Evaluation of Normothermic Flush and Perfusion Techniques, Using a Novel Normothermic Preservation Solution, in an Isolated Porcine Haemoperfusion Model

By

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The work on which this dissertation is based is my own independent work except where acknowledged

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Publications from this Work

Papers

Comparison of preservation solutions in an experimental model of organ cooling in kidney transplantation
M. D. Kay, S. A. Hosgood, A. Bagul and M. L. Nicholson
British Journal of Surgery 2009 Oct; 96(10):1215-21.

Static normothermic preservation of renal allografts using a novel non-phosphate buffered preservation solution.
M.D Kay, S.A Hosgood, S.J.F Harper, D. Rees, M.L Nicholson
Transplant International 2007 Jan; 20 (1): 88-92

Normothermic versus hypothermic ex-vivo flush. A comparison of a novel phosphate-free preservation solution and a standard hypothermic method in renal allografts.
M.D Kay, S.A Hosgood, S.J.F Harper, D. Rees, M.L Nicholson
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Poster Presentations

Static normothermic preservation of renal allografts using a novel non-phosphate buffered preservation solution. M.D Kay, S.A Hosgood, S.J.F Harper, D. Rees, M.L Nicholson.
Presented at British Transplant Society Belfast meeting April 2005
European Society of Transplantation, Geneva October 2005 and ASGBI 2006

Normothermic versus hypothermic ex-vivo flush. A comparison of a novel phosphate-free preservation solution and a standard hypothermic method in renal allografts.
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Synopsis

The basis of the work in this thesis is an assessment of renal preservation techniques. The first chapter comprises a review of the pathophysiology of ischaemic injury and the role of hypothermic preservation. In the second chapter a review of the various preservation solutions in use is presented as well as the constituents of preservation solutions. The role of the organ flush is then presented, comparing warm and cold flush. The effect of viscosity and pressure is then examined followed by a review of hypothermic and normothermic preservation methods.

The five main experimental chapters are then presented as a series of papers. The first of these aimed to assess the rate of organ cooling during back-table flush using different preservation solutions. In this paper, porcine kidneys were flushed with UW or Soltran solution, and the perfusion rate and cooling of the kidney was assessed. The second paper follows on comparing organ cooling using either a cold flush or a warm flush followed by a cold flush to compare flush rates and cooling times. The third paper assesses different flush techniques using a novel normothermic preservation solution, AQIX® RS-I, to assess the optimal flush conditions and functional outcomes.The fourth paper compares functional results of AQIX with the more commonly used cold flush solutions, Soltran and University of Wisconsin solution. The final experiment assesses the effectivness of complete normothermic preservation using AQIX® RS-I. An isolated organ perfusion system using cardiopulmonary bypass equipment was used for normothermic preservation and as a surrogate for transplantation. Porcine kidneys were used throughout all experiments as large animal models best reflect the human condition.

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Abbreviations

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
AMP	Adenosine Monophosphate
ATN	Acute Tubular Necrosis
CS	Cold Storage
CIT	Cold Ischaemic Time
DGF	Delayed Graft Function
EMS	Exsanguinous Metabolic Support
HBD	Heart Beating Donor
HOC	Hyperosmolar Citrate
IRI	Ischaemia Reperfusion Injury
IRR	Intra-renal Vascular Resistance
K^+	Potassium ions
LDH	Lactate Dehydrogenase
Mg^{2+}	Magnesium ions
MP	Machine Perfusion
MMP	Matrix Metalloproteinase
MPS	Machine Perfusion Solution
Na^+	Sodium ions
NHBD	Non-Heart-Beating Donor
NO	Nitric Oxide

PNF	Primary Non-Function
POPS	Portable Organ Perfusion System
SEC	Sinusoidal Endothelial Cell
UW	University of Wisconsin
WIT	Warm Ischaemic Time
WP	Warm Perfusion
XO	Xanthine Oxidase

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Section One

REVIEW OF THE LITERATURE

Section One: Introduction

Renal transplantation is the gold-standard treatment of end-stage renal failure. It is more cost-effective compared to dialysis, and gives a better quality of life to the patient. However the use of transplantation in renal failure is severely limited by the mismatch of supply and demand for kidney organs (Figure1) [1]. Donor sources have traditionally been from cadaveric donors, but there has been a vast expansion in the supply from live-donors over the last twenty years (Figure 2) [1]. There has also been a large increase in kidneys sourced from non-heart beating donors (NHBD), and these continue to remain a potential source of expansion. NHBD kidneys are known to have a significantly higher incidence of delayed graft function (DGF), whilst the long term allograft function is similar to kidneys from heart-beating donors (HBD) and live donors [2].

An important component of all organs sourced for transplantation is the need to flush the kidney at retrieval and for effective preservation thereafter. Preservation of organs is an important non-immunological factor that can influence graft function. By optimising organ retrieval and kidney preservation techniques, a reduction in both the incidence and duration of delayed graft function may be possible. Progress in preservation can only be achieved with an adequate understanding of mechanisms involved in ischaemia-reperfusion injury.

Figure 1. Deceased donor UK kidney programme. Number of donors, transplants and patients on the active transplant list 1 April 1999 - 31 March 2009

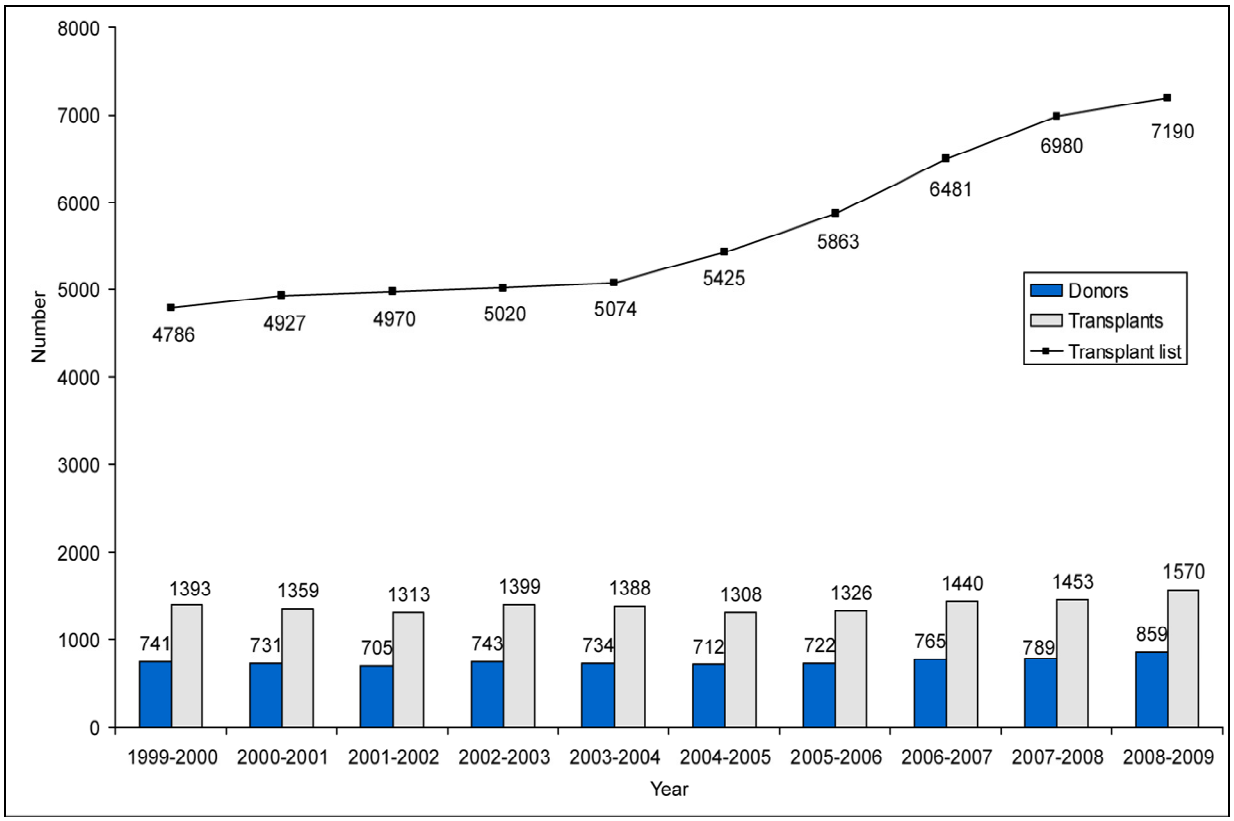
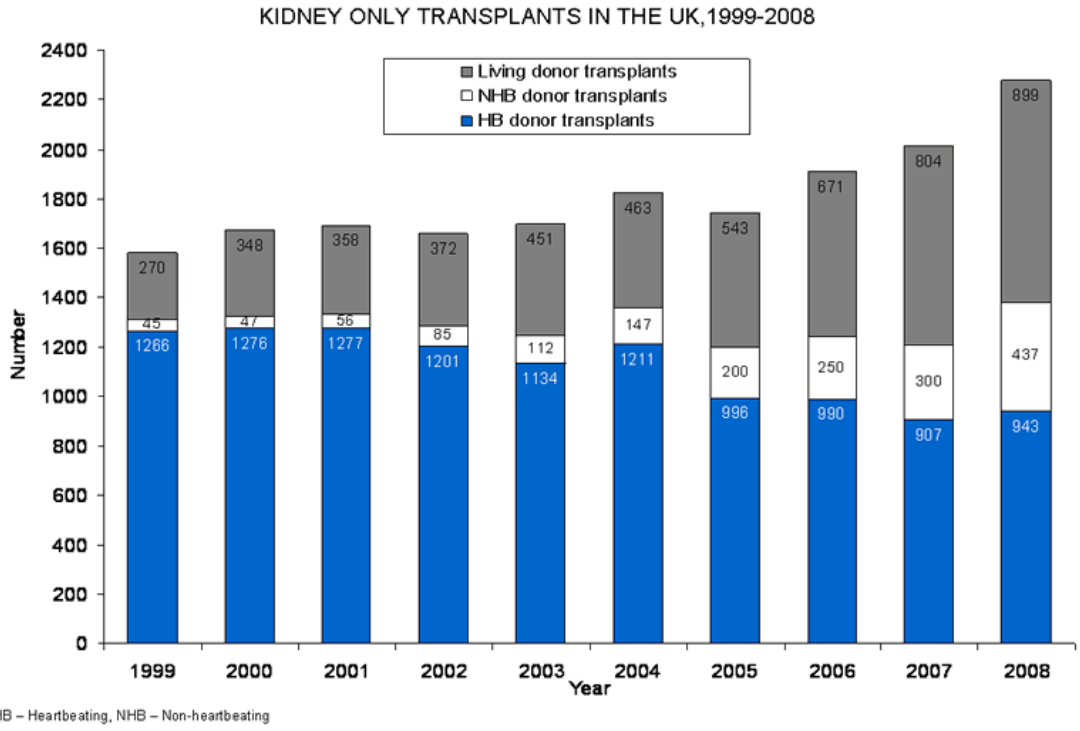


Figure 2. Kidney Transplants performed from live donor, heart-beating and non-heart beating donors 1999-2008.



HB – Heartbeating, NHB – Non-heartbeating

Pathophysiology of ischaemia, hypothermia and reperfusion

Under normal aerobic conditions, various processes occur to maintain ionic equilibrium across the renal cell membrane.

Passive processes include:

- Sodium influx and potassium and magnesium efflux from the cell along their concentration gradients
- Sodium influx, together with chloride that result in influx of water
- Calcium influx along its concentration gradient
- Passive movement of water by the Donnan effect.

The above processes would cause detrimental cellular swelling if they remained unchecked by active, ATP-dependent processes. The dominant ATP-driven membrane pump is the sodium-potassium ATPase pump that exchanges 3 sodium ions out for 2 potassium ions into the cell for each ATP used. This maintains ionic homeostasis across the cell membrane, resisting the influx of chloride, and also maintains a negative resting membrane potential. Calcium is exchanged with sodium via a co-transporter (antiporter) and also by a sodium-calcium dependent-ATPase to maintain their homeostasis. ATP is broken down to ADP and inorganic phosphate that is processed by oxidative phosphorylation in the mitochondria to ATP.

Effects of Ischaemia

Ischaemia results in hypoxia and nutrient depletion which has two key effects, firstly on aerobic metabolism and hence ATP dependent processes, and secondly it initiates anaerobic metabolism. When aerobic metabolism is interrupted, oxygen-dependent processes in the mitochondria cease and ATP levels fall dramatically. Anaerobic metabolism can generate only 2 ATP molecules per glucose molecule metabolised compared with 38 ATP molecules during aerobic metabolism [3]. The normal homeostatic mechanisms across the cell membrane described above thus cease to be effective, which is further exacerbated by the intracellular acidosis created by lactate formation during anaerobic metabolism. Without ATP the anoxic ischaemic cell can no longer maintain ionic stability. The net result is cellular swelling as water enters the cell with the influx of sodium, chloride and calcium ions, and loss of potassium and magnesium out of the cell. Acidosis decreases the stability of lysosomal membranes, activating lysosomal lytic enzymes, and disrupts the binding of transition metals, such as iron, to their binding proteins, that can later cause damage during reperfusion [3] (figure 3).

The resting membrane potential is also lost with the influx of chloride and sodium causing depolarisation. Depolarisation allows voltage-dependent calcium channels to open allowing an influx of calcium. The mitochondrial membrane potential is also lost allowing release of intracellular calcium stores into the cytosol. Calcium influx remains unchecked by the sodium-calcium ATPase system and thus diffuses into the cytoplasm.

High levels of intracellular calcium have detrimental effects:

- Activation of phospholipases that damage the bilayer cell membrane and increase its permeability to water and ions resulting in cellular oedema [4].
- Activation of proteases in lysosomes which may result in cytolysis [5].
- Calcium influx activates proteases, which convert xanthine dehydrogenase to the superoxide xanthine oxidase, involved in reperfusion injury.
- Binding of calcium to membrane proteins affects the cytoskeleton [6], causing cell membrane rigidness. This is of particular significance to erythrocytes as they need to be flexible to allow passage through capillaries. Rigid erythrocytes may become trapped causing sludging, that can inhibit perfusion and contribute to the "no-reflow" phenomenon [5].

The failure to synthesise ATP results in the accumulation of ADP. ADP is normally converted to ATP and AMP, the latter being further broken down to adenosine which moves freely from the cytosol to the extracellular space, where it is further broken down to inosine and hypoxanthine. Hypoxanthine is normally converted to xanthine and then uric acid. There is thus a net depletion of the precursors of ATP formation. During ischaemia, proteolytic enzymes convert the accumulating xanthine dehydrogenase to xanthine oxidase, which is a prominent generator of reactive oxygen species (ROS).

Effects of hypothermia and its complications

The principal effect of hypothermic preservation is to slow the metabolic rate. Cellular oxygen requirements fall exponentially with decreasing temperature [7], due to conformational changes in enzymes, altering their catalytic activity. This varies with each enzyme but as a guide, metabolism is slowed by a factor of 1.5-2 for each 10 ºC fall in temperature [8, 9]. Below 15°C, the Na^+/K^+-ATPase ceases to function [10], and metabolic demand is reduced by 90% [8]. Reducing the organ's core temperature below 4 ºC results in a decrease in metabolism to approximately 5-8 % in the majority of cells [11, 12] .This is beneficial in inhibiting ATP consuming processes such as gluconeogenesis, and slowing down the damaging processes resulting from ischaemia described above. However there are several potential complications of hypothermia that have been observed:

- ATP depletion and a loss of its precursors.

ATP can actually still be produced during hypothermia by the enzyme ATP synthetase which is impeded by hypothermia to a lesser degree. However the enzyme, adenine nucleotide translocase, (that transports ATP from the mitochondria to the cytosol and transports ADP back into the mitochondria) is cold sensitive and thus ADP builds up in the cytosol [7]. ADP is then converted to ATP and AMP, the latter being further broken down to adenosine which moves freely from the cytosol to the extracellular space, where it is further broken down to inosine and hypoxanthine, and is therefore lost from the cell. There is thus a net depletion of the precursors of ATP formation.

- Cellular oedema

During hypothermia, the ATP-dependent active processes described above will cease to function effectively and cell membrane homeostasis will be lost. Inactivation of the membrane bound sodium/potassium and calcium/magnesium pumps, results in influx of sodium (and hence water) leading to cellular swelling and cell death. Sodium also enters the cell due to the osmotic force of intracellular proteins. These anionic proteins create an osmotic force resulting in a passive transport of sodium (and hence water) across the cellular membrane culminating in cellular oedema [13].

- Compromised membrane function

During hypothermia damage to the cytoskeleton occurs due to the effects described above of calcium activating phopholipases, damaging the integrity of the cytoskeleton, and damaging mitochondria. Mitochondria are now no longer able to couple the electron transport chain with phosphorylation processes. There is also a loss in membrane lipid fluidity causing the protein and lipid components to separate making the membrane 'leaky'[14].

- Free radical formation

By the conversion of hypoxanthine to ROS as described above.

- Acidosis

During anaerobic glycolysis, metabolisation of 1 mol glucose only yields 2 mol ATP, but also two lactic acid molecules are formed as well [13]. Lactate accumulation during anaerobic metabolism results in acidosis. The acid pH affects the stability of lysosomal enzymes with release of lytic enzymes (as described above)

- Cellular trapping

Erythrocytes, polymorphonuclear leucocytes and platelets not removed by organ flush will be trapped and aggregate in the micro-circulation [3].

