20m	20	1-1-0	SGF	Yes
Pair 3						
CS	54	10h 38m	42	1-1-1	DGF	No
NP	54	16h 52m	30	1-1-2	IGF	No
Pair 4						
CS	28	10h 11m	36	2-1-0	IGF	No
NP	51	13h 22m	30	2-2-2	IGF	No

Table 8.35 Demographics and parameters. Age, cold ischaemic time (CI) in hours (h) and minutes (m), anastomosis time, HLA mismatch, graft function and episodes of acute rejection in the four paired kidneys under going cold storage (CS) or normothermic perfusion (NP).
Delayed graft function (DGF), slow graft function (SGF), initial graft function (IGF).

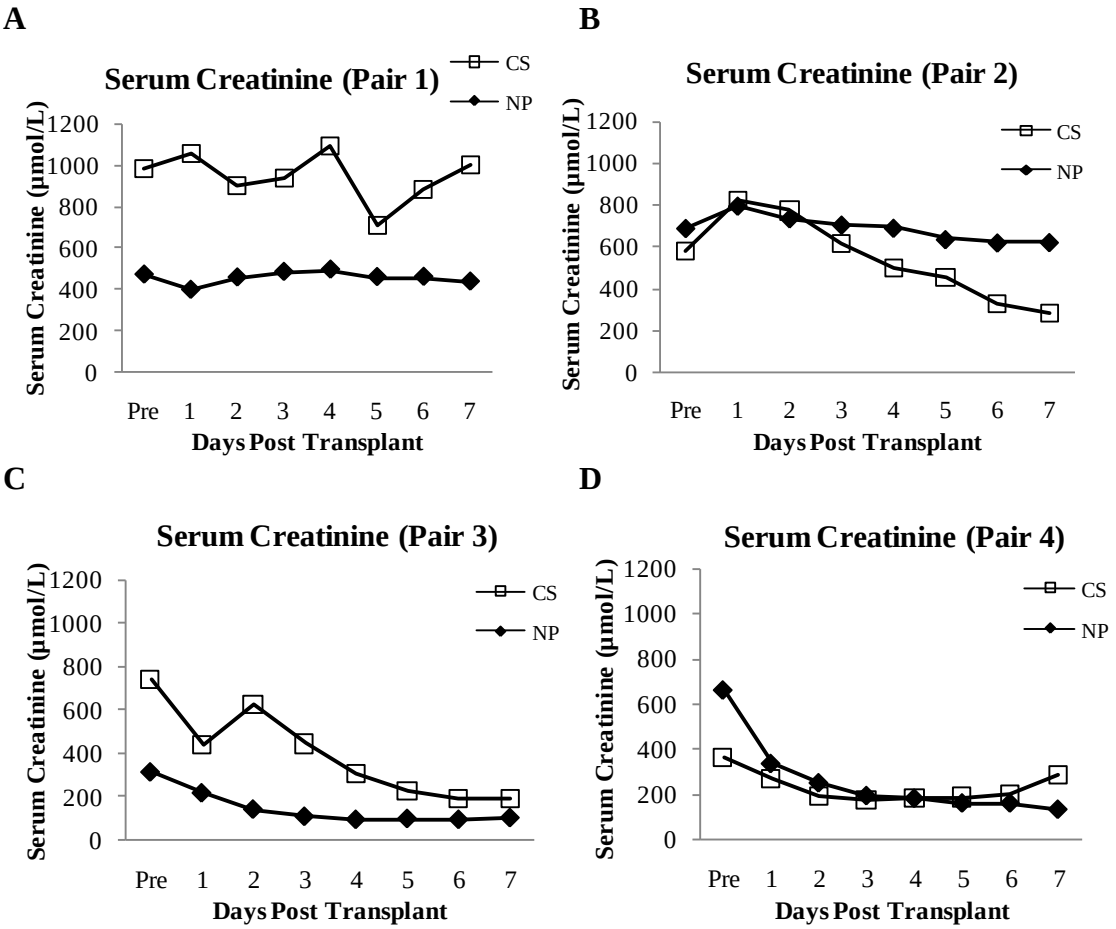


Figure 8.35 Serum creatinine levels pre and over the first 7 days post-transplant in patients receiving kidneys after cold storage (CS) or normothermic perfusion (NP). (A) pair 1 (B) pair 2 (C) pair 3 (D) pair 4.