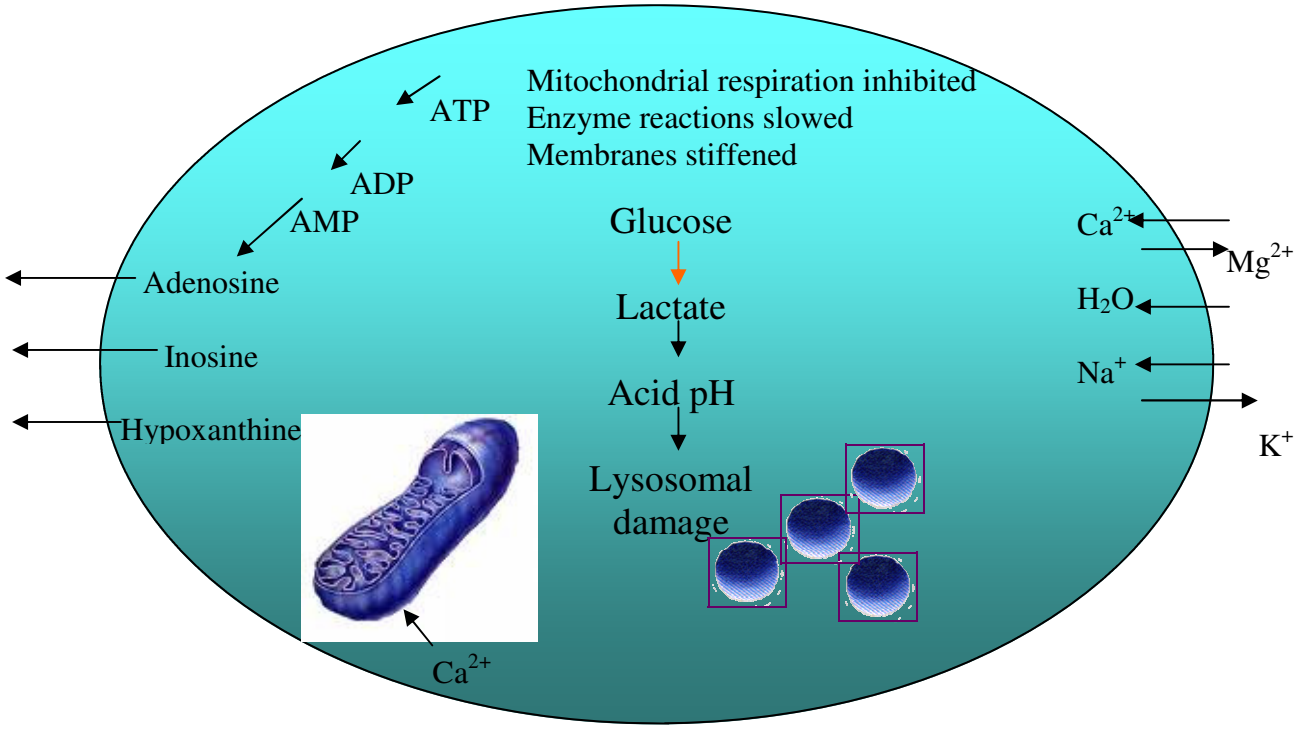


Figure 3. Effects of hypothermia and ischaemia: fuel depletion slowed, cellular homeostasis still impaired (Adapted from Marshall [3])

Effect on renal vasculature during ischaemia and hypothermia

The vascular endothelial cells and tubular cells are also affected by ischaemia and hypothermia. The endothelium is an important part of the renal microcirculation, necessary for perfusion and controlling vascular tone. The above processes result in endothelial cell swelling and increased intra-renal vascular resistance, due to increased endothelin-1 secretion [15], which is vasoconstrictive, and impaired production of the vasodilator, nitric oxide [16]. Tubular cells are damaged by ischaemia and slough off and may form casts that can obstruct the tubule lumen [3].

Finally swelling of renal tubule cells can cause extrinsic compression of blood vessels causing vascular congestion, which when combined with erythrocyte sludging further contributes to the "no-reflow" phenomenon during reperfusion [17, 18].

Reperfusion injury

The pathway of injurious processes described so far culminate during reperfusion causing organ injury that may result in primary non-function, or delayed graft function.

During ischaemia, xanthine dehydrogenase is converted to the enzyme xanthine oxidase as previously described. Upon reperfusion, xanthine oxidase converts hypoxanthine to xanthine, and during this process transfers an electron to oxygen, creating an oxygen free radical (instead of transferring it to NAD as happens in aerobic metabolism). These free radicals have potent oxidizing and reducing properties, which result in tissue injury [19, 20]. Hydroxyl ions that can react with free iron are also produced, and form highly reactive hydroxyl free radicals. These further damage the cell membrane, which already has lost ionic homeostatic control due to the effects of ischaemia and hypothermia.

Endothelial cell damage causes erythrocytes, leucocytes and platelets to adhere and cause obstruction and the no-reflow phenomenon. This injury is further exacerbated during reperfusion by neutrophils that release chemotactic agents such as oxygen free radicals, Leucotriene B4 and platelet activating factor [21], which amplify the response. The role of leucocytes in ischaemia reperfusion injury was demonstrated elegantly by Harper et al [22], who compared normothermic perfusion of porcine kidneys with both whole and leucocyte depleted blood, showing improved post-perfusion renal function, renal blood flow, acid-base homeostasis and amelioration of tubular damage with leucocytes removed. At a time of growing organ shortage, the role of preservation to ameliorate the damaging effects of ischaemia, hypothermia and reperfusion injury is paramount.

Chapter 2- Preservation Methods

Preservation Methods

The success of organ transplantation can be attributed to improvements in several areas, such as immunosuppression, tissue typing, surgical experience and preservation techniques. The first successful renal transplant was performed in 1954 by Murray, in a living related setting with implantation occurring immediately after explantation and thus preservation was not essential. Preservation is now essential to allow safe storage, transfer, and recipient preparation to transplant an organ that will function. Kidney perfusion existed as early as 1938 when Carrel and Lindbergh perfused kidneys with normothermic oxygenated serum [23]. In subsequent years work continued, with examples such as Calne et al who preserved canine kidneys for 8 to 12 hours by immersion in iced water [24], and Keeler et al in 1966 [25], who developed an intracellular-type preservation solution high in potassium and magnesium to prevent loss of these ions. It was in the late 1960's though, that landmark developments in preservation took place by two pioneers, Dr Folkert Belzer and Dr Geoff Collins.

In 1967, Belzer et al reported successful 3 day preservation of canine kidneys using pulsatile machine preservation at 6-8° C with a solution derived from plasma [26]. Initial problems with aggregation of lipoproteins in the perfusate were overcome by freeze-thawing the plasma and ultrafiltration of the the lipoproteins to produce cryoprecipitated plasma (CPP). Although initially successful in NHBD human and canine recipients [27], there was concern about infection as it was human-based. Thus the search for a synthetic substitute continued.

At the same time, Collins et al developed a new, simpler, machine-free preservation method, cold static storage. Static storage involves merely flushing the organ with cold solution to remove blood from the vasculature and then storing it in ice at 4 °C. “Collins solution”, as it became known was an intracellular- type solution, based on its high potassium and low sodium content, to help maintain intracellular ionic balance. Glucose was also added in high concentration to make it isosmotic and helped prevent hypothermic-induced cell swelling. Using this solution, Collins et al reported successful preservation of canine kidneys for 30 hours at 4 °C [28].

Subsequently modifications were made to both Belzer’s perfusion solution and Collins’ static storage solution. A plasma-based perfusion solution that removed lipoproteins by silica gel filtration, that had improved shelf stability was tested [29], as was a Ringer’s- type solution containing albumin for colloidal osmotic support [30]. None of these showed significant advance over Belzers original solution, until the 1980’s when Belzer et al developed a synthetic perfusion solution that contained hydroxyl-ethyl starch for colloidal support instead of albumin. It was synthetic, thus abrogating the concerns of infection, had shelf stability [31] and was shown to increase preservation time of kidneys up to seven days [32, 33].

Collins solution contained concentrations of magnesium and phosphate above their solubility product (Mg 30 mmol/L, PO_4 57.5 mmol/L) causing a precipitate and crystal formation. Although never proved to be damaging, the original solution was modified by removing the magnesium, resulting in a solution known as EuroCollins (EC) solution [34].

An Australian based group developed a cold storage solution known as hyperosmolar citrate (HOC) or Marshall’s solution to overcome some of the limitations of Collins solution. A phosphate-buffered sucrose solution (PBS) was

developed by Coffey and Andrews that proved to be comparable to other solutions, but it was not until Belzer and Southard developed University of Wisconsin solution (UW), that advances were made for longer term storage and multi-organ preservation.

Principal components of flush solutions

Currently used preservation solutions were designed to allow effective organ flush, organ cooling and to minimize the negative effects of ischaemia and hypothermia. The constituents of preservation solutions have changed over time as a reflection of increased knowledge of their effects, and how they may ameliorate the damage caused by reperfusion injury. There are however core components which are outlined in table 1.

Intracellular vs extracellular ionic composition

The maintenance of equilibrium between the intravascular, interstitial and intracellular fluid compartments is important to prevent cellular oedema and maintain membrane function. The Donnan effect of the negatively charged intracellular proteins, and distribution of electrolyte ions helps to maintain equilibrium [35]. In the early years of organ preservation it was believed that an intracellular-type solution that mimicked the intracellular ionic balance (ie high potassium, low sodium) was effective in minimising exchange of fluid thus preventing cellular swelling.

Extracellular solutions (high sodium/low potassium) are now more commonly used as they have been shown to be equally effective [36, 37], and have the added advantage of minimising the vasoconstrictive effect of potassium [38, 39]. Reduced

vasoconstriction should reduce intravascular resistance and allow a better washout of the microcirculation.

Impermeable solutes

Impermeants counteract the osmotic effect of proteins and anions inside cells. The effectiveness of saccharide impermeants is based on their molecular weight. Glucose (used in EC) has the smallest molecular weight (180mW) and thus can pass through the cell membrane resulting in cellular oedema. Thus larger saccharides have superceded such as sucrose (342mW) in PBS, and raffinose (594mW) in HTK.

Non-saccharide impermeants use their size and anionic charge to counter swelling. The most described is lactobionic acid, used in UW, which has a high molecular weight (358mW) and has been added to other preservation solutions with effective results [36, 37].

Colloids in preservation solutions

UW was originally designed as a MP solution and as such contained the colloid hydroxyethyl starch (HES). Colloids are an important component in MP techniques to counteract extravasation of fluids due to hydrostatic pressure. There has been much debate concerning the role of colloids in CS solutions. HES-free UW has shown equivalent results in rat livers [40], and kidneys [41]. Omission of colloid in a randomised controlled trial showed equivalent and satisfactory preservation of kidneys with HES-free UW solution compared to the more expensive UW [42]. However studies on protein synthesis in the rat liver, suggest that HES plays an important role in limiting proteolysis [43]. Finally colloids increase the viscosity of

solutions making UW a slower initial flush solution, which may increase the initial ischaemic period.

Free radical scavengers

Free radical scavengers such as allopurinol [44, 45], superoxide dismutase [46], mannitol and glutathione [47], have been shown to be beneficial by inhibiting ROS generating enzymes such as xanthine oxidase and acting as oxygen free radical scavengers.

Buffers

Buffers such as phosphate (UW, PBS), citrate (HOC) and histidine (HTK) are important to prevent cellular acidosis. Some buffers have beneficial secondary effects for example histidine can chelate calcium and inhibit matrix metalloproteinases [48].

	EC	PBS	HOC	HTK	UW	Celsior	Collins
Physical environment							
Na/K ratio	↑K ↓Na	↑Na ↓K	↑Na ↓K	↑Na ↓K	↑K ↓Na	↑Na ↓K	
Buffer	*Phosphate*	*Phosphate*	*Citrate*	*Histidine*	*Phosphate*	*Phosphate*	
Impermeants	*Glucose*	*Sucrose*	*Mannitol Citrate Magnesium*	*Mannitol Histidine*	*Raffinose Lacto-bionate*	*Mannitol Lacto-*	
pH	*7.3*	*7.2*	*7.1*	*7.3*	*7.4*	*7.3*	
Osmolality	*340*	*310*	*486*	*310*	*320*	*360*	
Electrolytes (mM)							
Calcium	-	-	-	-	-	*0.26*	-
Chloride	*15*	-	-	*32*	*20*	*41.5*	*15*
Magnesium	-	-	*35*	*4*	*5*	*13*	*30*
Phosphate	*50*	*60*	-	-	-	-	*50*
Potassium	*115*	-	*80*	*9*	*100*	*15*	*115*
Sodium	*10*	*125*	*80*	*15*	*25*	*100*	*10*
Sulphate	-	-	*40*	-	*5*	-	*30*
Buffers (mM)							
Citrate	-	-	*55*	-	-	-	-
Histidine	-	-	-	*198*	-	*30*	-
K_2HPO_4	*15*	-	-	-	-	-	*15*
KH_2PO_4	*42.5*	-	-	-	25	-	*42.5*
Na_2HPO_4	-	*56*	-	-	-	-	-
$NaHCO_3$	*10*	-	-	-	-	-	*10*
NaH_2PO_4	-	*13*	-	-	-	-	-
Impermeants (mM)							
Glucose	*195*	-	-	-	-	-	*25*
Histidine	-	-	-	*198*	-	*30*	-
Lactobionate	-	-	-	-	*100*	*80*	-
Mannitol	-	-	*185*	*30*	-	*60*	-
Raffinose	-	-	-	-	*30*	-	-
Sucrose	-	*140*	-	-	-	-	-
Colloids (g/l)							
HES	-	-	-	-	*50*	-	-
ROS scavengers (mM)							
Allopurinol	-	-	-	-	*1*	-	-
Glutathione	-	-	-	-	*3*	*3*	-
Mannitol	-	-	*185*	*38*	-	*60*	-
Tryptophan	-	-	-	*2*	-	-	-
Substrates (mM)							
Adenosine	-	-	-	-	*5*	-	-
Glutamate	-	-	-	-	-	*20*	-
Ketoglutarate	-	-	-	*1*	-	-	-

EC: EuroCollins, PBS: Phosphate Buffered Solution, HOC: Hyperosmolar Citrate, HTK: Histidine-Tryptophan-Ketoglutatate, UW: University Wisconsin solution.

Table 1: Components of commonly used preservation solutions

University of Wisconsin Solution

The advent of UW solution permitted the expansion of transplantation, both because it could be used for multi-organ preservation, especially kidney, liver and pancreas, and because it allowed preservation times to be extended, facilitating transfer of organs, donor preparation time and organ use. UW was shown to be effective for three day preservation of canine kidney [49], and two day canine liver preservation [50]. In a prospective randomised trial of 695 cadaveric renal transplants comparing kidneys preserved in UW with EC, the incidence of DGF was significantly less in recipients recieving kidneys preserved in UW compared to EC (23% compared to 33%, p=0.0003) [51]. UW is more expensive than EC but in the longer term the costs are more than recovered because of improved kidney function, reduced rates of DGF and dialysis requirements [52]

UW has proved to be superior to other solutions with CIT in excess of 24 hours, and also in NHBD [53]. In a retrospective analysis of 323 cadaveric renal transplants, DGF rates were comparable between UW and HTK in kidneys with a CIT of <24 hours, but significantly different (23.9% vs 50% respectively) with a CIT >24 hours [54]. UW preservation has been demonstrated to result in less organ weight gain [55] and less endothelial cell damage [56]. Currently UW is considered the gold-standard preservation solution.

Gluconate was initially used as the impermeant anion, to help reduce translocation into the cell when the membrane potential was disrupted. Subsequently lactobionate was exchanged for gluconate due to its higher molecular weight (MW = 358 Da), after a study suggested that the impermeant ion was the most important

protective factor in UW [57]. Lactobionate is lactose that has been oxidised and has a net negative charge. This together with the trisaccharide, raffinose, are thought to suppress hypothermically induced cell swelling [58]. Lactobionate can chelate calcium and iron, potentially reducing oxidative injury [59]. UW contains a colloid, hydroxyethyl starch (HES) thought to be protective against cell swelling. There are several other added ingredients thought to be protective. Glutathione was added for its potential antioxidant role in the cell, and has been shown to be beneficial in both liver [60] and kidney preservation [61]. The high adenosine and phosphate concentrations help prevent loss of high energy phosphate bonds during preservation and stimulate ATP synthesis [62]. Dexamethasome was added as steroids are believed to act as a membrane stabiliser [63], whilst allopurinol is a xanthine oxidase inhibitor.

Hydroxytryptophan Ketoglutarate

Hydroxytryptophan ketoglutarate (HTK) solution, was originally developed as a cardioplegic solution by Dr Bretschneider in Germany, but has also been effective for renal [64] and liver [65]CS preservation. It is an extracellular solution consisting of histidine as the buffer, tryptophan as a membrane stabiliser and ketoglutarate as the energy substrate. HTK is the preservation solution of choice for NHBD kidneys in Maastricht [64]. It was approved in the US for kidney preservation in 2001 and liver preservation in 2002 [66].

HTK has been shown to reduce the incidence of DGF [52, 67] and its use results in higher rates of initial graft function and graft survival than kidneys stored in EuroCollins solution [52]. There have been numerous studies comparing HTK and UW,

assessing rates of DGF, graft survival, cost and viscosity. HTK and UW have shown comparable results in HBD kidneys regarding DGF and 3-year graft survival [68, 69, 70]. Equivalent results using HTK and UW have been observed in both living-donor liver grafts [71, 72], and cadaveric liver grafts [73]. However, experimental data suggests that UW is superior to HTK in the preservation of ischaemically-damaged kidneys [52]. Similar efficacy between the two has been demonstrated in renal transplants with a CIT exceeding 24 hours [74], whilst other authors have found UW to be superior with extended cold times [54, 75].UW has been demonstrated to have a greater antioxidative potential than HTK by reducing the reactive oxygen species in rat kidneys [76]. Both UW and HTK have been shown to preserve endothelial cell function superiorly compared to other commonly used solutions [77], although other papers suggest UW is superior to HTK [78].

Other authors [79] have assessed a combination of HTK followed by UW flush and observed superior results with a HTK flush followed by UW storage compared to the single use of either UW or HTK solution, as measured by portal venous pressure, bile flow, liver enzymes released into the effluent perfusate, glycerol leakage, and histological examinations. These data were consistent with a rat liver transplantation study after 24 hours CS by the same authors [79], and confirmed by other authors [80]. Choosing lower-viscosity HTK solution for the initial organ flush followed by UW storage may represent a feasible option in preservation, which also further reduces the required amount of UW solution and hence has cost implications. UW is more expensive than HTK (£172 vs £129 per litre), however a greater volume of HTK is recommended for flush than UW and thus the cost difference may not be significant [81, 82]. The issue of viscosity is further addressed in chapter 3.

Celsior Solution

Celsior has a formulation similar to extracellular fluid with a lower concentration of potassium (replaced with sodium). It uses mannitol and lactobionate as its impermeants to counteract cell swelling, glutathione and histidine to prevent oxygen-derived free radical injury and high magnesium to limit calcium overload [83]. Clinically Celsior has predominately been used in cardiac transplantation with good results [84, 85].

Celsior has also shown equal efficacy to UW in the context of renal transplantation regarding rates of DGF, acute rejection [86, 87], 3 year [86] and 5 year survival rates [88]. Similar results have been demonstrated in liver grafts [89], and pancreas transplants [90, 91], when compared to UW.

Phosphate-buffered sucrose (PBS)

PBS is an extracellular solution, with a high sodium content and no potassium, developed by Andrews and Coffey. PBS is an isotonic solution (300 mOsm) that uses sodium phosphate as its buffer. Its impermeant sucrose is not transported across cell membranes, nor broken down by sucrase activity in the nephron, and so remains in the extracellular space during CI and WI [92], thus reducing the risk of cellular oedema. Sucrose has been shown to be as effective in the prevention of cellular swelling as raffinose and the anion lactobionate used in UW, and a more effective impermeant than glucose (EC) or mannitol (HOC) [92]. In a porcine auto-transplant model comparing PBS, HOC and UW after 24 hours cold storage, PBS demonstrated

significantly better renal function, higher GFR, higher renal plasma flow and improved tubular function [93].

In an *in-vivo* NHBD model, Ahmad et al [94] induced warm ischaemia in the rat kidney for 45 minutes by crossclamping the left renal pedicle and performing a contralateral nephrectomy. Assessment took place during 2 hours reperfusion without any cold storage. PBS proved to be at least as effective or superior to UW with no animal deaths, and better than EC or HOC which demonstrated poor function and weight gain. PBS has also been demonstrated to give superior preservation in small bowel grafts, when compared to HTK, EC, and UW [95]. PBS is a simple solution in design and thus is theoretically cheaper than UW.

Hyperosmolar Citrate (HOC)

Hyperosmolar citrate (HOC) or Marshall's solution was developed by an Australian group. HOC is an intracellular solution with a high potassium content that uses mannitol as the impermeant ion and citrate as its buffer. Citrate can chelate calcium and magnesium, and may aid in the extrusion of calcium from the cell as well as the maintenance of pH [2]. It uses a hypertonicity of 400 mOsm in a bid to prevent fluid entry into cells whose isotonic environment is approx 285-300 mOsm [92]. HOC has been shown to be effective for 72 hour canine kidney preservation [96], and has been used extensively for clinical transplantation in the UK and Australia [1].

POLYSOL

Polysol is a newer preservation solution designed at the Academic Medical Center in Amsterdam, for machine perfusion and also cold storage. Polysol is based on the composition of tissue culture media. It is an extracellular type solution based on its sodium and potassium balance, contains the colloid, polyethylstarch, and is isosmolar (320 mOsm/l). Its added impermeants to resist cellular oedema are raffinose, trehalose and gluconic acid. The anti-oxidants glutathione, ascorbic acid, selenium and alpha-tocopherol were added to counter free radical activity.

In a series of papers on liver perfusion published by the Dutch group of Bessems and van Gulik, polysol has been assessed against cold storage with UW, HTK, Celsior and machine perfusion with UW-Gluconate (UW-G). UW-G is a modified UW solution that contains sodium gluconate, potassium gluconate and magnesium gluconate as its impermeants rather than lactobionate. It also has an extracellular composition of sodium: potassium (125mmol/l: 25mmol/l). As a wash-out solution, polysol had equal results to HTK with less weight gain [97]. As a preservation solution in NHBD rat livers, 24 hours MP with polysol was significantly better than cold storage in UW, but not significantly better to MP with UW-G [98]. In a porcine liver perfusion model with 24 hours cold followed by normothermic reperfusion, MP with polysol was significantly improved compared to CS with Celsior, with lower AST levels and reduced intravascular resistance. There were no differences in weight gain, LDH or pH between the 2 groups [99].

Chapter 3 - Flush of Organ

Flush of organ

Successful organ preservation starts with an effective blood washout of the donor organ. Aggregation of blood cells can impede satisfactory washout of the microcirculation, and thus the initial flush of an organ can have a significant impact on graft viability. Initial flush is followed by flush of a preservation solution designed to cool the organ and prevent cellular swelling and acidosis.

Role of flush

- Vascular washout and remove RBC
- Organ cooling
- Counter cellular swelling
- Prevent cellular acidosis

Pre- transplant flush

There are several studies that have examined the role of a pre-flush, after storage and just prior to reperfusion. This has been attempted with a variety of solutions, both warm and cold. A pre-flush is used with UW preservation to prevent cardiac complications secondary to hyperkalaemia [100].

Cold flush

In an attempt to prevent the detrimental effects of reperfusion injury, Parrott et al [101] suggested the use of a second flush of preservation solution immediately prior to implantation. They compared 106 renal transplants using standardized in-situ flush, cold storage and transplantation with 39 kidneys that received a pre-flush with 500mls of Travenol flush solution prior to transplantation. Delayed graft function occurred in 61 of 106 patients who did not receive a late flush (57.5%) compared to 12 of 39 (30.8%) kidneys that did receive a late flush. The patient groups had been matched although 13 patients in the no-reflush group had azathioprine and prednisolone only as immunosuppression, with cyclosporine used for the other patients. They thus suggested late perfusion as a simple and safe method to reduce the incidence of DGF.

There was criticism of Parrotts study design owing to the use of historical comparisons as controls. Thus Lodge et al [102], conducted a prospective randomized trial to assess the result of the "reflush effect", using HOC and PBS solutions. Kidneys with less than 24 hours cold storage were randomized to either no reflush or a reflush with 500 mls of HOC or PBS prior to transplantation. Reflushing the kidneys reduced the post-operative dialysis requirement from 40% to 15%, but there was no significant difference in renal function between the three groups at one week or one year. There was minimal difference between HOC and PBS re-flush, only PBS having a higher concentration of sodium and potassium loss from the kidneys. The beneficial mechanism of late reflush was unclear but felt to be due to a reduction of the calcium paradox and free radical formation by the release of calcium and ATP breakdown products in the venous effluent prior to implantation. The numbers in this trial however were small with a total of only 30 patients.

A larger randomized controlled trial with 99 patients by Jos et al [103], found no significant difference in rates of delayed graft function between no-reflush (20.4%) and re-flushed (24%) groups. Furthermore a prospective randomized study by Roake et al [104] was terminated after recruitment of only 18 patients (9 to each arm), because of fear that reflushing was harmful. Post-reperfusion biopsies of reflushed kidneys showed unusual histological features of cellular debris in renal tubules and eosinophilic proteinaceous material within Bowman's capsule. These changes were not apparent in pre-flush biopsies or in controls. Acute tubular necrosis and biopsy-proven rejection was also more common in the reflushed kidneys, although there was no significant difference in graft function at one year. The use of a cold late re-flush did not therefore become incorported into widespread clinical practice

Warm flush

Van Wagensveld [105] observed that pre-flush with UW at 37°C attenuated sinusoidal endothelial cell (SEC) and hepatocellular damage during reperfusion. Following cold flush and storage in UW solution, rat livers were given a pre-flush with UW at either 4°C, 20 °C or 37 °C prior to 90 minutes reperfusion at 37°C. Control livers were reperfused at t0 with no pre-flush.

Uptake of hyaluronic acid (HA) by the SEC decreased with increasing CIT reflecting hypothermic damage. Uptake of HA after 90 minutes reperfusion was only evident in control livers, and after 8 hours CIT in combination with a flush at 20°C or 37°C. Release of endogenous HA was significantly lower in the 37°C group suggesting less SEC damage. Pre-flush at 37 °C also gave a significantly lower AST release (as a measure of hepatocellular damage). Uptake of hyaluronic acid is a useful

marker as it is taken up and metabolised exclusively by SEC of the liver. SEC damage is known to induce leucocyte and platelet adherence, potentiating microcirculatory damage [105].

Rentsch et al [100] compared pre-flushing using Ringers lactate (RL) at 4°C or 37°C in rat livers following 24 hours cold storage in UW. Compared to grafts rinsed with cold RL, acinar and sinusoidal perfusion was found to be significantly increased after rinsing the grafts with warm RL. The warm rinse decreased WBC adherence in sinusoids and especially in postsinusoidal venules decreased significantly. Kupffer cell activation was markedly reduced and bile flow was enhanced when compared to cold RL. Other studies have also observed superior survival, reduced liver enzyme release and normalized histology with a warm RL flush compared to cold flush [106].

Gonzalez et al [107] assessed the role of a warm pre-flush with Carolina solution to reduce microvascular reperfusion injury in rat liver transplantation. They found that the use of warm Carolina rinse significantly increased sinusoidal perfusion and improved bile flow compared to cold CR. Leucocyte adherence was not affected by temperature, and thus they speculate that the contents of solutions may be a more important factor for leucocyte adherence.

Furthermore the same authors have previously found that cold CR reduced WBC endothelial adherence compared to cold Ringers lactate[108] (Post- CR)
Other authors have demonstrated Carolina rinse to be a more effective pre-flush solution than RL, with reduced sinusoidal endothelial cell damage, diminished graft swelling and improved postoperative hepatic microcirculation [109].

Schilling et al [110], observed that uptake of adenosine, raffinose and glutathione were significantly lower at 4°C compared to 37°C in rat livers and kidneys. Thus

application of these cytoprotectants may be most beneficial during a warm pre-flush, to allow their subsequent protective action during reperfusion.

Initial Warm flush

There is a relatively small amount of research examining the role of an initial warm flush. The use of a warm flush is a rational attempt to achieve more rapid clearance of blood from the microcirculation. Warm preservation solution may reduce vasoconstriction and prevent stiffening of the cell membranes in both endothelial cells and the cellular components of the blood. This may then allow a uniform flush of the microcirculation and ameliorate the no-reflow phenomenon, which is an important mechanism in ischaemia-reperfusion injury. Increasing the temperature of the flush solution has been associated with a decrease in the vascular resistance of isolated organs perfused continuously [106]. Furthermore, pre-warming the donor liver to 10°C prior to reperfusion significantly increased survival rates in rats one week post-liver transplantation, and increased bile flow [111]

A porcine liver transplant experiment conducted by Otto et al [112] in 1986 compared an initial flush of RL at 4°C (group 1) to RL at 15°C (group 2). After hepatectomy, all grafts were perfused with cold RL, and group 1 kidneys were further perfused with either Collins solution or plasma protein fraction, whilst group 2 had no other flush. Total CIT was 6 hours. Survival time was significantly increased in the warmer flushed group (mean survival time was 8.3 hours in group 1 whilst the warmer flushed pigs survived a mean of 41.6 days). Histology showed marked sinusoidal damage in the cold-flushed group but only minor sinusoidal alteration after warm flush.

In a NHBD rat liver transplant study, Tojimbara et al [113] assessed the role of an initial flush after 30 minutes WIT, with either UW at 4°C or UW at 37°C and no flush as controls. All livers were subsequently flushed and preserved in cold UW. No rats in the no-flush group survived more than 3 days. One rat survived more than 3 days with cold UW whilst 5 out of 6 rats in the warm UW survived more than 7 days. The ALT level was significantly lower in the warm-flushed group and their histology revealed only mild injury.

In a further study, Tojimbara et al [114] found that both the temperature and viscosity of the flush solution influenced allograft function in NHBD organs. After 30 minutes WIT, an initial flush was performed just before donor hepatectomy with cold or warm UW, UW without HES, sodium lactobionate sucrose solution, or RL solution as the control group. A separate group also used as control received no initial flushing. The livers were cold flushed and cold stored prior to orthotopic liver transplantation. The 7-day survival rate was improved by initial warm flush (83% and 100% in the cold and warm sodium lactobionate sucrose solution groups, 60% and 50% in the warm and cold lactated RL groups, 67% and 0% in the warm and cold UW groups, respectively). In the no-initial-flush group, rats did not survive. Serum ALT levels were also significantly lower in the warm-flushed groups and histology showed evidence of only mild injury.

Interestingly eliminating the hydroxyethyl starch from the cold UW improved the survival from 0-67%, suggesting the importance of the flush viscosity. The viscosity of UW fell (86.2 cp to 34.7cp) when warm but was still higher than all the other groups. Vascular resistance was also lower in the other groups regardless of temperature.

In an extended NHBD study with 120 minutes WIT, Sannomiva et al [115] flushed porcine livers with 500 ml of UW solution at either 4°C or 37°C prior to hepatectomy. The livers were subsequently flushed with 500mls of cold UW and cold stored for 6 hours prior to orthotopic liver transplantation. The tissue blood flow was significantly higher in the warm-flushed group, with significantly lower serum ALT, hyaluronic acid (HA) and LDH levels, although all animals died within 3 days of transplantation.

A series of papers by Minor et al has examined the role of an initial warm pre-flush, enhanced with the fibrinolytic, streptokinase. NHBD rat livers (30 minutes WIT) were flushed with warm RL (25°C) or warm RL with streptokinase, both then followed by cold UW, and compared to cold UW flush. Using in-situ microscopy, they observed marked microperfusion deficits in UW flushed livers (approximately 30% of acini and sinusoids perfused), associated with elevated hepatic enzyme release. The use of warm RL with streptokinase significantly reduced the inflow time of subsequent UW flush, attenuated hepatic enzyme rise, and significantly improved acinar and sinusoidal perfusion (>70%). This pattern was not observed with warm RL only. The addition of a fibrinolytic to the initial flush may thus help to improve microvascular perfusion and allow subsequent graft equilibration of UW [116].

Minor's group then took this a stage further in rat livers[117] with 60 minutes WIT, and compared an initial flush with either, UW, HTK or warm RL (22°C) with streptokinase followed by UW flush. Rat livers were then stored for 24 hours at 4°C followed by in-vitro 37°C reperfusion. Using MRI scanning and histological analysis, they again found poor tissue perfusion with UW, which was slightly improved in the HTK group. Histology revealed intravascular fibrin microthrombi, and clotted erythrocytes in the liver sinusoids, with areas of liver not adequately perfused. The

initial warm flush with RL and streptokinase normalized hepatic tissue perfusion with preservation solution, and effectively eliminated thrombus formation. Hepatic enzyme release was high in all 3 groups but recovered best in the RL group, which also displayed a three-fold increase in bile production.

In a further renal NHBD study [118], Minor's group assessed an aortic flush of cold UW, warm RL (25°C) followed by cold UW (4°C) or warm RL with streptokinase and cold UW, then cold UW preservation for 24 hours. Rat kidneys were then transplanted and assessed for 4 hours. HBD rat kidneys were used as controls. Again a normalization of tissue perfusion was observed after use of RL with streptokinase. The length of perfused capillaries or functional capillary density (FCD) in NHBD perfused with UW was half of the values in HBD. In the RL/streptokinase group, the FCD was statistically similar to the HBD group. Interestingly there was a higher leucocyte infiltration in the RL/streptokinase group at 4 hours reperfusion. The authors hypothesise that this was related to the reflow-paradox, whereby leucocyte infiltration has occurred after sufficient microvascular perfusion as part of reperfusion injury.

Viscosity of flush solution

There has been much debate regarding the relative benefits of colloids in flush solutions due to concern of their effect on viscosity. Viscosity is influenced by the type, size, and the temperature of the solute-solvent combination [119]. A temperature rise decreases the surface tension of the solution, resulting in reduced viscosity. The inclusion and exclusion of the colloid HES, in UW, has been most disputed. HES prevents interstitial oedema [120], and has a beneficial effect on MMP [121]. However it increases viscosity and has been shown to increase RBC aggregability. This

aggregation enhances the tendencies of leucocytes to adhere to the post-capillary endothelium, and as neutrophils are known to be involved in I/R injury [122, 123], it could potentially obstruct the microcirculation resulting in the no-reflow phenomenon [124].

Morariu et al [125] assessed the effect of HES on RBC aggregability *in vitro* at 4°C with human blood. They found that both UW and a combination of high molecular weight HES (MW =200kDa) had a potent hyper-aggregating effect, while low molecular weight HES (MW=130 kDa) had a hypo-aggregating effect on RBC. Furthermore the RBC aggregates were of large size and their resistance to dissociation by flow-induced shear stress was high, such that normal gravity induced hydrostatic flush pressures would be unlikely to be adequate to dissociate the RBC aggregates.

Van der Plaats et al [119] demonstrated that the viscosity of blood at 4°C is two times higher than at 37°C. A UW/blood mixture had a higher viscosity and is 1.3-times more viscous than blood at 37°C. They also observed that HES markedly increased the aggregation formation of RBC's. The aggregates were large (30 μm long and 18μm wide) compared to the diameter of liver sinusoid (10μm) and potentially could cause obstruction of the sinusoids, resulting in a poor initial washout. The amplitude of aggregation increased with the concentration of UW used. In comparison, HES-free UW did not display this effect on aggregation. Thus a lower viscosity flush may be beneficial for the initial organ flush.

Pressure of flush

The optimum pressure for organ flush has yet to be established and varies for different organ types. In-situ kidney perfusion is commonly given by a gravity-induced hydrostatic pressure at a height of 1 metre equating to100 cmH_2O [126]. A high pressure flush has been advocated by some investigators, the rationale being to create a mean pressure similar to that present in the arterial system in normal conditions, with the aim to perfuse the microcirculation more effectively. There are concerns however that the higher pressures potentially could cause endothelial cell damage.

Tisone et al [127] compared in-situ perfusion in human renal grafts with either gravity perfusate infusion from 1 metre height, or at 1 metre height with an additional 100 mm Hg pressure applied by a pressure bag. They observed no statistical difference between the two groups regarding DGR, PNF or serum creatinine levels. Acute rejection episodes were higher with the higher pressure flush but not significantly. Using liver grafts, the same authors observed better early graft function, and graft and patient survival with a higher pressure flush, although the numbers were small with only 38 patients [128].

Tokunaga et al [129] showed in rat livers that perfusion pressure was optimal below 15 mmHg, whilst Hart et al [130], showed better results with higher perfusion pressures. They compared low-pressure perfusion with Viaspan-CS (12 mmHg), which is the routine method in clinical practice, high-pressure perfusion with Viaspan-CS (100 mmHg) and low-pressure perfusion with modified HES-free Viaspan solution (12 mmHg). After procurement all livers were preserved in Viaspan for 0, 24 or 48 h, followed by reperfusion in oxygenated Williams Medium E for 24 hours at 37 °C. The high pressure group and modified Viaspan group both showed better clearance of erythrocytes from the microcirculation than standard Viaspan.

ATP levels were highest in the high pressure flush group, which could be accounted for by the faster flush (less than 1 min in contrast to 15 min in groups A and C) and hence reduced warm ischaemic injury. Furthermore, the livers showed a rapid decrease to 10–15 °C during washout, but a slow decline to 4°C. However they also observed decreased hepatocyte integrity with the higher pressures and modified Viaspan, although the authors conclude this was unlikely to be related to the effects of pressure as the modified Viaspan group received low-pressure flush.