	Pre Cr	1mth	3mth	6mth	12mth
Pair 1					
CS	988	673	236	216	186
NP	471	183	147	118	117
Pair 2					
CS	574	201	204	204	
NP	689	262	220	157	
Pair 3					
CS	743	189	174	-	
NP	315	131	122	-	
Pair 4					
CS	359	136	-	-	
NP	665	143	-	-	

Table 8.351 Serum creatinine levels in pairs of kidneys undergoing static cold storage (CS) or normothermic perfusion (NP) pre-transplant and 1, 3, 6 and 12 months post-transplant.

8.4 Summary

This is the first report of a series of *ex vivo* NP, using a plasma-free red cell based perfusate, being applied in human renal transplantation. This series of 15 cases demonstrates that NP is feasible and can be performed without apparent deleterious effects to the donor kidney or the recipient.

8.5 Discussion

Clinical Study of normothermic perfusion in marginal donor kidney transplantation

This series of 15 patients demonstrated NP to be a safe and practicable method of preservation. Only one out of 15 patients had DGF (6.7%) although a further 2 had slow graft function during the first week after transplantation. Four patients had an episode of acute rejection, (2 cell mediated, 1 antibody mediated and 1 borderline) within the first month and were treated successfully. The patient who had DGF had an episode of rejection at 3 months post transplant. All patients had a functioning graft at 1, 3 and 6 months post-transplant.

The outcome of kidneys from ECDs in terms of early graft function and longer term survival is significantly inferior to that of DBD donors (291). The incidence of DGF in ECD kidneys has been reported to be as high as 61% (291). Furthermore, a high percentage of kidneys retrieved from ECDs are deemed unsuitable for transplantation with discard rates reported at 40% in 2005 (291). The increased risk of graft failure is mainly because of an age related decline in renal function, reduced capacity for repair, a higher degree of immunogenicity, increased sensitivity to nephrotoxic drugs, especially calcineurin inhibitors (CNI) and increased susceptibility to ischaemic injury (292). Therefore, in marginal donor kidney transplantation it appears essential to minimise the CI time and ensure that donor and recipients are well matched. A significant improvement in graft survival was found in the Eurotransplant Senior Program, an allocation scheme based on the concept of age-matching between donors

and recipients over 65 years of age, when the CI time was reduced from 19 to 12 hours, 1 year graft survival increased from 79% to 86% (293). In this present clinical series, the average duration of CI was approximately 12 hours and therefore likely to be of benefit.

The incremental development and design of the *ex-vivo* perfusion circuit using clinical grade paediatric cardiopulmonary bypass technology has allowed the translation of the experimental work into clinical practice with relative ease. The *ex-vivo* system was used in conjunction with CS was and designed to re-establish circulation and restore metabolism and kidney function for a short period immediately before transplantation. It was carried out in the operating theatre whilst the recipient was being prepared for surgery to avoid prolonging the preservation interval.

It is clear that further study is required to elucidate the exact mechanisms responsible for the potential resuscitative effect of NP. This research has shown the advantage of a short period of NP and early clinical evidence is extremely promising. The continuous flow of blood through the microcirculation of the kidney may be both protective to the endothelium and facilitate the removal of toxic metabolites that accumulate during cold storage.

No firm conclusions can be drawn from the four pairs of kidneys undergoing either NP or CS however the results of the NP kidneys in comparison to the CS kidneys are again encouraging. In two of the pairs the CS kidney had DGF with the patients requiring dialysis compared to initial graft function in the NP kidneys. There was evidence in only one case where the initial graft function was reduced in comparison to the paired

cold stored kidney. The incidences of acute rejection were within the range for marginal donor kidneys. Furthermore, there was no evidence of increased immunogenicity in the NP kidneys. In two of NP cases that had episodes of rejection the recipients of the CS paired kidneys also had rejection.