In cardiac grafts, a higher washout pressure of 120 mmHg with Viaspan has been demonstrated to be beneficial compared to lower pressure (60 mmHg) giving a complete wash out the blood components, distribution of the solution, better ATP content without any obvious ultrastructural endothelial cell damage [131].

Sasaki et al [132] compared low and high pressure washout in rabbit lungs, and found that high pressure flushing (20-25 mmHg) induced vasoconstriction and thus did not give a uniform washout. Low pressure (5 mmHg) flush failed to reach the peripheral vascular beds due to the slow flow rate, and resulted in incomplete flushing out of blood. A flushing pressure of 10-15 mmHg (which is slightly lower than normal pulmonary arterial pressure) gave the best washout and subsequent pulmonary function. Maintenance of the endogenous nitric oxide-producing ability in preserved lungs, has also been shown to be optimal with a flushing pressure less than 20 mmHg [133].

CHAPTER 4

Advantages and disadvantages of current methods

Cold Machine preservation versus static cold storage

There is a significant body of research comparing cold static storage (CS) and machine perfusion (MP) in HB and NHB kidneys, but debate still remains. This is in part due to fact that there are few, good quality randomised controlled trials, and existing evidence is often based on small numbers with relatively short follow up times. There have also been many advances in the field of transplantation with immunosuppression use, tissue typing, preservation solutions, and more modern perfusion equipment. A number of studies have shown no advantage of MP over CS [134, 135, 136], and this together with the simplicity, lower cost and ease of transport, make cold storage the method of choice for the majority of renal transplant centres. Machine preservation has several potential advantages such as providing oxygen and nutrients capable of meeting the reduced metabolic demands of hypothermic organs. MP is said to supply or regenerate metabolic substrates lost during warm ischaemia such as adenine nucleotides and glutathione [137, 138].

MP can also wash away toxic metabolic waste products, reverse the vasospasm of ischaemia [139], and improve perfusion of the microcirculation and hence clearance of blood cells [140]. MP allows some viability assessment with flow rates and intra-renal vascular resistance. Theoretical disadvantages of MP are that the perfusate may wash away energy substrates such as adenosine, thus reducing the capacity for ATP regeneration post-perfusion. There are concerns regarding endothelial damage

[141], which can be minimised by maintaining perfusion pressures of 60 mmHg or lower [142, 143]. Logistically, MP is more complex and requires expensive machinery and thus may be more expensive in the short term

Wight et al [144] and Fuller et al [145] have conducted comprehensive review articles of evidence comparing the two methods, showing no advantage of MP over CS regarding primary non function (PNF), acute rejection (AR), patient and graft survival. However there is evidence suggesting machine perfusion of kidneys gives lower rates of delayed graft function compared to cold storage[146, 147,]. A review of 85,000 renal transplants in North America showed a significant reduction in DGF with MP compared to CS (19.6% versus 27.6%) [148].

In a randomised comparison of paired HBD kidneys, Alijani et al [149] found that dialysis requirements post-transplantation were 63% for CS grafts and 17% for MP grafts. Light et al [150] report 95% immediate function with MP for HBD kidneys compared to the national figure of 75% for non-perfused kidneys [151]. Southard and Belzer [152], reported only 18% ATN for MP of NHBD compared to the reported rates of 70-100% for CS NHBD.

The use of MP in extended criteria donors (ECD) has been demonstrated to be of benefit by several authors. Kidneys from extended criteria donors that were older, had a higher BMI, had an increased incidence of cerebrovascular brain death and pre-existing donor hypertension, and had a lower estimated creatinine clearance (CrCl, all $P < 0.01$) compared with standard criteria donors, were demonstrated to have similar graft survival to CS kidneys from standard criteria donors [153] and a significantly lower incidence of DGF (26% vs. 36%, $p < 0.001$)[154]. Stratta et al [155] have found that despite being subjected to longer cold ischemia times, recipients of ECD kidneys managed with MP had similar survival and functional outcomes to CS, but experienced a

marked reduction in the rate of delayed graft function. This demonstrates the potential of MP to overcome some of the damage conferred by cold ischaemia

The latest evidence comes from a large European multi-centre randomised controlled trial that compared 336 MP and 336 CS kidneys from consecutive deceased donors [156]. MP kidneys were perfused with KPS solution using pulsatile flow at 30 mm Hg perfusion pressure. MP kidneys had a significant reduction in the incidence of DGF (20.8 % vs 26.5 %, P = 0.05) compared to CS kidneys. The primary non-function rate was also lower in MP kidneys (2.1% vs 4.8%, P = 0.08). One-year graft survival in the MP group was significantly higher than in the CS group (94% vs 90%, P = 0.04). There were no significant differences in length of hospital stay for recipients, acute rejection rate or creatinine clearance in the first 14 days post-transplantation.

Overall therefore it seems there is evidence of reduced delayed graft function rates with machine perfusion. DGF has both clinical and economic implications with increased hospitalisation, dialysis requirements and overall cost. The use of MP may thus be justified, particularly if it allows for extension of the donor pool with extended criteria donors. However more randomised studies with higher study numbers and longer follow up are required to confirm this. The inherent difficulties posed are highlighted by Wight et al [144], that in order to detect a 5% change in graft survival from 70-75%, at a 5% significance level, would require a study of at least 1256 patients followed up for 10 years.

Potential Advantages of warm preservation

There are several potential advantages to warm perfusion as a means of preservation:

1. Warm perfusion provides a means for maintaining an organ in physiological condition, thus reducing the level of injury typically sustained by an organ during cold ischaemia and reperfusion. Warm Perfusion may also be able to resuscitate organ function, which could have major potential clinical implications, particularly with marginal donors, and may hence allow expansion of the donor pool.

2. Normothermic preservation could allow viability assessment, by measuring functional parameters that correlate with post transplant function. Currently there are no reliable pre-transplantation tests of viability that are routinely used, with transplantation being the only measure of outcome, as cold preservation renders metabolic studies difficult to interpret.

3. The preservation period could be potentially extended without causing added organ damage, bringing logistical benefits including long distance transport of organs to the best matched donors.

4. *Ex-vivo* warm preservation offers the possibility of manipulation of the organ prior to transplantation, to try and minimise the effects of ischaemia-reperfusion injury. This may be achieved pharmacologically or with novel therapies such as gene transfer. *Ex-vivo* warm preservation has the advantage of easy accessibility and allowing direct manipulation of the graft thus reducing systemic side-effects.

Results of Normothermic preservation

Brasile et al have published a series of papers of normothermic preservation in a canine model using an acellular perfusate, EMS solution, with approximately 70 ingredients based on cell culture medium, supplemented with pyridoxylated bovine haemoglobin to provide adequate oxygen and support metabolism. They used chemically modified haemoglobin as the oxygen carrier because of concerns over the mechanical damage to circulating red blood cells over time and its effect on oxygen tension.

In an autotransplant model using foxhound kidneys with 30 minutes WIT [157], they compared CS at 4 °C with those warm perfused with EMS solution for 18 hours. Statistically significant improvement in serum creatinine (AUC) was observed with WP, with histologically less renal tubule damage, less interstitial oedema and neutrophil aggregates. Eighteen-hour CS kidneys were then resuscitated with WP for either 3 hours or 18 hours, with amelioration of damage by 3 hours and normal creatinine levels after 18 hours WP.

These authors have also demonstrated resuscitation of kidneys with an extended WIT of 2 hours in a canine auto-transplant model [158]. The kidneys were either immediately reimplanted, hypothermically machine perfused at 4°C with Belzers UW machine preservation solution, or warm perfused at 32°C for 18 hours with EMS solution. The warm perfused kidneys provided life-sustaining function after transplantation unlike the other 2 groups, with continual urine flow and normal creatinine levels by days 3-10. All controls were anuric and suffered primary non-function. The warm perfused kidneys histologically had normal vessels and glomeruli.

Brasile et al compared extended preservation times of 24 hours and 48 hours by WP, cold MP and static CS in canine renal autotransplantation model [159]. The WP group displayed significantly better function with normal serum chemistries, urine output and normal histology. They also demonstrated in human kidneys, released for research after cold storage for 38 hours, maintenance of function with stable oxygen consumption, re-establishment of urine flow and no significant abnormalities histologically after 48 hours WP.

Stubenitsky et al [160] formulated a pre-transplantation prognostic test using foxhound kidneys in an auto-transplant model. The kidneys were removed and subjected to 30 mins warm ischaemia followed by 24 hrs CS in Viaspan (UW). The kidneys were then warm perfused for 3 hours using EMS technology and viability assessment carried out. A viability score was formulated based on a combination of oxygen consumption, perfusion index (ability to regain normal perfusion pressures and flow rates), and vascular integrity (ratio of platelet concentrate in perfusate after 15 mins and 3 hours). The sum of the 3 created a viability index as a prognostic index to predict the severity of ATN (<2 severe ATN, >2 mild ATN). Decreasing viability score was associated with an increase in the severity of ATN, both in terms of the peak serum creatinine level and length of organ recovery time.

A series of elegant studies by Friend et al in Oxford has shown evidence of resuscitation and viability assessment for both HBD and NHBD livers. In a HBD model [161], they compared 24 hours preservation with either static cold storage in UW solution or oxygenated normothermic blood perfusion. Following preservation all livers were placed on an extracorporeal circuit for 24 hours with whole blood, so that assessment of viability (enzyme levels, bile production, factor V levels, glucose

consumption, and histology) could be made. The warm perfused livers displayed significant improvement in bile and factor V production, did not gain weight and showed significantly less necrosis on histology

In a NHBD porcine model [162], Friend et al compared static cold storage for 24 hours in UW solution with warm perfusion using heparinised whole blood at 38°C in porcine livers with 60 minutes WIT. To simulate the transplant model both groups were flushed with 2 litres non-oxygenated cold Eurocollins solution and left for 45 mins before being flushed with warm plasma substitute. Upon reperfusion, none of the cold stored livers demonstrated signs of viability, whereas the warm perfused livers showed significantly lower enzyme levels, continued bile production and metabolic activity (glucose utilization) and maintenance of portal flow. The benefits of being able to assess viability and the restriction of ischaemic injury with continuous warm perfusion are clearly evident.

In view of the practical constraints of transporting warm perfusion equipment and the logistical requirements of a multi-organ retrieval, a further study [163] aimed to mimic a clinical scenario of a NHBD liver using both cold and warm ischaemia. Porcine livers subjected to 60 minutes WIT were either warm perfused for 24 hours, or cold perfused for 4 hours followed by 20 hours warm perfusion. Viability was compared on the ex-vivo normothermic circuit over 20 hours. Normothermic preservation failed to resuscitate livers with 4 hours of cold storage and 60 minutes WIT, with greater necrosis and destruction of architecture on histology. In contrast the purley normothermic perfused livers had superior bile production, less evidence of hepatocellular damage (alanine aminotransferase and aspartate aminotransferase levels), better metabolic activity (base deficit and glucose level), and preserved tissue

architecture with minimal necrosis. This highlights the detrimental combination of CIT and WIT.

Brasile et al [157] found the impact of cold ischaemia was more pronounced when cold ischaemia followed warm perfusion, showing a time-dependent increase in damage with raised mean peak serum creatinine and an increase in the number of days needed to normalize the serum chemistries. This suggests that the relative degree of damage between warm and cold ischaemia may in fact be additive.

The relative effects of CI and WI are also demonstrated elegantly by the Nicholson group here in Leicester. In an experimental model of HBD and controlled/uncontrolled NHBD kidneys, porcine kidneys with warm ischaemic times ranging through 0, 10 and 25 mins were subjected to cold storage for either 2 or 18 hours, and viability assessed *ex-vivo* at normothermic temperatures with autologous blood for 3 hours [164]. Renal functional parameters were not significantly different between 0, 10, 25 WI with 2 hours CS. After 18 h CS, renal function was severely impaired in the 10 and 25 WI groups compared to 0 min WI. This data suggests that limiting the cold storage period is of paramount importance when transplanting ischaemically damaged kidneys from non-heartbeating donors.

In a controlled NHBD model of porcine kidneys using autologous blood [165], the Leicester group have shown that kidneys subjected to 16 hours of CS then resuscitated with 2hrs NP had comparable haemodynamic results to those with only 2 hrs CS regarding renal blood flow and AUC for serum creatinine. Normothermically resuscitated kidneys also had improved renal tubular function (as measured by fractional excretion of sodium), and significantly higher ATP levels.

In earlier studies, normothermic autologous blood perfusion and hypothermic pulsatile perfusion were compared in a controlled NHBD model [166]. Porcine kidneys

with 2 hours cold static storage were either warm perfused at 37°C on a POPS system or cold perfused on RM3 system for 16 hours, thus simulating the average clinical situation of 18 hours ischaemic time. *Ex-vivo* function was then assessed with blood perfusion on the POPS apparatus for 2 hours. The warm preserved kidneys were significantly better at concentrating creatinine and conserving sodium than those hypothermically perfused. Creatinine clearance was higher, but vascular resistance, proteinuria and glycosuria were lower in the warm perfused kidneys.

Optimal conditions for *ex vivo* preservation have not yet been established. Thus a further study [167] examined the effects of arterial pressure on renal preservation using isolated *ex-vivo* haemoperfused porcine kidneys. Groups of kidneys (n = 6) were perfused at a mean arterial pressure of 95, 75 or 55 mmHg, with the higher perfusion pressures demonstrating improved renal function, acid-base homeostasis and improved renal haemodynamics.

The role of leucocytes in ischaemia-reperfusion injury has also been demonstrated by the Leicester group [22]. After 2 h cold storage, porcine kidneys were perfused normothermically with autologous whole blood or leucocyte depleted blood for 6 hours. Depletion of leucocytes resulted in improved renal blood flow, oxygen consumption and acid-base homeostasis, and histological tubular damage was ameliorated. This study highlights the important role that white cells play in renal ischaemia-reperfusion injury.

In a HBD porcine liver transplantation study, Schon et al [168] compared direct transplantation with transplantation after 4 hours cold preservation in UW solution or 4 hours of normothermic extracorporeal liver perfusion (NELP) using 2 litres of heparinised whole blood and 1 litre of a balanced electrolyte solution, with no cold

flush administered. All the warm perfused animals survived more than 7 days post transplant and displayed function similar to those immediately transplanted, as demonstrated by histological analysis and transaminase production.

The same 3 groups were then compared in a NHBD model after 1 hour of warm ischaemia. Of great interest is that after 1 hour of warm ischaemia all cold preserved animals died within 24 hours unlike the warm perfused animals, who survived and whose histology showed no irreversible damage and had lower release of transaminases. Although the 4 hour preservation period was not a true reflection of the clinical setting, the ability to resuscitate ischaemically damaged organs is evident.

In contrast, Hellinger et al [169] compared normothermic preservation with cold storage of porcine livers subjected to 1or 3 hours warm ischaemic time. They found that the results after cold storage were superior to those after normothermic perfusion as judged by bile production and glutamic oxaloacetic transaminase levels.

Normothermic preservation may allow "safe" extension of the preservation period thus giving logistical benefits for organ transportation. Butler et al [170] successfully maintained porcine livers with 60 minutes warm ischaemic time for 72 hours using extracorporeal warm perfusion. Brasile et al [159] reported successful perfusion of isolated canine and human kidneys *ex vivo* at near physiologic temperatures for 48 hours, with normal renal function upon reimplantation of the canine kidneys.

Koostra et al [171] have shown successful resuscitation of canine kidneys subjected to 6 days cold machine preservation with only 3 hours of normothermic blood perfusion on day 3. Nine of the eleven kidneys showed life-sustaining function

after 6 days preservation and had histologically well preserved kidney tubules compared to hypothermically perfused controls.

Normothermic Recirculation

Normothermic recirculation entails cannulation of the donor via a large vessel with connection to cardiopulmonary bypass equipment to allow organ perfusion. In a clinical study of NHBD, Valero et al [172] compared 3 different preservation techniques, *in situ* preservation with cold Collins solution, total body cooling through cardiopulmonary bypass and normothermic recirculation at 37 degrees for 60 minutes before cooling. Of the 56 patients, 8 underwent normothermic perfusion, 8 total body cooling and 40 *in situ* cooling. Warm ischaemic time was lowest in the *in-situ* group. The normothermic recirculation group had a significantly reduced incidence of DGF and PNF with 7 of the 8 kidneys functioning immediately. Serum creatinine levels also returned to normal in shorter time. It should be noted however that the warm perfusion was only for 1 hour, the groups were not randomised and the kidneys were cooled with Collins solution rather than the gold standard UW solution.

Valero et al [173] have also evaluated the use of cardiopulmonary bypass (CPB) and normothermic recirculation (NR) before total body cooling, and its relationship with recipient survival in a porcine NHBD model. Survival rate was 100% with 20 minutes WI, 70% with 30 minutes WI, and 50% with 40 minutes WI. Control group survival with 20 minutes WI was 0%.

The same authors have suggested that NR has a pre-conditioning effect by maintaining adequate adenosine and xanthine levels. In a porcine NHBD model with 20 minutes WI [174], they observed that hepatic adenosine levels increased and xanthine

levels decreased during NR. Blocking the adenosine-2 receptor abolished the effect of NR, whereas the blocking of adenosine-1 receptors further protected the liver. Adenosine administration prior to WI simulated the effect of NR (20% survival with no treatment, 90% with adenosine and 100% with NR).

Organ Manipulation

Ex-vivo warm preservation offers the possibility of manipulation of the organ prior to transplantation, to try and minimise the effects of ischaemia-reperfusion injury and rejection. This may be achieved pharmacologically or with novel therapies such as gene transfer,

Ex-vivo warm preservation has the advantage of easy accessibility and allowing direct manipulation of the graft thus reducing systemic side-effects.

Valero et al [175] have examined the potential role of S-adenosyl-L-methionine (SAMe) as a protective agent during normothermic recirculation. SAMe metabolism increases cysteine, and consequently the anti-oxidant glutathione, and also generates adenosine production. After donor cardiac arrest, cardiopulmonary bypass and normothermic recirculation (NR) were performed for 30 min, during which (SAMe) was administered. There was a significant reduction in apoptosis of hepatocytes and sinusoidal endothelial cells at reperfusion and at 5 days. SAMe was also associated with higher portal blood flow and lower levels of α-glutathione-S-transferase after reperfusion

Imber et al [176] found that native porcine bile acids were decreased to 30% of their original value by 20 hours of preservation. However the addition of taurocholate during normothermic perfusion maintained bile production.