The anaphylactic event in one of the intended transplant recipients, which occurred whilst the donor kidney was being perfused, is an excellent example of the unpredictability of transplantation and how NP can be adapted to fit clinical requirements. The decision had to be taken at that time whether the organ was suitable for transplantation into a different recipient and what the effect would be if the organ were cooled for a further period of time. This extended period of CI after NP appeared to have no detrimental effect and once transplanted the kidney had immediate graft function. An intermediate period of NP has been previously studied in a canine model. Rijkmann *et a* (203,204) and Maessen *et al* (221,222) both demonstrated that kidneys normothermically perfused for a short period in between prolonged periods of hypothermic preservation had improved graft function and survival compared to kidneys stored under hypothermic conditions alone. The reasons for the improved outcome are unknown but it has been suggested that an intermittent period of NP may afford protection by a number of mechanisms. It may protect the kidney by recovering energy metabolism and adenine nucleotides, restoring circulation to the endothelium, enhancing the tolerance to hypothermia or protecting the lipid bilayers and preventing the loss of membrane fluidity.

The perfusate conditions in this clinical series varied from the experimental model used in the pre-clinical studies. Clinically, perfusion was based on compatible packed red blood cells suspended in crystalloid solution and modified by the addition of an electrolyte and amino acid solution containing multivitamins, mannitol, dexamethasone, and prostacyclin as a vasodilator rather than sodium nitroprusside. This combination was chosen to prevent inflammation and oxidative damage and to minimize cellular swelling and enhance renal blood flow. The mean arterial pressure in the system was set at the lower end of the physiological range at which autoregulation of renal blood flow occurs (294,295). This was chosen in an attempt to prevent endothelial injury. The reperfusion temperature was kept below normal body temperature (34°C) to slightly lower the metabolic rate and avoid the depletion of energy substrates.

Conclusion

A straightforward technique for *ex-vivo* NP with a plasma free red cell based solution is described for the first time in human renal transplantation. This technique worked well and seemed to have advantages over conventional CS. These 15 cases have demonstated NP to be a feasible and safe method of kidney preservation.

Chapter 9

Conclusion

9.1 Conclusion

This research has shown that prolonging the hypothermic preservation period even after a minimum WI insult causes a significant deterioration in renal and tubular cell function. The severity of injury was also detected by early urinary biomarkers of endothelial and inflammatory injury (Endothelin-1, IL-6, TNFα). These may prove beneficial in the clinical setting to determine early graft injury and allow the patients treatment to be tailored accordingly.

Limiting the hypothermic preservation time to reduce the severity of injury is not always logistically possible and therefore alternative techniques of preservation that can reverse the detrimental effects of CI injury and improve early graft function are advantageous. Using adapted paediatric cardiopulmonary bypass technology a technique of NP has been developed to resuscitate the kidney and restore function after a period of WI and CI injury. NP improved blood flow and oxygenation to the kidney during reperfusion. Renal function was also improved and kidneys maintained better acid base homeostasis. When the effects on early graft function were assessed using an autotransplant model, NP was demonstrated to be a safe and feasible method of preservation. The use of clinical grade equipment made the transition into clinical practice relatively simple. The results of this first clinical series of marginal kidneys are extremely encouraging with 14 out of 15 patients having initial graft function and graft and patient survival at 6 months was 100%. The successful perfusion and transplantation of all of the kidneys validates the safety and efficacy of the technique in marginal donor kidney transplantation.

9.2 Experimental model: strengths and limitations

The porcine model used in these studies provides clinically relevant information and aided the translation of this research in clinical practice. The pigs used were of similar size to humans and their physiology and organ development is comparative. The anatomy of porcine and human kidneys has many similarities in that they are multilobular and have a multipapillary structure. In contrast, mice, rats, rabbits and dogs have unilobular, unipapillary kidneys (296). These similarities with along with the ability to concentrate urine, sensitivity to ischaemia and comparative reference values for creatinine and urea make the pig an ideal pre-clinical model on which to assess the effects of kidney injury (296). Furthermore, their similar size allowed for clinical grade perfusion equipment to be adapted for their use.

A disadvantage of using pigs is that because of their rapid growth we are restricted to using young healthy animals and this clearly does not mirror the clinical situation. Nevertheless, porcine kidneys have been used in many studies as models of renal injury to determine the effects of therapeutic agents, assess preservation techniques and ischaemic injury. The autotransplant model is particularly advantageous in assessing the effects of preservation techniques as it avoids an immunological response allowing a direct assessment of the preservation technique.