Heat shock proteins (HSP), such as heme oxgenase-1 (H0-1) and HSP 70, have received much attention due to their proposed protective benefit on ischaemia-reperfusion injury [177]. Their upregulation by various substrates such as cobalt-protoporphyrin administered during WP [178, 179], heat preconditioning [180], ergothioneine [181], and tin-protoporphyrin [182] have been demonstrated with beneficial results.

Improved renal function, reduced tubular damage and increased superoxide dismutase and HSP 72 expression has been observed with use of taurine [183]. Glutamine increases HSP70 expression in rat renal grafts and attenuates tubular cell apoptosis [184].

Brasile et al [185] assessed foxhound and human kidneys subjected to 2 hours of warm ischaemic damage for evidence of ex-vivo repair. Foxhound kidneys were perfused for 24 hours in the presence or absence of fibroblast growth factors (FGF) and assessment of cytoskeletal integrity and DNA repair was made by the up-regulation of proliferating cell nuclear antigen (PCNA). The markers of cytoskeletal damage (ZO-1 protein expression) were significantly reduced and PCNA expression up-regulated with 88% of cells staining positive, thus confirming evidence of *ex-vivo* repair.

Gene transfer

Gene transfer involves the delivery of single or multiple genes and the sequences that regulate their expression, into a target cell by use of a vector. These vectors may be retroviral (RNA), adenoviral (DNA) or non-viral. (eg liposomes).

Brasile et al [185] transfected human kidneys (deemed not suitable for transplantation) with adenovirus to assess expression of green fluorescent protein

during 24 hour *ex-vivo* perfusion. Histologic assessment showed positive expression of this protein in the intima of blood vessels, thus confirming that *de-novo* protein synthesis had occurred during *ex-vivo* perfusion.

Abunasra et al [186] have demonstrated *ex-vivo* gene transfer of superoxide dismutase and beta-galactosidase, using an adenoviral vector during experimental I/R and transplantation in rat hearts. Four days post-transplantation, the hearts were given 6 hours of ischaemia followed by 1 hour of reperfusion. Effective gene transfection was found for all groups, with percentage recovery of pre-ischaemic left ventricular developed pressure being greatest with manganese superoxide dismutase. Recovery of pre-ischaemic left ventricular function was superior with a continuous infusion rather than bolus infusion [187].

Viral IL-10 is structurally homologous to mouse and human IL-10 except it lacks the ability to stimulate T-cells. Retroviral-mediated gene transfer and expression of IL-10 has been demonstrated to significantly prolong cardiac allograft survival [188, 189], and inhibit graft rejection [190].

Conclusions

The potential advantages of normothermic preservation are evident. Maintaining an organ in a condition equivalent to its physiological environment optimises enzymatic activity, allows aerobic metabolism, allows viability assessment and can repair some of the detrimental effects of hypothermic preservation. There is a possible opportunity to modify the organ by pharmacological or genetic means and improve its function.

The disparity between the availability of organs and the number of patients in need of organ transplantation has resulted in a resurgence in the use of NHBD organs

as well as a rise in live donation from related, un-related and altruistic donors. Expanded criteria or marginal organs are subject to both CI and WI damage. The effect of combining the two injuries appears to be additive with the risks of delayed graft function and primary non-function

The importance of WP as a mechanism to resuscitate ischaemically damaged kidneys is thus paramount to allow successful transplantation of functioning organs, and should help to expand the non-heart beating part of the donor pool.

However, normothermic perfusion is at present a complex undertaking requiring expense, training and expertise in its set-up and monitoring. The equipment is not currently easily portable which may hinder its widespread application. The added costs involved in normothermic preservation will have to prove to be justified by the improvement in preservation, and expansion of the donor pool. A preliminary period of cold preservation may be necessary due to the practical constraints of having the equipment available and organs may have to be transferred to a transplant centre before being warm perfused.

Despite the disadvantages of cold preservation, it is an easily portable method, that is simple to perform and relatively inexpensive and thus may continue to render it the preservation method of choice. Renewed interest in cold MP, its portability, and the reduced incidence of DGF particularly with marginal organs may make this a more realistic preservation method. In the meantime, research in normothermic preservation will continue to expand.

Section Two

METHODS

Circuit

The Isolated Organ Perfusion System (IOPS) consisted of cardio-pulmonary by-pass technology, (Medtronic, Watford, UK), incorporating a centrifugal blood pump (550 Bio-pump), speed controller, TX50P flow transducer and pressure transducer. A heat exchanger (Grant, GD120, Cambridge, UK), temperature probe (Cole-parmer, London, UK) and two PC- 2 Gemini infusion pumps (Alaris, Basingstoke, UK) were added to the system. The disposable circuit consisted of a 5L venous reservoir container (Medtronic), polyvinylchoride tubing 1/4 and 3/16 inch (Medtronic), minimax plus membrane oxygenator (Medtronic), and a urine meter (Bard, Crawley, UK) (Fig 4).

The circuit was primed with a solution containing Ringer's solution 500ml, mannitol 10mg (Baxter, healthcare, Norfolk, UK), dexamethasone 10mg, (Organon labs Ltd, Cambridge, UK) and cefuroxime 750mg (Britannia pharmaceuticals Ltd, Surrey, UK). 15ml sodium bicarbonate 8.4% (Fresenius kabi, Warrington, UK) was added to the blood to meet physiological conditions. 500ml of heparinized whole blood was added to the circuit after priming and allowed to circulate at a temperature of 37ºC.

The perfusate was also supplemented with a nutrient solution (Nutriflex ; B. Braun Sheffield, UK) infused at 20ml/hr to which 100 units insulin (Actrapid : Novo Nordisk, Denmark) and 25ml sodium bicarbonte 8.4% (Fresenius kabi) was added.

A vasodilator, Sodium Nitroprusside 25 mg, (Mayne pharma PLC, Warwickshire, UK) was administered during the first hour of reperfusion at 25ml/hr. 5% glucose solution (Baxter) was infused at 7ml/hr once the sodium nitroprusside had been discontinued. Ringer's solution (Baxter) was used to replace urine output ml for ml. The perfusate was 'spiked' with creatinine (Sigma, Germany) to bring the initial circulating concentration to 1000µmol/L.

Isolated organ perfusion system (IOPS)

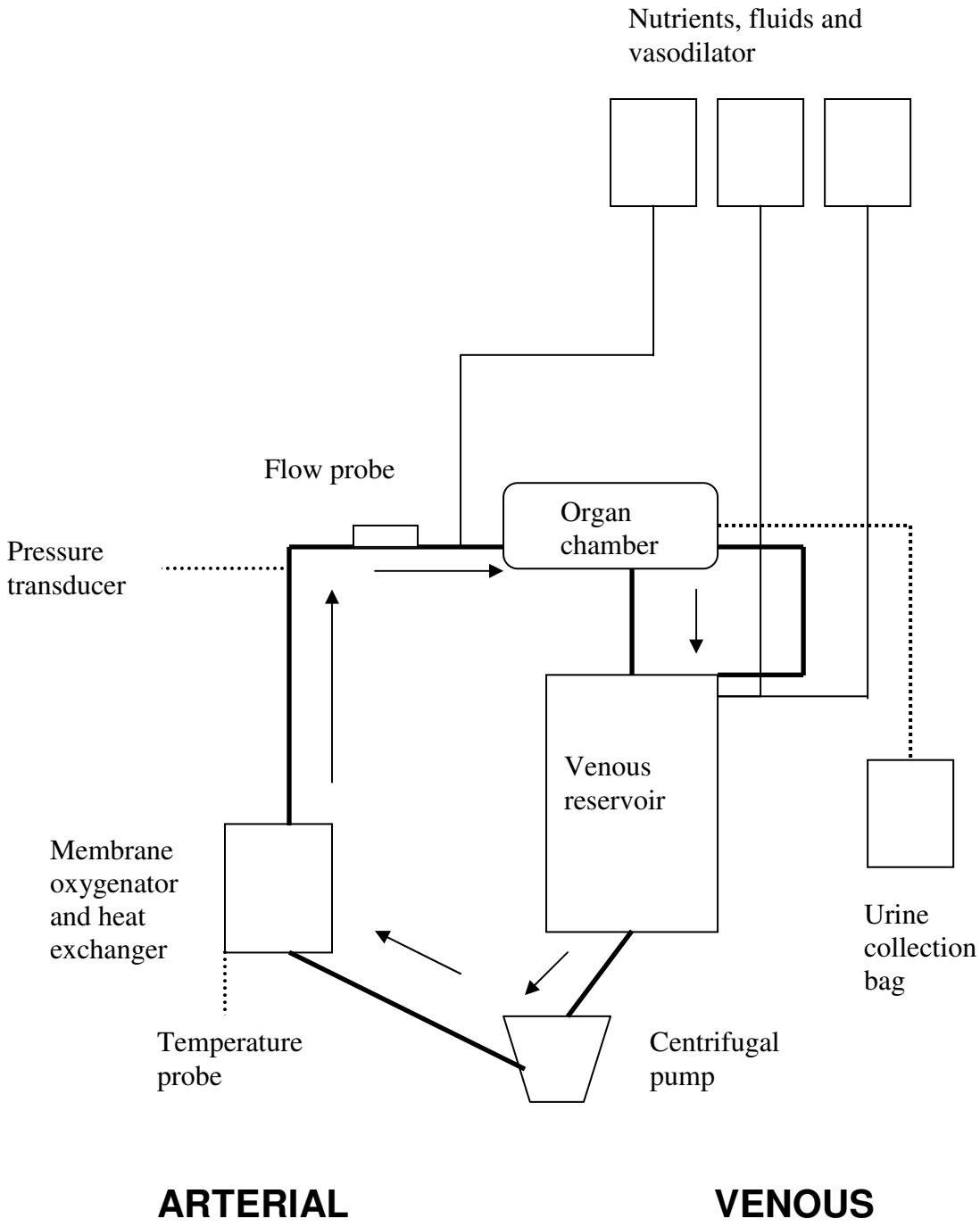


Figure 4: Diagram of the Isolated Organ Perfusion System (IOPS) outlining the direction of blood flow.

Isolated Organ Preservation System

Figure 5: The two IOPS machines used for experiments

AQIX- A Normothermic preservation solution

AQIX has been designed as a universal normothermic organ preservation solution that would reflect physiological ionic concentrations, osmolarity and ion conductivity, thereby maintaining the ionized status of the cell membrane and the function of enzymatic and receptor moieties. The osmolarity of AQIX is similar to that of serum at 286 mOsmoles/l, and its ionic concentrations have been chosen to maintain it as an isosmotic solution. The ionic conductivity is also comparable to human serum, namely, 12.6 mS cm-1.

AQIX is a non-phosphate buffered solution that utilizes a combination of the bicarbonate system ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$) and Good's BES as its buffer which, because of its ideal pk_a value allows the pH to be maintained within a range of 7.2 – 7.46 over a temperature range of 4 – 37 ºC thereby facilitating the transition from hypothermic to normothermic conditions and vice versa. Its constituents are displayed in Table 2.

AQIX is capable of carrying oxygen in solution or by incorporating red blood cells during machine preservation. Initial studies using AQIX were focused on isolated, small animal tissues and organs preserved and perfused in an organ bath system using oxygenated AQIX at 15 – 37 ºC. Such studies demonstrated functional viability of rat jejunum for 9 days, rat colon for 5 days and rabbit uterus for 7 days [9].

All the experiments presented in the following chapters used AQIX RS-I (x10) concentrate perfusate solution.

Table 2. Composition of AQIX

Sodium	110
Potassium	5.0
Magnesium	0.45
Sodium bicarbonate	25.0
BES	5.0
D-glucose	10.0
Calcium chloride	1.25
Glycerol	0.11
L – Glutamate	0.30
L – Glutamine	0.40
L – Aspartate	0.02
L – Carnitine	0.05
Choline Chloride	0.01
Thiamine pyrophosphate TPP (co-carboxylase)	40.0 nmol/l
Insulin	28.0 mIU

*All units in mmol/l unless specified

SECTION 3

EXPERIMENTS

1. Cooling Experiment 1
2. Cooling Experiment 2
3. Optimal Flush conditions for AQIX
4. Normothermic versus hypothermic flush
5. Normothermic flush and preservation

Comparison of preservation solutions in an experimental model of organ cooling in kidney transplantation

Background

Viscous preservation solutions such as University of Wisconsin solution (UW) may be less effective at rapid removal of blood from an organ so that cooling takes longer. This study assessed the temperature changes of kidneys flushed with UW and hyperosmolar citrate (HOC).

Methods

Porcine kidneys were retrieved and flushed with 500 ml UW or HOC at 4°C while monitoring kidney temperature at depths of 5 and 20 mm. Renal function was measured on an isolated organ preservation system.

Results

The mean (s.d) rate of temperature fall was slower with UW (at 20 mm: 0.64(0.11) *versus* 1.01(0.56)°C per min per 100 g; $P = 0.016$)**.** The amount of preservation solution required to reduce the temperature to less than 10ºC was lower in the UW group ($P = 0.002$). Kidneys flushed with HOC gained a significant amount of weight compared with those flushed with UW (mean (s.d.) 50(8) *versus* 7(13) per cent; $P = 0.002$). Flushing with UW was associated with less histological injury but there were no significant differences in renal function parameters between the groups.

Conclusion

UW cooled kidneys more slowly than HOC, but with no adverse effect on renal function. UW resulted in less oedema and histological injury than HOC.

Introduction

Despite decades of clinical experience, there are many unanswered questions in the field of organ preservation. Hypothermic storage, the mainstay of current clinical practice, is used deliberately to slow metabolism in order to abrogate the deleterious cellular effects of warm ischaemia. Organs are stored at 4°C based on the principle that metabolism is slowed by a factor of 1.5–2 for each 10° drop in temperature [8,9]. At temperatures below 15°C, Na^+/K^+-adenosine 5′-triphosphatase ceases to function[10] and metabolic demand is reduced by 90 per cent [2]. Hypothermic preservation solutions are designed to allow an effective organ flush to remove blood, cool the organ, and minimize the negative effects of ischaemia and hypothermia. They maintain equilibrium between the intravascular, interstitial and intracellular fluid compartments. This is important in the prevention of cellular oedema and in maintaining membrane function [35].

University of Wisconsin solution (UW) is a widely used organ preservation solution and is particularly beneficial for organs from less optimal donors or for prolonged periods of hypothermic storage[5]. UW contains a colloid, hydroxyethyl starch (HES), which is thought to be protective against cell swelling, as well as several other ingredients that protect against oxidative damage [60,61], stimulate adenosine 5′-triphosphate synthesis and stabilize cellular membranes [62,63].

Hyperosmolar citrate (HOC), or Marshall's solution, is used extensively for clinical transplantation in the UK and Australia [2,3]. HOC is an intracellular solution with a high potassium content that uses mannitol as the impermeant ion and citrate as its buffer to aid in the extrusion of calcium from the cell and to maintain the pH [3]. As a hypertonic solution, HOC prevents the entry of fluid into cells [92] and has been shown to be effective for 72-h canine kidney preservation [96].

There has been much debate regarding the relative benefits of colloids in preservation solutions owing to their increased viscosity. Viscous solutions reduce the rate at which blood is flushed from the organ, and it would be logical to suggest that this would prolong the rate at which organs cool, potentially increasing the initial warm ischaemia period and causing damage to the organ.

The aims of this study were to assess the relative efficiency of cold flushing with UW and HOC in cooling porcine kidneys, and to determine the effects on subsequent renal function during isolated organ perfusion with autologous blood.

Methods

Large White pigs (60–70 kg) were killed by electrocution followed by exsanguination. Kidneys were removed surgically after 10 min warm ischaemia, weighed and placed in a dish at room temperature for 5 min to allow for preparation (figure 7). Two K-type thermocouple probes (Hanna Instruments, Leighton Buzzard, UK) were inserted into either the upper or lower pole of kidney at depths of 5 and 20 mm, and the temperature was recorded at 1-min intervals with a thermocouple logging thermometer (Hanna Instruments) during the 5 min at room temperature and also during the cold flush (figure 7).

The renal artery was cannulated using a Tibbs arterial cannula (Downs Surgical, Sheffield, UK) connected to a 4.0-mm blood set (Codan, Lensahn, Germany). UW (DuPont Pharmaceuticals, Wilmington, Delaware, USA) or HOC (Baxter Healthcare, Sheffield, UK), 500 ml, was used to flush the kidneys (n = 6 per group), delivered at a hydrostatic pressure of 100 cmH_2O at 4ºC via the blood set. The refrigerated solution was stored on ice during transportation in a polystyrene retrieval box and removed

during the 5-min preparation period. After the preparation period, kidneys were placed in a dish containing cold HOC preservation solution and the flush was started. The perfusate was allowed to drain freely from the renal vein into the dish. The infusion rate was recorded accurately using a chart recorder (Gould TA240; Gould Instrument Systems, Ilford, UK) (Figure 8). Kidneys were reweighed after flushing.

The specific heat capacity of the two solutions was measured. A volume of UW and HOC at a recorded temperature was added to a volume of water at a known mass and temperature ($n = 6$). The specific heat capacity was calculated using the following equation: (Δ temperature $\times$ mass of water)/(Δ temperature $\times$ mass of solution).

Separate experiments were performed to assess the effects of UW and HOC on renal function. Porcine kidneys were retrieved in the same manner and flushed with 500 ml solution after a minimal warm ischaemia time of 10 min. The duration of flush was recorded. This amount of solution has been found to be an adequate volume to flush the kidney before static storage and to reduce the temperature optimally [22]. One litre of blood was also collected from the animal into a sterile receiver containing 25 000 units heparin (Multiparin®; CP Pharmaceuticals, Wrexham, UK). Kidneys were flushed with either UW or HOC at a hydrostatic pressure of 100 cmH_2O at 4ºC, then stored on ice and transported back to the laboratory. The total cold ischaemia time was standardized to 2 h.

Kidneys were then prepared for reperfusion with autologous blood on an isolated organ preservation system, as described previously [22]. Creatinine (Sigma-Aldrich, Steinheim, Germany) was added to the perfusate to achieve an initial circulating concentration of 1000 µmol/l. The renal artery, vein and ureter were cannulated and any residual preservation solution was removed by flushing the

kidneys with 90 ml plasma substitute (Gelofusine; B. Braun, Sheffield, UK) at 4ºC. The kidneys were weighed immediately before and after perfusion using a balance accurate to 1 g. Weight change was recorded.