In this research, an *ex-vivo* reperfusion model was used to assess the effects of WI and CI injury and the NP technique, before NP was translation into a pre-clinical autotransplant model and then into clinical practice. The *ex-vivo* reperfusion model was designed using commercially available clinical grade paediatric cardiopulmonary bypass technology to simulate the acute reperfusion phase of a renal transplant. This

model is reliable and reproducible, allowing the continuous measurement of physiological parameters to investigate the pathophysiology of ischaemic injury. While this system yields useful hemodynamic and renal functional data, it is accepted that the reperfusion period was quite limited, being a relatively short reperfusion interval of 3 hours. Although longer haemoperfusion has been described in the liver (245,246,297), the need for prolonged perfusion was not required to study the acute effects of I/R injury in this model. The WI insult was also relatively limited in the *ex-vivo* NP model and it would be interesting to examine the effects in a model of uncontrolled DCD donor.

In the *ex-vivo* models HOC was used as the CS preservation solution. UW has long been regarded as the gold standard cold preservation solution, which contains additional ingredients, such as adenosine, to support metabolism and allopurinol and glutathione to scavenge ROS and is widely used. HTK which was used in the autotransplant model is a popular preservation solution and is used in the preservation of abdominal organs as an alternative to UW. It is popular in the US and Europe. HOC is one of the most basic preservation solutions and perhaps not the most suitable solution for DCD kidneys, however the evidence for this is sparse. In a recent laboratory study, we found no benefit with the use of UW or HTK in terms of renal function compared with HOC and therefore justified in these experimental studies (298). Furthermore, there have been recent concerns about the efficiency of HTK to protect against CI injury in kidney, pancreas, and liver transplantation (119,299,300). In an analysis of HTK preserved kidneys from marginal donors, Stevens *et al* (121)

found that HTK solution was associated with a higher risk of graft loss and PNF. In a large retrospective series, Stewart *et al* (300) also found that HTK solution was associated with a 20% increase risk of graft loss in NHB donors. Therefore, it has been suggested that careful consideration should be given to the use of HTK in kidneys from marginal donors.

The use of clinical grade equipment and compatible crossed match packed red blood cells made the transition into clinical practice relatively straightforward. Whilst the system is suitable for use in the operating theatre to deliver a short period of NP it is not practical for transporting the kidney under normothermic conditions. If longer NP periods are used a more compact, portable system would be required.

The evidence for NP is extremely encouraging and this technique offers many advantages over hypothermic techniques with the potential to limit the effects of cold preservation, restore function, and recondition an organ in preparation for transplantation. Nonetheless, NP has the disadvantage of requiring additional technical expertise, hardware and consumables and also has the potential to damage the kidney through cannulation of the vessels or by introducing infection during perfusion. Therefore, it remains to be determined if other centres will readily adopt this technique of preservation considering the additional requirements and financial cost.

9.3 Future work

The aim of this thesis was to develop and introduce a technique of NP into clinical practice to improve the condition of marginal kidneys. Nonetheless, many unanswered questions regarding the effects of NP remain. Research is ongoing in the transplant laboratory to establish the processes of protection afforded by a brief period of NP and the molecular impact during reperfusion. Early evidence suggests that after 60 minutes of NP heat shock protein (HSP) 70 is upregulated and further enhanced during reperfusion. HSP 70 is known to be induced under stress conditions and has a role in regeneration and repair (301,302). Several inflammatory cytokines (Il-β, IL-6, IL-8) were also found to be elevated after NP. Although a measure of inflammatory injury, these cytokines may also have a role in mediating repair. Therefore NP may have a conditioning effect that modulates the inflammatory process during reperfusion. Nonetheless, more research is needed to determine the mechanisms of protection.
The perfusion conditions are also being streamlined to optimise oxygen delivery and improve the protective environment. Due to the shortage of available compatible blood, alternatives to a blood based solutions which have suitable oxygen carriage and delivery properties, such as perfluorocarbons and artificial haemoglobin solutions, need to be explored. Other novel oxygen carriers such as the previously mentioned Hemarina-M101 (M101) and micro bubbles derived from Dodecafluoropentane (DDFPe) may also be applied in the future.