Kidneys (n = 6 per group) were perfused for 6 h at a mean arterial pressure (MAP) of 55 mmHg. Renal blood flow (RBF) and MAP were recorded continuously and intrarenal vascular resistance (MAP/RBF) calculated. Serum and urine samples were taken hourly for biochemical analysis, and whole blood for haematology. Creatinine clearance and fractional excretion of sodium were calculated, and serum creatinine levels recorded hourly. Blood gases were measured for the calculation of oxygen consumption in millilitres per minute per gram and acid–base balance. Arterial pH was measured before perfusing the kidneys, then at 1-, 3- and 6-h intervals for assessment of acid–base homeostasis.

Needle core biopsies were taken before and 6 h after perfusion with autologous blood, fixed in 4 % formalin, dehydrated and embedded in paraffin wax. Sections were cut at 4 µm, and then stained with haematoxylin and eosin for evaluation under light microscopy. Seven morphological parameters (tubular debris, tubular dilatation, vacuolation, epithelial shredding, epithelial flattening, condensed tubular nuclei and red blood cell presence) were each scored over five fields using a semiquantitative scale (0, normal; 1, mild; 2, moderate; 3, severe). Training in histological assessment was taken from the Professor of histopathology, and although it was a subjective scoring system the results were reproducible.Two people independently scored the slides, and both were blinded to the sample type.

Figure 6. Set up of equipment pre-organ harvest.

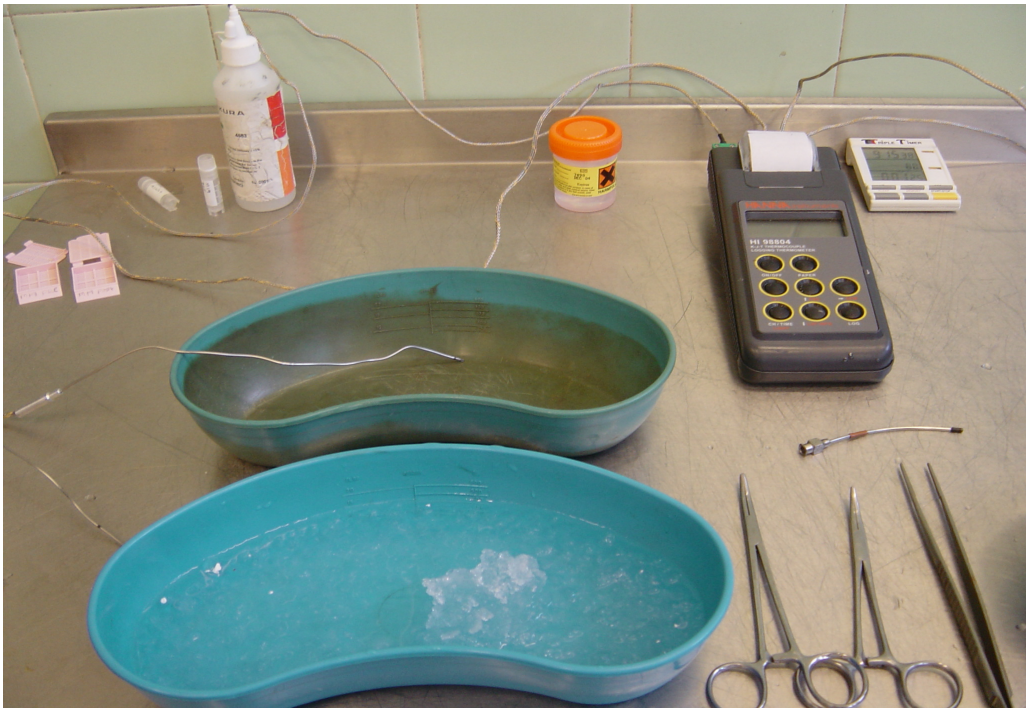


Figure 7. Temperature Measurements being recorded during kidney flush with thermocouple logging thermometer

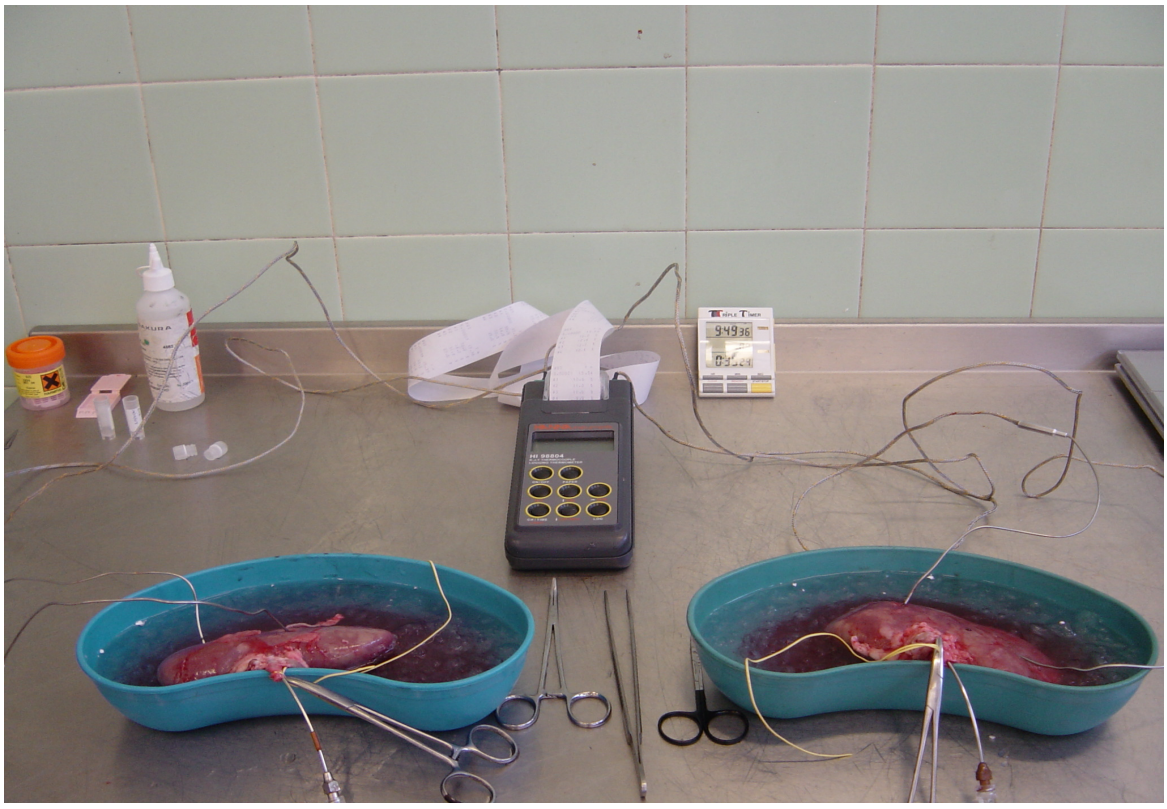
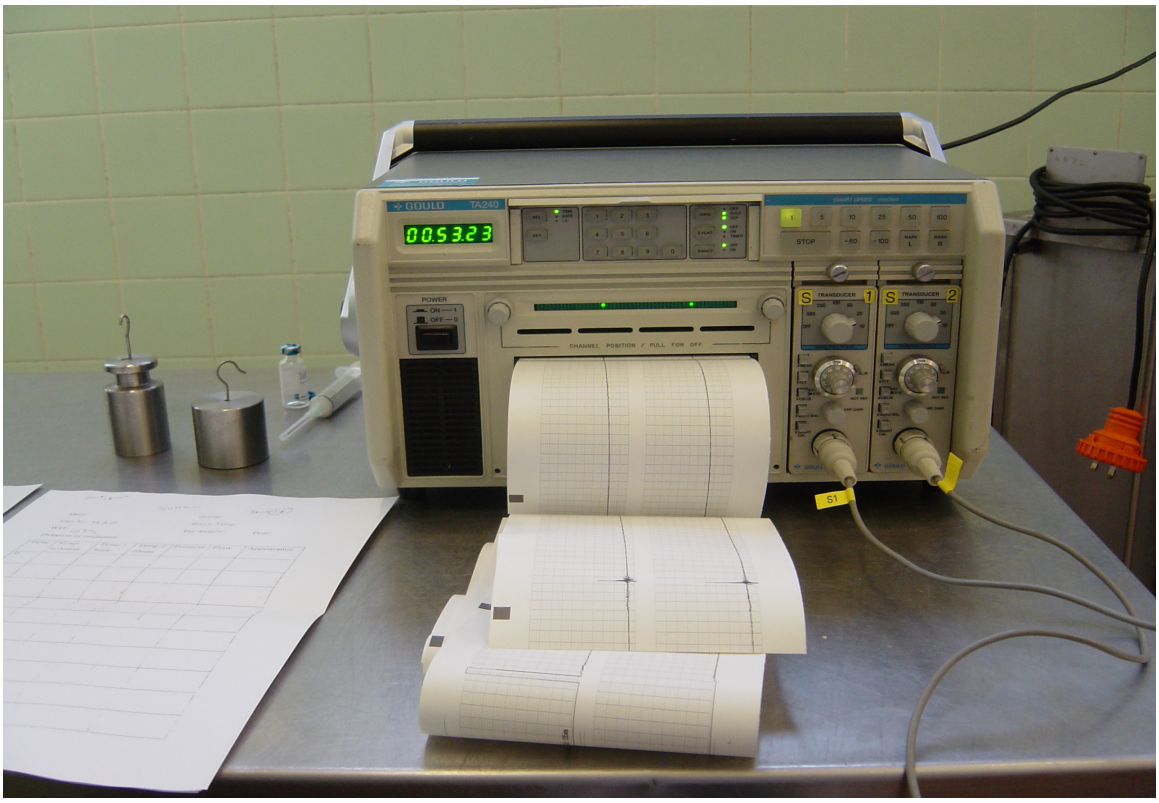


Figure 8. Chart Recorder measuring perfusate flow rates



Statistical analysis

For flush experiments, the flow rate of the preservation solution and the temperature of the kidneys at depths of 5 and 20 mm were plotted against time, and the area under the curve (AUC) calculated using GraphPad Prism® (version 3.00; GraphPad Software, San Diego, California, USA). The temperature drop at depths of 5 and 20 mm was determined by the following parameters: time to reach less than 10°C, perfusion flow rate to reach less than 10°C (millilitres per minute per 100 g tissue) and amount of perfusate needed to reach below 10°C. The slope of temperature fall to reach less than 10°C was calculated by means of linear regression (GraphPad Prism®).

For renal function, the continuous variables creatinine clearance, RBF, intrarenal resistance, urine output and fractional excretion of sodium were plotted against time, and the AUC for individual perfusion experiments was calculated using Microsoft® Excel software (Microsoft, Reading, UK) and GraphPad Prism®.

Raw data were compared with the Mann–Whitney *U* test (GraphPad InStat® version 3.00; GraphPad Software). Values are presented as mean (s.d.). $P \leq 0.050$ was taken as statistically significant.

Results

There was no difference between the two groups in the initial temperature of the preservation solution used to flush the kidneys (4.7(0.8) *versus* 4.6(0.6)°C for UW and HOC respectively; $P = 0.522$), or in the temperature of preservation solution in the kidney dishes at the start of perfusion (3.1(0.8) *versus* 3.0(0.8)°C respectively; $P = 0.573$). The temperature of solution in the dishes did increase significantly to a similar level in both groups by the end of the flush period (UW: 5.7(1.9)°C, $P = 0.026$; HOC: 5.5(1.1)°C, $P = 0.002$), but there was no difference between the groups ($P = 0.936$). The room temperature range was 10.4–12.4°C.

Immediately after retrieval, kidney temperatures at depths of 5 and 20 mm were comparable in the two groups, and fell to similar levels during the 5-min preparatory period (*Table 3*).

Table 3. Temperature of porcine kidneys at depths of 5 and 20 mm after excision and after 5 min preparation at room temperature in University of Wisconsin solution and hyperosmolar citrate

	UW	HOC	*P**
After excision			
5 mm	34.2(1.3)	34.4(1.7)	0.688
20 mm	36.6(1.5)	37.0(0.5)	0.630
After 5 min preparation			
5 mm	30.9(3.1)	30.5(3.7)	0.630
20 mm	33.2(1.9)	33.7(2.0)	0.999

Values are mean(s.d.). UW, University of Wisconsin solution; HOC, hyperosmolar citrate. *Mann–Whitney *U* test.

There was no difference between the initial weight of kidneys in either group (206(49) *versus* 191(27) g for UW and HOC respectively; $P = 0.574$). However, kidneys in the HOC group gained a significant amount of weight during the flush compared with those flushed with UW (50(8) *versus* 7(13) per cent respectively; $P = 0.002$).

The 500-ml flush with UW took significantly longer than that with HOC (38.7(7.0) *versus* 25.0(6.8) min respectively; $P = 0.009$), and thus the flow rate was significantly slower (4.8(1.1) *versus* 10.8(3.1) ml per min per 100 g; $P = 0.002$) and the AUC for total flush significantly greater (9574(2537) *versus* 6476(1017) ml/min; $P = 0.026$).

The time needed for the kidney temperature to fall below 10°C at both 5 and 20 mm was greater in the UW group, but not significantly different from that in the HOC group ($P = 0.180$ and $P = 0.522$ respectively) (*Table 4*, *Fig. 9*). The rate of temperature fall at both depths was significantly lower in the UW than in the HOC group (5 mm, $P = 0.041$; 20 mm, $P = 0.016$) (*Table 4*). In addition, there was no significant difference between the two depths in either group in the time taken to reach a temperature of less than 10ºC (UW, $P = 0.228$; HOC, $P = 0.936$). The amount of preservation solution required for the temperature to fall below 10°C in the UW group approached significance at the 20-mm depth and 5 mm depth compared to the HOC group ($P = 0.054$ and $P = 0.064$ respectively)(*Table 4*). The perfusion flow rate to reach less than 10°C was significantly lower in the UW group at both 20 and 5 mm ($P = 0.002$ and $P = 0.004$ respectively) (*Table 4*). For either depth, the slope of temperature fall to less than 10°C was not significantly different between groups (5 mm, $P = 0.937$; 20 mm, $P = 0.589$) (*Table 4*, *Fig. 9*).

At the end of the 500-ml flush, there was no difference between the groups in temperature at 5 mm (5.5(0.9) *versus* 6.2(1.4)°C for UW and HOC respectively; P = 0.377). However, at 20 mm the temperature was significantly lower in the UW group (7.1(0.9) *versus* 8.3(0.8)°C for HOC; P = 0.041). At the end of infusion, the temperature at the 5-mm depth was significantly lower than at 20 mm in both groups (P = 0.004 and P = 0.008 for UW and HOC respectively).

Mean (s.d.) specific heat capacity was similar for the two solutions (0.81(0.06) *versus* 0.87(0.07) for UW and HOC respectively; P = 0.109).

The initial flush of the kidneys after retrieval took significantly longer with UW than with HOC (flow rate 4.9(1.6) *versus* 10.0(1.6) ml per min per 100 g respectively; P = 0.002). This rate was similar to that recorded in the flush experiments (P = 0.818 and P = 0.873 for UW and HOC respectively).

Table 4. Time taken, temperature change, amount and rate of solution required for kidney temperature to fall below 10°C at 5 and 20 mm**.**

	UW	HOC	*P**
Time to < 10ºC (min)			
5 mm	16.0(3.2)	13.7(5.5)	0.180
20 mm	18.3(3.0)	13.8(5.3)	0.522
Rate of temperature fall (°C per min per 100g)			
5 mm	0.61(0.11)	0.98(0.29)	0.041
20 mm	0.64(0.10)	1.01(0.56)	0.016
Amount of solution (ml)			
5 mm	155.0(105.5)	263.3(65.9)	0.064
20 mm	170.0(49.4)	269.2(116.9)	0.054
Perfusion flow rate (ml per min per 100 g)			
5 mm	4.46(1.76)	11.15(3.98)	0.004
20 mm	4.68(1.38)	10.83(3.58)	0.002
Slope to < 10ºC			
5 mm	−1.42(0.86)	−1.85(0.88)	0.589
20 mm	−1.43(0.32)	−1.89(1.30)	0.937

Values are mean(s.d.). UW, University of Wisconsin solution; HOC, hyperosmolar citrate. *Mann–Whitney *U* test**.**

Fig. 9 Mean (s.d.) reduction in temperature over time to achieve a temperature of less than 10ºC at a depth of **a** 5 mm and **b** 20 mm in kidneys flushed with University of Wisconsin solution (UW) or hyperosmolar citrate (HOC). **a** $P = 0.041$, **b** $P = 0.016$ (Mann–Whitney U test)

Figure 9a

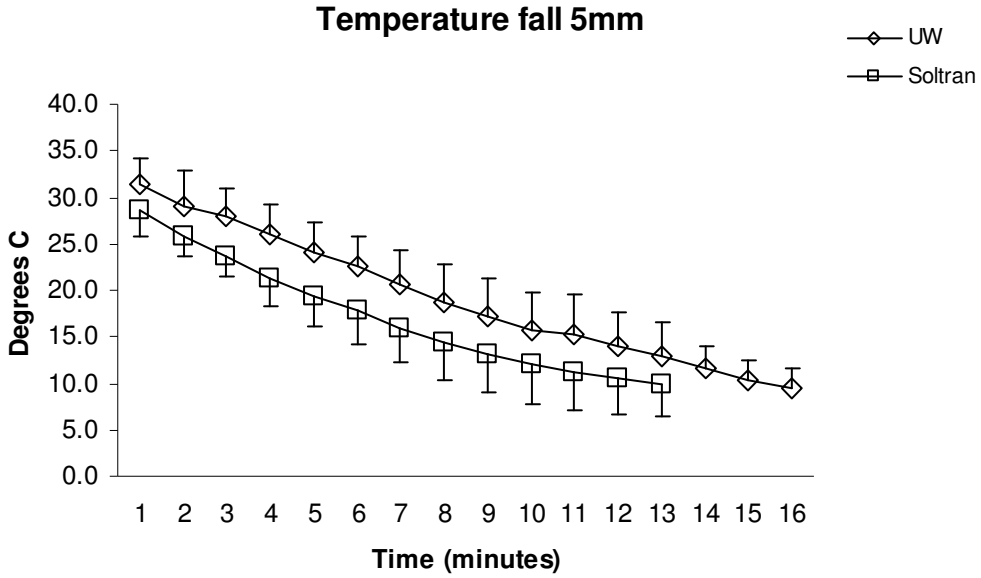
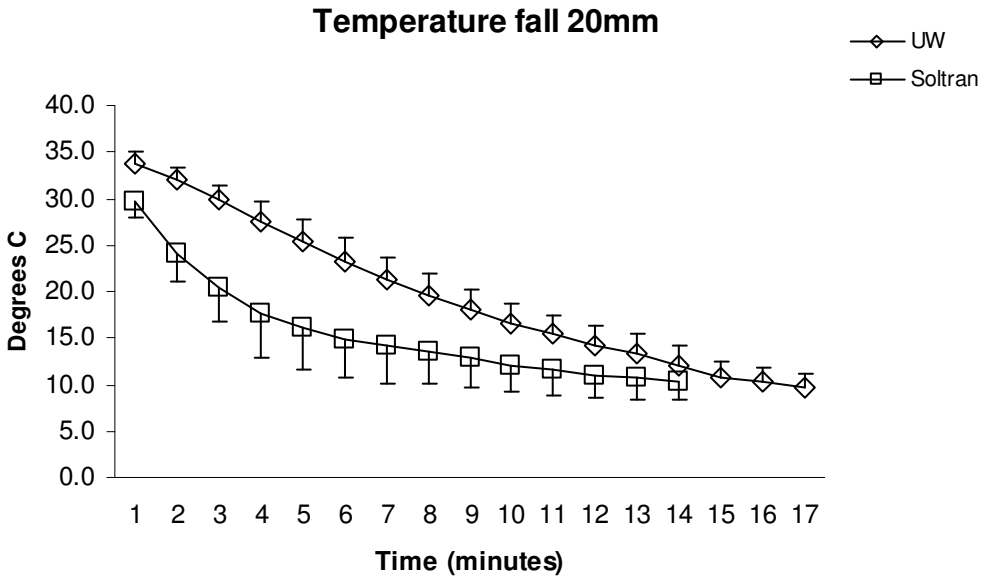


Figure 9b



Kidneys in both groups gained a significant amount of weight after perfusion compared to baseline weight (29.7(3.4) *versus* 21.2(7.7) per cent for UW and HOC respectively; $P = 0.078$).

Intrarenal resistance was significantly lower in the HOC group throughout the 6 hours of reperfusion, but both RBF and renal function were similar (*Table 5*). The total urine output was also similar (410(153) *versus* 536(221) ml for UW and HOC respectively; $P = 0.394$).

By the end of reperfusion, arterial blood pH was reduced in both groups (7.23(0.12) *versus* 7.21(0.09) for UW and HOC respectively; $P = 0.936$). Oxygen consumption levels remained similar throughout perfusion, with no significant difference at 6 h (33.7(15.1) *versus* 31.0(6.3) ml per min per g respectively; $P = 0.999$).

Table 5. Renal function parameters over 6 h of reperfusion

	Area under the curve		
	UW	HOC	*P**
RBF ((ml per min per 100 g)·h)	215(69.1)	226.2(54.7)	0.818
Intrarenal resistance (mmHg·h)	6.31(3.05)	3.39(0.37)	0.026
CrCl ((ml per min per 100 g)·h)	17.2(9.8)	14.4(11.9)	0.818
Fractional excretion of sodium (%·h)	63.2(29.6)	82.3(28.3)	0.626

Values are mean(s.d.). UW, University of Wisconsin solution; HOC, hyperosmolar citrate; RBF, renal blood flow; CrCl, creatinine clearance. *Mann–Whitney *U* test.

The HOC group showed most evidence of damage after flushing and storage (preperfusion biopsies) with significantly more tubular dilatation, epithelial flattening and tubular debris than in the UW group (*Table 6*). After reperfusion with autologous blood, there was a significant degree of histological change in both groups, with increased tubular dilatation, epithelial flattening, tubular debris vacuolation and condensed tubular nuclei in the UW group, and condensed tubular nuclei and red blood cell presence in the HOC group. After 6 h of reperfusion histological changes in the two groups were similar.

Table 6. Histological scores for biopsies taken before and 6 h after perfusion with autologous blood

	UW			HOC		
	Before	After	*P**	Before	After	*P**
Tubular dilatation	0.03(0.46)	2.27(0.45)	0.001	1.80(0.81)†	2.17(0.59)	0.102
Epithelial flattening	0.30(0.46)	1.48(0.70)	0.001	1.47(0.78)†	1.77(0.73)	0.220
Epithelial shedding	0.97(0.18)	0.85(0.36)	0.587	1.10(0.31)	1.10(0.30)	0.994
Tubular debris	0.33(0.48)	0.56(0.51)	0.011	0.93(0.36)†	0.80(0.48)	0.380
Vacuolation	0.03(0.18)	0.41(0.57)	0.021	0.13(0.45)	0.30(0.47)	0.195
Condensed tubular nuclei	0.40(0.49)	0.96(0.65)	0.002	0.30(0.47)	0.87(0.73)	0.004
Presence of RBCs	0.30(0.47)	0.63(0.74)	0.112	0.13(0.35)	0.50(0.51)	0.013

Values are mean(s.d.). UW, University of Wisconsin solution; HOC, hyperosmolar citrate; RBC, red blood cell. *Between time points in each group; †$P \leq 0.050$ *versus* UW before perfusion (Mann–Whitney *U* test).

Discussion

Although UW reduced the core temperature of porcine kidneys at a slower rate than HOC, a smaller volume was needed to reduce the temperature to less than 10°C . This finding can be explained by the different flow rates of the two solutions. The specific heat capacity of the solutions was similar, so the lesser volume of UW required probably resulted from the slower transit time of this more viscous solution, leading to a greater degree of heat transfer per unit volume. Flushing with UW was associated with less histological injury, but there was no difference in renal functional outcome when kidneys were reperfused *ex vivo* with autologous blood. The slower renal perfusate flow rate and cooling achieved by UW may therefore be advantageous in reducing endothelial injury.

In theory, the slower flush rate and hence longer time to cool to below 10ºC should increase the degree of warm ischaemic injury. However, rapid cooling has been implicated as a cause of endothelial cell damage. Winchell and Halasz [191] found that rapid initial cooling and storage of rabbit kidneys at 0ºC markedly diminished function after 24 h of cold storage. When the rate of cooling was slowed by using a warmer flush (8ºC *versus* 2ºC), a twofold increase in creatinine clearance was observed. Creatinine clearance was further increased by both flush and storage at 8ºC. Otto and colleagues[112] compared warm flushing (15ºC) and cold flushing (2ºC), using Ringer's solution in a porcine liver transplant model with 24 h of cold storage. A marked difference was observed, with pronounced endothelial damage after cold flushing but minor damage with the warmer flush. They concluded that the velocity of cooling was important and that rapid cooling was detrimental to endothelial cells. Although a warmer flush was not used in the present experiment, the principle of a slower rate of cooling was achieved in the UW group.

Storage temperature and duration of storage are also important factors. Astarcioglu and co-workers[192] observed that survival of livers stored in UW at 10ºC was similar to that for 4ºC when a short ischaemia time (6 h) was employed.

The HOC-flushed kidneys gained a significant amount of weight during the flush compared with those in the UW group, suggesting that UW is superior at minimizing ionic changes across the cell membrane. This finding is consistent with the work of Lodge *et al*.[93] in a comparison of porcine renal preservation using an autotransplant model, and may be due to the higher molecular weight impermeants (lactobionate and raffinose) and the presence of the colloid HES in UW acting to counter cellular oedema.

Kidneys flushed with HOC showed a significant amount of histological change after flushing, which may have been caused by rapid cooling. Although the two study solutions flushed and cooled kidneys at different rates, this had no effect on renal function measured during isolated organ perfusion with autologous blood. It is worth noting, however, that this study used a short preservation period and thus involved minimal cold ischaemic injury. A more prolonged storage period might have revealed the consequences of the histological change. Intrarenal resistance was higher throughout reperfusion in the UW group, which is perhaps unsurprising in view of the increased viscosity of this solution and its known effect of increasing red blood cell aggregation[123]. This did not, however, have a detrimental outcome on renal function.

This experimental study provides a model of the controlled non-heart-beating donor with a fairly short period of warm ischaemia. However, the relatively small volume of perfusate introduced directly into the cannulated renal artery and the short period of static hypothermia is not such a good model of the live donor situation in humans. Porcine kidneys are anatomically and physiologically similar to human

kidneys[193,194] and, importantly, their response to warm and cold ischaemic insults is very similar to the human situation[195,164].

The retrieval of kidneys from deceased donors after both brain and cardiac death is typically performed *in situ*, often with more than 5 litres of perfusate being infused through the abdominal aorta before a final flush on the back-table. The volume of preservation solution used in organ retrieval is certainly a consideration, with the majority of centres opting for cheaper, less viscous, solutions. This study has provided evidence that UW is more effective than HOC in reducing organ temperature, with a lesser amount needed to reach the optimal temperature, albeit at a slower rate. A future area of research may also address the efficiency of UW and HOC in removing blood from the kidney, preventing thrombosis of the microcirculation.

UW improves the results of renal transplantation from organs stored for prolonged cold ischaemic intervals[196]. In current practice, the use of UW has been reserved largely for marginal organs, especially those from non-heart-beating donors[53]. The results of this experimental study suggest that, despite its slower perfusion flow rate, UW may have a role in reducing oedema and histological injury in live donor kidney transplantation. Further clinical studies are required to address this.

The Rate of Organ Cooling – Using a Warm Flush Followed by a Cold Flush

Introduction

The importance of slowing metabolism by reducing the temperature through adequate perfusion is a fundamental paradigm of organ preservation. This study aimed to measure the efficiency of cooling using different preservation solutions and to determine whether flushing an organ at a normothermic temperature prior to hypothermia had any overall beneficial effects on the rate of cooling.

Methods

Porcine kidneys (n=4) were retrieved after 10 minutes of warm ischaemia (WI) and two thermocouple probes ((Hanna Instruments, Leighton Buzzard, UK) were inserted into either the upper or lower pole of the kidney at depths of 5mm and 20 mm, and the temperature was recorded at 1-min intervals with a thermocouple logging thermometer. Temperature monitoring was started 5 minutes before the onset of perfusion forming a baseline level at normal room temperature. The renal artery was cannulated using a Tibbs arterial cannula and connected to a 4.0-mm blood set. The kidneys were then flushed at a hydrostatic pressure of 100 cm H_2O with either 500ml of UW at 4°C, Soltran at 4°C or 250ml AQIX RS-I at 30°C followed immediately by 250ml AQIX at 4°C. The AQIX solution was pre-warmed in a temperature regulated water-bath and maintained at 30°C during transport in an insulated organ retrieval carrier; temperature was monitored with a temperature probe. The infusion rate was recorded accurately using a chart recorder (Gould TA240; Gould Instrument Systems,

Ilford, UK). The temperature and flow were recorded at minute intervals throughout. Values presented are the mean ± SD.

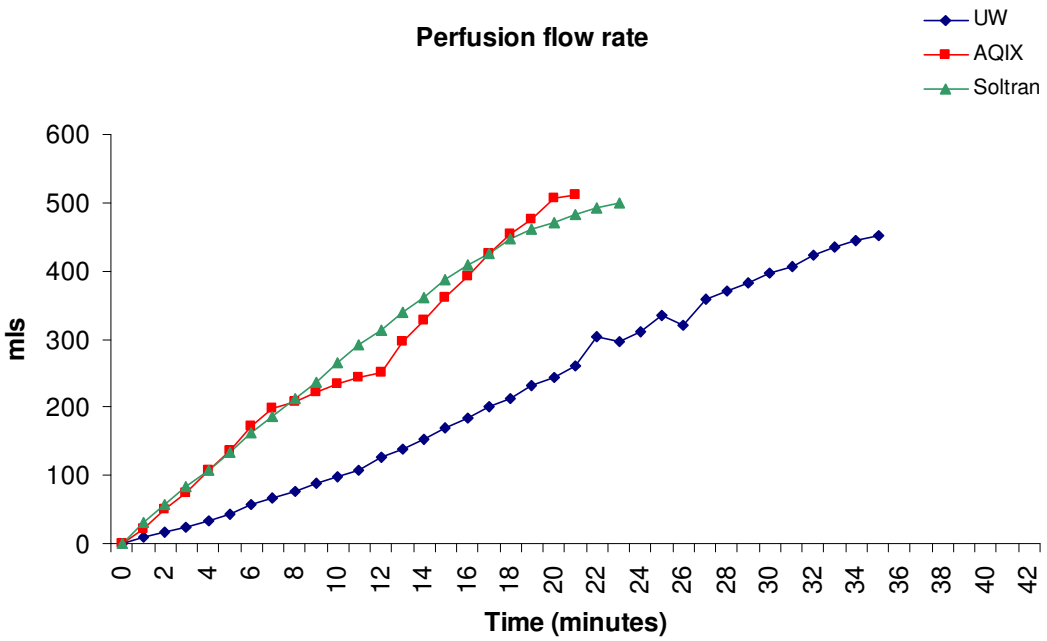
Results

The rate of perfusion was significantly lower with UW solution compared to both Soltran and AQIX (5 ± 1.3 vs 12 ± 3.9 vs 15.45 ± 0.25 ml/min/100g respectively) (table 7, figure 10). Warm AQIX flush was fastest at 23 ± 2.3 ml/min/100g followed by the cold Aqix flush at 15.5 ± 2.5 ml/min/100g (table 7).

Table 7: Rate of flush ml/min/100g for kidneys flushed with 500mls of cold UW or HOC, or 250mls of warm AQIX followed by 250mls of cold AQIX

	Warm	Cold	Total
UW		5 ± 1.3*	5 ± 1.3*
AQIX	23 ± 2.3	15.5 ± 2.5*	15.45 ± 0.25 *
SOLTRAN		12 ± 3.9	12 ± 3.9
P value (Aqix Vs UW)		0.018	0.021

Figure 10: perfusion flow rate ml/min/100g for kidneys flushed with 500mls of cold UW, 500mls of cold Soltran and 250mls of warm AQIX followed by 250mls of cold AQIX.



The time taken to reach 10°C was fastest in the Soltran group compared to AQIX and UW- flushed kidneys at depths of both 20mm (11.5 ± 4.9 vs 21.5 ± 4 vs 16.8 ± 2.2 minutes respectivley), and 5mm (10.8 ± 4.4 vs 21.8 ± 4.4 vs 16 ± 3.7 minutes respectively)(Table 8, Fig 11, 12). Soltran reached 10°C significantly quicker than Aqix at 20mm (P=0.029) and at 5mm (P= 0.026).

There was no significant difference in the rate of temperature fall °C per minute between the groups during perfusion at 20mm (1.34 ± 0.2 Soltran vs 1.06 ± 0.3 Aqix vs 0.93 ± 0.1 UW; P= 0.115) or 5mm (1.3 ± 0.2 Soltran vs 0.99 ± 0.3 Aqix vs 0.83 ± 2.4; P= 0.118) (Table 9).

Table 8: Mean ± SD Time taken (minutes) for the kidney to reach <10°C at a depth of 20 mm and 5 mm in kidneys flushed with University of Wisconsin solution (UW), AQIX or Soltran.

Group	20mm	5mm
UW	16.8 ± 2.2	16 ± 3.7
AQIX	21.5 ± 4.0*	21.8 ± 4.4*
SOLTRAN	11.5 ± 4.9*	10.8 ± 3.6*
*** P value (Aqix vs Soltran)**	0.029	0.026

Table 9: Rate of temperature fall (ºC per minute) at depths of 20 mm and 5 mm during flush of kidneys with University of Wisconsin solution (UW), AQIX or Hyperosmolar Citrate (HOC).

Group	20mm	5mm
UW	0.93 ± 0.1	0.83 ± 2.4
AQIX	1.06 ± 0.3	0.99 ± 0.3
SOLTRAN	1.34 ± 0.2	1.3 ± 0.2
P value (all groups)	0.115	0.118

Figure 11: Rate of cooling at 20mm depth in kidneys flushed with 500mls of University of Wisconsin solution (UW), 250mls of warm AQIX followed by 250mls of cold AQIX or 500 mls of cold Hyperosmolar Citrate (HOC).

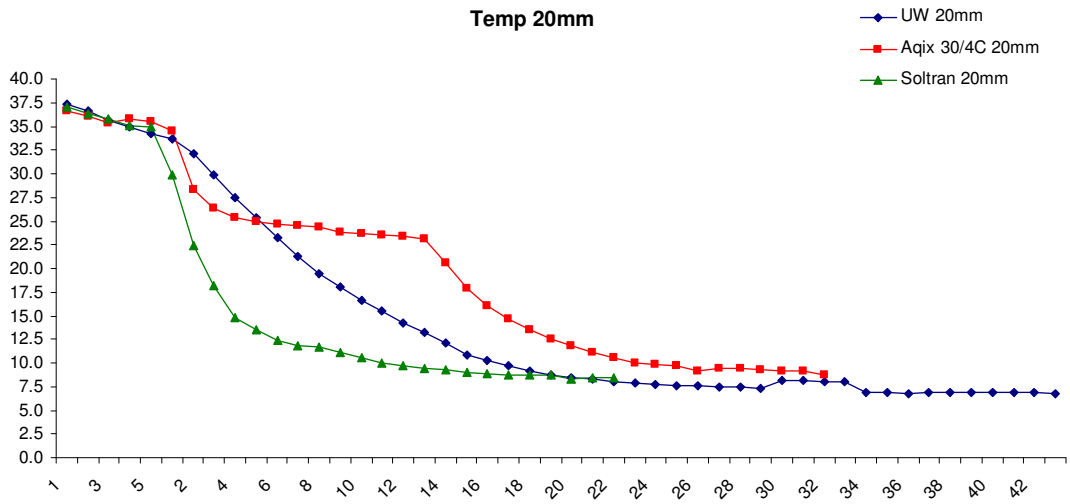
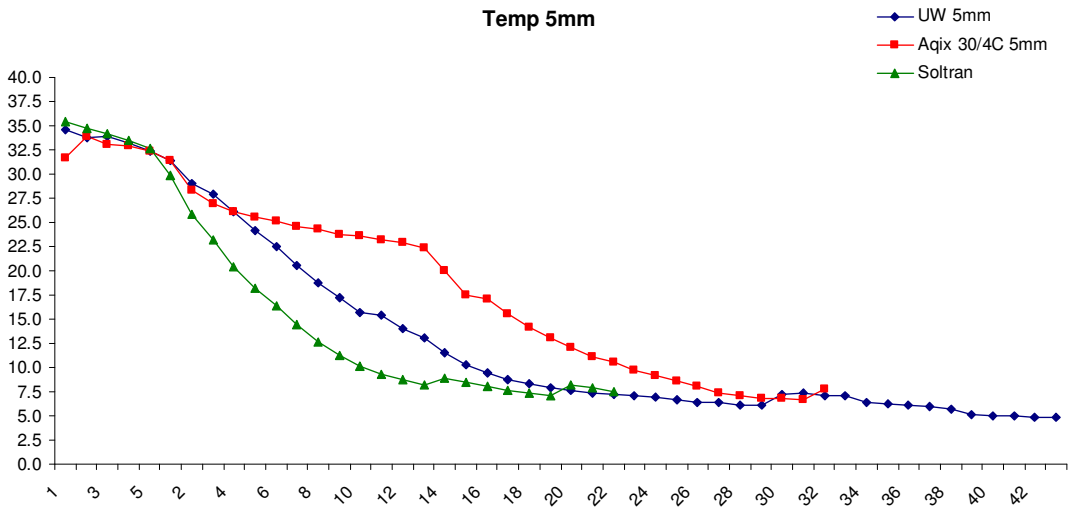


Figure 12: Rate of cooling at 5mm depth in kidneys flushed with 500mls of University of Wisconsin solution (UW), 250mls of warm AQIX followed by 250mls of cold AQIX or 500 mls of cold Hyperosmolar Citrate (HOC).