The clinical application of NP holds many potential advantages over hypothermic conditions. Restoring function *ex-vivo* may also prove to have a role in viability testing of marginal organs. The novel urinary biomarkers used to determine the severity of ischaemic injury during reperfusion in this research could be adopted to judge the quality of the kidney during NP. This would allow an organ to be transplanted on the basis of its quality rather than relying on the current system of donor characteristics and ischaemic intervals to judge its suitability. An application for ethical approval to use the NP system to perfuse disused human kidneys to determine viability and to study the potential of NP to recover function has been made. An array of injury markers will be used to measure injury and recovery.

In the future, NP may also prove to be a useful adjunct in the delivery of gene therapy and stem cells in an attempt to effect *ex-vivo* tissue repair and modulation targeting I/R injury, the immune response and reduction of early fibrotic gene expression. Brasile *et al* have previously used their EMS system to introduce fibroblast growth factors to the kidney to recover and repair warm ischaemically damaged kidneys (232,251). Most recently, they have treated canine kidneys with a bioengineered interface consisting of a nano-barrier membrane to modify the antigenicity of the vascular wall to delay the onset of acute rejection (303). Such therapies targeting injury and repair and graft antigenicity would have important clinical consequences by potentially expanding the organ donor pool, allowing more marginal kidneys to be utilised, reducing the discard rate and improving the outcome.

Nonetheless, the most important question is if NP provides any advantage over traditional hypothermic preservation techniques. In answering this question a large randomised multicentre trial is required to assess the outcome in marginal donor kidney transplantation.

Chapter 10

Appendix

10.1 Reperfusion outcome measurements *ex-vivo* and autotransplant models

Assessment	
Renal function	Serum creatinine, creatinine clearance, serum potassium
Tubular function	Fractional excretion of sodium, protein fraction ratio, urine output
Acid base balance	pH, bicarbonate, base excess,
Haemodynamics	Renal blood flow, intra-renal resistance
Metabolism	Oxygen consumption
Cellular injury	AST, LDH
Endothelial injury	Endothelin-1,
Oxidative damage	8-isoprostane, protein carbonyl (ELISA)
Inflammation	IL-6, TNFα,
Neutrophil infiltration	Myeloperoxidase (Immunohistochemistry)
Cellular structure	Histology score (Hematoxylin & Eosin)

Table 10.1 Summary of the functional and injury parameters measured during *ex-vivo* reperfusion using the isolated organ perfusion system. Asparatate transaminase (AST) and lactate dehydrogenase (LDH), interleukin-6 (IL-6), tumour necrosis factor alpha (TNFα).

10.2 Histology: The effect of warm and cold ischaemic injury in an *ex-vivo* model of kidney transplantation

Post cold storage

Group	Tubular dilatation	Epithelial loss	Tubular Debris	Vacuolation	Glomerular shrinkage
0 (2)	2.3 ± 0.4	1.0 ± 0.2	1.0 ± 0.1	0.2 ± 0.5	1.7 ± 0.5
0 (18)	1.6 ± 0.4	1.0 ± 0.0	0.9 ± 0.2	0.0 ± 0.0	1.1 ± 0.2
10 (2)	1.7 ± 0.4	1.2 ± 0.2	0.9 ± 0.3	0.1 ± 0.1	0.3 ± 0.2
10 (18)	1.7 ± 0.7	1.1 ± 0.3	1.0 ± 0.0	0.0 ± 0.0	1.3 ± 0.5
25 (2)	1.9 ± 0.7	1.1 ± 0.2	1.1 ± 0.1	0.0 ± 0.0	1.8 ± 0.6
25 (18)	1.5 ± 0.7	1.0 ± 0.3	0.8 ± 0.4	0.0 ± 0.0	1.2 ± 0.5

Table 10.2a Histology score in the post-storage biopsies. Wedge biopsies were taken after cold storage and stained with H&E for evaluation using light microscopy after warm ischaemic (WI) periods of 0, 10 and 25 minutes and 2 and 18 hours of cold storage (CS) [0 (2), 0 (18), 10 (2), 10 (18), 25 (2), 25 (18)] . Sections were semi-quantitatively scored blinded over ten fields, assessing changes in five morphological variables, tubular dilation, tubular epithelial loss, tubular debris, vacuolation, and glomerular shrinkage. Samples were scored from 0 to 3 according to the level of damage; 0 representing normal, 1 mild, 2 moderate and 3 severe morphological changes.

Post reperfusion

Group	Tubular dilatation	Epithelial loss	Tubular Debris	Vacuolation	Glomerular shrinkage
0 (2)	2.1 ± 0.3	1.1 ± 0.2	0.9 ± 0.2	1.0 ± 0.5	1.5 ± 0.4
0 (18)	2.2 ± 0.3	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.6	1.4 ± 0.3
10 (2)	2.5 ± 0.4	1.3 ± 0.3	0.9 ± 0.4*	0.1 ± 0.1	1.8 ± 1.0
10 (18)	1.7 ± 0.7	0.9 ± 0.2	0.9 ± 0.4	0.3 ± 0.7	1.5 ± 0.5
25 (2)	2.0 ± 0.5	1.2 ± 0.3	1.0 ± 0.1	1.6 ± 0.4**	1.5 ± 0.5
25 (18)	2.3 ± 0.3	1.0 ± 0.1	1.0 ± 0.1	0.4 ± 0.7	1.4 ± 0.4

Table 10.2b Histology score in the post-reperfusion biopsies. Wedge biopsies were taken after reperfusion on the isolated organ perfusion system and stained with H&E for evaluation using light microscopy after warm ischaemic (WI) periods of 0, 10 and 25 minutes and 2 and 18 hours of cold storage (CS). Sections were semi-quantitatively scored blinded over ten fields, assessing changes in five morphological variables, tubular dilation, tubular epithelial loss, tubular debris, vacuolation, and glomerular shrinkage. Samples were scored from 0 to 3 according to the level of damage; 0 representing normal, 1 mild, 2 moderate and 3 severe morphological changes. * P = 0.037, 10 (2) vs 25 (2), ** P = 0.003, 25 (2) vs 10 (2).

10.3 Histology: The effects of *ex-vivo* normothermic perfusion

Post Preservation

Group	Tubular dilatation	Epithelial loss	Tubular Debris	Vacuolation	Glomerular shrinkage
2h CS	1.7 ± 0.4	1.2 ± 0.2	0.9 ± 0.2	0.1 ± 0.1	0.3 ± 0.2
18h CS	1.7 ± 0.7	1.1 ± 0.3	1.0 ± 0.0	0.0 ± 0.0	1.3 ± 0.5
HMP	2.2 ± 0.4	1.1 ± 0.2	0.8 ± 0.4	0.0 ± 0.0	1.6 ± 0.3**
NP	1.8 ± 0.7	1.2 ± 0.4	0.8 ± 0.4	0.1 ± 0.1	1.6 ± 0.3*

Table 10.3a Histology score in the post-preservation biopsies. Wedge biopsies were taken after preservation and stained with H&E for evaluation using light microscopy after preservation by 2and 18 hours of cold storage (CS), hypothermic machine perfusion (HMP) or normothermic perfusion (NP). Sections were semi-quantitatively scored blinded over ten fields, assessing changes in five morphological variables, tubular dilation, tubular epithelial loss, tubular debris, vacuolation, and glomerular shrinkage. Samples were scored from 0 to 3 according to the level of damage; 0 representing normal, 1 mild, 2 moderate and 3 severe morphological changes. (* P = 0.03, NP vs 2h CS, ** P = 0.03, HMP vs 2h).

Post reperfusion

Group	Tubular dilatation	Epithelial loss	Tubular Debris	Vacuolation	Glomerular shrinkage
2h CS	2.5 ± 0.4*	1.3 ± 0.3	0.9 ± 0.4	0.13 ± 0.2	1.3 ± 0.9
18h CS	1.7 ± 0.4	0.9 ± 0.2	0.8 ± 0.4	0.3 ± 0.7	1.5 ± 0.5
HMP	2.0 ± 0.3	1.0 ± 0.0	0.5 ± 0.5	0.7 ± 0.8	1.0 ± 0.6
NP	1.9 ± 0.4	0.8 ± 0.3	0.9 ± 0.2	1.5 ± 0.6	1.8 ± 0.6**

Table 10.3b Histology score in the post-reperfusion biopsies. Wedge biopsies were taken after 3 hours reperfusion on the isolated organ perfusion system and stained with H&E for evaluation using light microscopy after preservation by 2 and 18 hours of cold storage (CS), hypothermic machine perfusion (HMP) or normothermic perfusion (NP). Sections were scored blinded over ten fields, assessing changes in eight morphological variables, tubular dilation, tubular epithelial loss, tubular debris, vacuolation, and glomerular shrinkage. Samples were semi-quantitatively scored from 0 to 3 according to the level of damage; 0 representing normal, 1 mild, 2 moderate and 3 severe morphological changes. (* P = 0.032, 2h CS vs 18h CS, ** P = 0.14, NP vs 18h CS)

10.4 Histology: A pilot study assessing the feasibility of a short period of normothermic perfusion in a porcine autotransplant model

	Baseline	Reperfusion	End
Tubular dilatation			
HMP	0.4 ± 0.64	1.2 ± 1.01	2.2 ± 0.68¶
NP	0.6 ± 0.65	1.3 ± 0.91	1.9 ± 0.82¶
Tubular debris			
HMP	0.6 ± 0.50	1.4 ± 0.73	2.4 ± 0.68¶
NP	1.3 ± 0.61*	1.6 ± 0.86	2.4 ± 0.76¶
Vacuolation			
HMP	0.5 ± 0.95	0.3 ± 0.46	1.2 ± 1.18¶
NP	0.4 ± 0.77	1.1 ± 0.64*	1.1 ± 0.66¶
Infiltration			
HMP	0.9 ± 0.32	0.9 ± 0.29	2.2 ± 0.92¶
NP	0.9 ± 0.28	1.2 ± 0.43	1.9 ± 0.73¶

Table 10.4 Histology score. Baseline, reperfusion and end biopsies were blinded and semi-quantitatively scored using 4 parameters over 5 fields, tubular dilatation, tubular debris, vacuolation and infiltration. Scores ranged from 0 - 3, 0 the least injury and 3 the most severe. Values are mean ± SD. * P value <0.05 between groups
¶ P value <0.05 between end time point and pre-perfusion biopsies within each group.

Pt	Age	Gender	IGF	DGF	Hospital stay	Acute Rejection	Pre Cr (μmol/L)	Cr 7 Day	Cr 1 Mth	Cr 3 Mth	Cr 6 Mth	Cr 12 Mth
1	55	F	Yes (SGF)	No	28	Yes	471	437	189	147	118	117
2	59	M	Yes	No	9	No	566	236	185	173	218	150
3	48	M	Yes	No	6	No	783	136	118	137	151	_
4	47	M	Yes (SGF)	No	23	Yes	689	622	262	151	157	_
5	68	F	Yes	No	6	Yes	1140	129	194	165	146	_
6	72	M	No	Yes	19	Yes	761	547	285	555	246	_
7	74	F	Yes	No	6	No	327	96	78	89	84	_
8	58	M	Yes	No	6	No	791	125	116	130	159	_
9	51	M	Yes	No	10	No	698	149	102	118	122	_
10	54	F	Yes	No	7	No	315	105	131	122	111	_
11	43	M	Yes	No	7	No	717	137	184	178	193	_
12	69	M	Yes	No	7	No	998	138	120	112	_	_
13	41	M	Yes	No	6	Yes	885	317	160	131	_	_
14	51	M	Yes	No	7	No	665	133	143	136	_	_
15	64	F	Yes	No	7	No	770	124	179	140	_	_

Appendix 10.5 Table 10.5 Patient demographics and outcome. Patient age, gender, initial graft function (IGF), slow graft function (SGF), delayed graft function (DGF), length of hospital stay (Days), Episodes of biopsy proven acute rejection, serum creatinine (Cr) levels pre and 7 days, 1, 3, 6 and 12 months post-transplant.

Chapter 11

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