Discussion

The initial warm Aqix flush gave the most rapid flush 23 ± 2.3 ml/min/100g, suggesting the lack of resistance to flow using normothermic temperatures without a vasoconstrictive effect. When Aqix was then flushed cold it also then had the highest rate of flush compared to Soltran and UW (15.5 ± 2.5 vs 12 ± 3.9 vs 5 ± 1.3 ml/min/100g respectively). However the increased rate of flush did not translate into a faster cooling rate. Indeed the total rate of flush of AQIX (combining warm and cold flush) was not significantly faster than Soltran (15.45 ± 0.25 vs 12 ± 3.9 ml/min/100g respectively). The time taken to reach 10°C was significantly faster in the Soltran group compared to the AQIX at depths of both 20mm (11.5 ± 4.9 vs 21.5 ± 4 minutes respectively) and 5mm (10.8 ± 4.4 vs 21.8 ± 4.4 minutes respectively)(Table 8, Fig 11, 12). Interestingly the temperature at the end of the Aqix warm flush was 23.7 ± 1.1 ºC at 20mm depth and 26.5 ± 0.83 ºC at 5mm depth.

The rate of perfusion was significantly lower with UW solution compared to both Soltran and AQIX (total combined warm and cold flush); (5 ± 1.3 vs 12 ± 3.9 vs 15.45 ± 0.25 ml/min/100g respectively; P = 0.018) (Table 7, Figure10). This would be in keeping with the higher viscosity of UW compared to the other 2 solutions. However UW- flushed kidneys reached 10 ºC faster than AQIX- flushed kidneys at both 20mm and 5mm depths (16.8 ± 2.2 vs 21.5 ± 4 minutes and 16 ± 3.7 vs 21.8 ± 4.4 minutes repectively). Despite the viscosity of UW and the slower rate of perfusion, the rate of temperature fall was equivalent to the less viscose solutions, Soltran and AQIX.

This study thus suggests that flushing kidneys at normothermic temperatures prior to a hypothermic flush does not increase the rate of cooling compared to a hypothermic

flush with traditional solutions. This study did not include a warm flush with UW or Soltran but this would be a useful future experiment to compare the three groups more accurately.

Assessment of flush conditions using a novel non-phosphate buffered preservation solution in a renal transplant model.

Background

Normothermic preservation may improve the quality of warm ischaemic damaged kidneys. AQIX RS-I is a novel non-phosphate buffered preservation solution that can be used over a range of temperatures and conditions to preserve organs. The aim of this study was to test AQIX RS-I solution under different retrieval conditions to ascertain the optimal conditions for its use.

Methods

Porcine kidneys were flushed with AQIX RS-I solution under 3 different conditions (n = 6), A: 4°C flush followed by 2hrs cold storage, B: 30°C flush followed immediately by a 4°C flush then 2hrs cold storage and C: 30°C flush followed by 2 hrs cold storage. An isolated preservation system (IOPS) was used to assess renal viability over a period of 6 hours after storage.

Results

Kidneys flushed with AQIX at 30°C followed by cold storage demonstrated overall improved renal function and improved acid-base homeostasis compared to the 4°C or 30°C followed by 4°C flush groups (AUC serum creatinine: AQIX 30°C; 2114 ± 1031, AQIX 4°C; 3676 ± 896, AQIX 30°C/4°C 3948 ± 328 μmol/L.h; P = 0.015).

Conclusion

This study demonstrates that simply flushing kidneys normothermically then storing them on ice with RS-I solution proved to be a better method of preservation. No beneficial effects were found when the solution was used solely under hypothermic conditions or combination with a normothermic flush.

Background

Hypothermic flush and static storage of organs for transplantation is the current preferred method of organ preservation in the UK [197]. The last decade however has seen a revival of interest in machine preservation and perfusion at normothermic temperatures [161,11]. University of Wisconsin solution remains the gold standard preservation solution, against which emerging solutions are compared. We have previously published work using a novel normothermic preservation solution, AQIX, showing its capability of maintaining acid-base balance after warm flush and 2 hours of warm ischaemic time [198]. The aim of this study was to assess AQIX under different flush conditions, and to ascertain the optimal conditions for its use, in a large animal model using normothermic perfusion.

AQIX

AQIX has been designed as a universal normothermic organ preservation solution that would reflect physiological ionic concentrations, osmolarity and ion conductivity, thereby maintaining the ionized status of the cell membrane and the function of enzymatic and receptor moieties. The osmolarity of AQIX is similar to that of serum at 286 mOsmoles/l, and its ionic concentrations have been chosen to maintain it as an isosmotic solution. The ionic conductivity is also comparable to human serum, namely, 12.6 mS cm-1.

AQIX is a non-phosphate buffered solution that utilizes a combination of the bicarbonate system ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$) and Good's BES as its buffer which, because of its ideal pk_a value, allows the pH to be maintained within a pH of 7.2 – 7.46 over a temperature range of 4 – 37 ºC thereby facilitating the

transition from hypothermic to normothermic and vice versa. The constituents of AQIX are displayed in Table 14.

Table 14. Composition of AQIX

Sodium	110
Potassium	5.0
Magnesium	0.45
Sodium bicarbonate	25.0
BES	5.0
D-glucose	10.0
Calcium chloride	1.25
Glycerol	0.11
L – Glutamate	0.30
L – Glutamine	0.40
L – Aspartate	0.02
L – Carnitine	0.05
Choline Chloride	0.01
TPP (co-carboxylase)	40.0 nmol/l
Insulin	28.0 mIU

*All units in mmol/l unless specified

Methods

Kidney Retrieval

Large white pigs (60–70kg) were sacrificed by electrocution followed by exsanguination and approximately 1 litre of blood was collected into a sterile receiver containing 25,000 units of heparin (Multiparin; CP Pharmaceuticals, Wrexham, UK). The kidneys were surgically removed with minimal warm ischaemic time and immediately flushed with 400 ml of preservation solution at a hydrostatic pressure of 100 cmH_20. The time taken for the total of 400 ml of solution to run into the kidney (the flush time) was recorded along with the exact warm ischaemic interval. Perfusate flow rates were calculated and expressed as ml/min/100g of kidney. After flushing, all kidneys were stored on ice in a polystyrene retrieval box for 2 hours (CIT) to allow transfer to the laboratory.

Three different preservation techniques were compared (n=6 kidneys per group): AQIX flush at 4°C; AQIX (200ml) flush at 30°C followed by AQIX (200ml) flush at 4°C; and AQIX flush at 30°C. For warm flushing, the AQIX solution was pre-warmed in a temperature regulated water-bath and maintained at 30°C during transport in an insulated organ retrieval carrier; temperature was monitored with a temperature probe.

Isolated Organ Perfusion

The Isolated Organ Perfusion system has been described in detail previously [10] but in brief consists of a centrifugal blood pump, a heat exchanger, a venous reservoir and a membrane oxygenator. The circuit hardware includes a speed

controller, a flow transducer, a pressure transducer and a temperature probe. Two infusion pumps and a urinometer were incorporated into the system.

The circuit was primed with 500 ml Ringer's solution containing mannitol 10mg (Baxter, Healthcare, Norfolk, UK), dexamethasone 10mg (Organon Labs Ltd, Cambridge, UK), cefuroxime 750mg (Britannia Pharmaceuticals Ltd, Surrey, UK) and 15ml sodium bicarbonate 8.4% (Fresenius Kabi, Warrington, UK). 500ml of heparinized whole blood was added to the circuit after priming and allowed to circulate at a temperature of 37ºC.

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

Experimental Protocol

Pairs of kidneys retrieved from the same animal were flushed by the same technique and then simultaneously perfused with autologous blood using two separate isolated organ perfusion circuits. The renal artery, vein and ureter were cannulated and any residual preservation solution was removed by flushing the kidneys with 90mls of plasma substitute (Gelofusine; B. Braun, Sheffield, UK) at 4 ºC.

Kidneys (n=6 per group) were perfused for 6 hours at a mean arterial pressure of 55 mmHg. Renal blood flow (RBF), and mean arterial pressure (MAP) were continuously recorded and intra-renal vascular resistance calculated (MAP/RBF). The addition of 1000 μmol creatinine to the circuit enabled an accurate measurement of renal function to be made. Serum and urine samples were taken hourly for biochemical analysis and whole blood for haematology. Creatinine clearance (U_{cr} x U volume / P_{cr}) and fractional excretion of sodium [(urinary sodium x urine volume) / (glomerular filtration rate x plasma sodium) x 100] were calculated and serum creatinine levels recorded hourly. Blood gases were measured for the calculation of oxygen consumption in ml/min/g [(PaO_2 art – PaO_2 ven) x flow rate/weight] and acid-base balance. Arterial pH was measured prior to perfusing the kidneys, then at 1, 3 and 6 hourly intervals for assessment of acid-base homeostasis

Needle core biopsies were taken pre- and 6 hours post-perfusion, fixed in 4% formalin, dehydrated and embedded in paraffin wax. Sections were cut at 4μm then stained with haematoxylin and eosin for evaluation using light microscopy. Seven morphological parameters were each scored over 5 fields using a semi-quantitative scale (0 normal; 1 mild; 2 moderate; 3 severe).

Statistical analysis

Values are presented as mean ± SD. Continuous variables, such as serum creatinine were plotted against time and the area under the curve for individual perfusion experiments calculated using Microsoft ®Excel software (Reading, UK). Mean areas under the curve were then compared using the Kruskal-Wallis test and post testing was performed with Dunn's test for serum Creatinine, Creatinine clearance, renal

blood flow and urine output (table 4). A P value ≤ 0.05 was taken as statistically significant.

Functional Results

There was no significant difference in the duration of warm ischaemia between the groups (AQIX 4ºC 6.3 ± 0.4, AQIX 30ºC/4ºC; 6.2 ± 1.2, AQIX 30ºC 6.2 ± 0.41 minutes; P = 0.704). The time taken to flush the kidneys immediately after retrieval was significantly shorter in the AQIX 30ºC group compared to the other two temperatures (AQIX 30ºC; 7.4 ± 0.9, AQIX 4ºC; 14.8 ± 3.5, AQIX 30ºC/4ºC; 12.6 ± 2.9 minutes; P = 0.003).

Serum creatinine levels fell to a significantly lower level in the 30 ^{o}C flushed AQIX group after 6 hours of reperfusion compared to the other groups (P = 0.012; Figure 13, Table 10). Levels of creatinine clearance were higher in this group, although this did not reach statistical significance (P = 0.610; Table 10, Figure 14). Renal blood flow was again significantly improved in the AQIX 30ºC group (P =0.036).

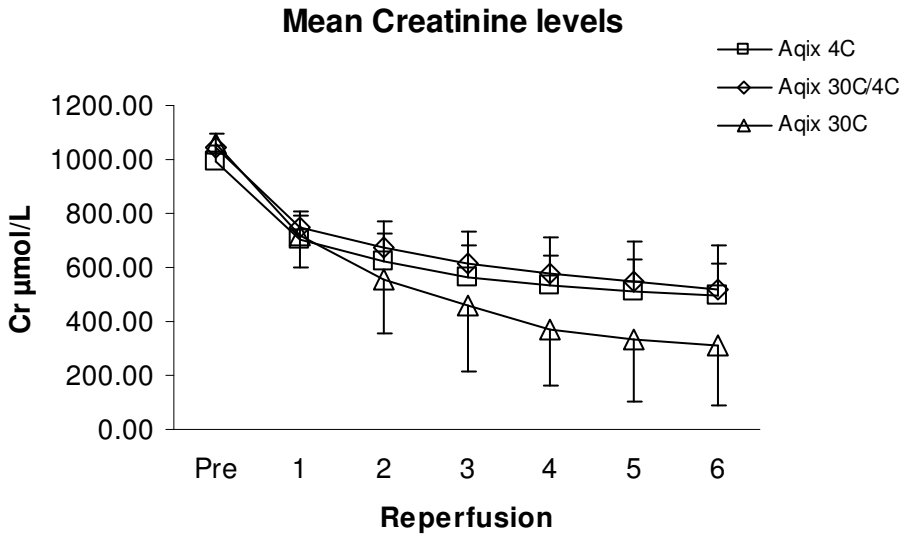


Figure 13: Serum creatinine levels; AQIX 4 ^{o}C flush, AQIX 30 ^{o}C/4 ^{o}C flush and AQIX 30^{o}C flush. Values are the mean ± SD.

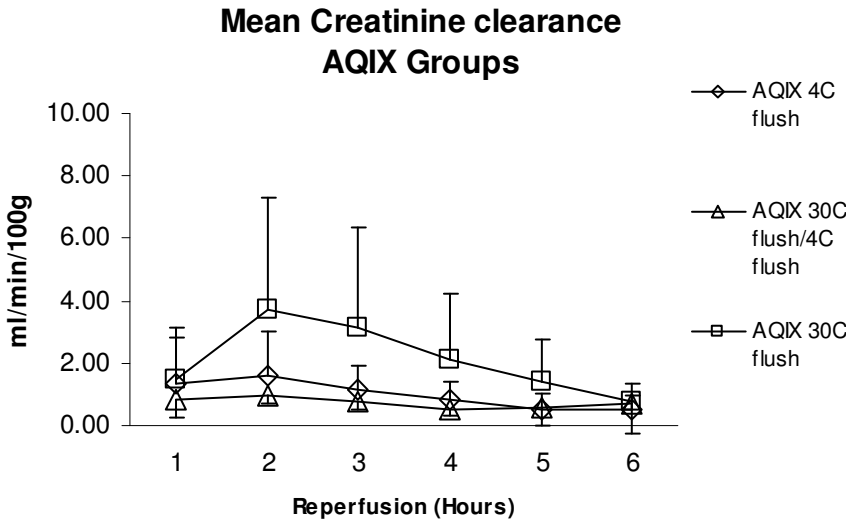


Figure 14. Mean creatinine clearance vs time for kidneys flushed with AQIX solution using 3 different temperature protocols.

	AQIX 4°C	AQIX 30 °C/4 °C	AQIX30 °C	P value
SCr (μmol/l.h)	3676 ± 896	3948 ± 328	2114 ± 1031	0.015*
CrCl (ml/min/100g.h)	5.1 ± 3.9	3.7 ± 1.6	11.6 ± 11	0.610
RBF (ml/min/100g.h)	108 ± 70	100 ± 25	172 ± 51	0.036*
Urine flow (ml/hr)	289 ± 195	297 ± 174	354 ±224	0.796

Table 10: Renal function, renal blood flow and urine output after flushing with AQIX solution using 3 different temperature protocols. Values are mean ±SD area under the curve for each parameter vs time. (Kruskal-Wallis test).

SCr serum creatinine; CrCl Creatinine clearance; RBF renal blood flow

Levels of fractional excretion of sodium were significantly lower in the AQIX 30 °C group and remained relatively stable throughout reperfusion compared to a steady increase in the levels in AQIX 4 °C and 30 °C/4 °C groups (AUC: 68.3 ± 39.4 vs 169.7 ± 102.4 and 195.7 ± 34.2%.h; respectively: P = 0.028; Figure 15). Levels of total urine output were similar in all groups (AQIX 4 °C; 289 ± 195, AQIX 30 °C/4 °C; 297 ± 174, AQIX 30ºC; 354 ± 224ml; P = 0.796).

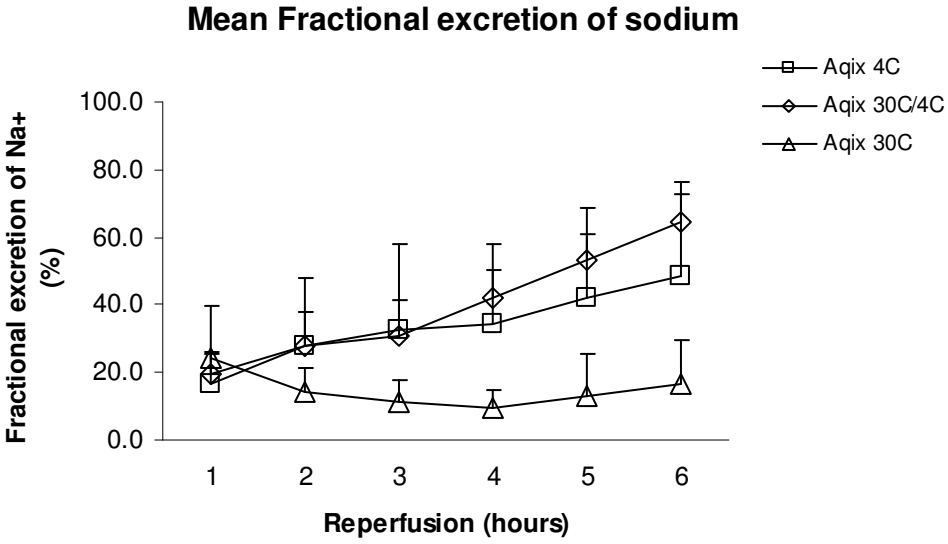


Figure 15. Fractional excretion of sodium; AQIX 4 °C flush, AQIX 30 °C/4 °C flush and AQIX 30 °C flush. Values are the mean ± SD.

Acid-Base Homeostasis

The pre-perfusion arterial pH, bicarbonate and base excess measurements were similar between the 3 AQIX groups. Acid base homeostasis deteriorated in all 3 groups, although kidneys flushed with AQIX at 30°C alone displayed the best acid-base homeostasis after 6 hours of reperfusion (Table 11, figure 16; P <0.05).

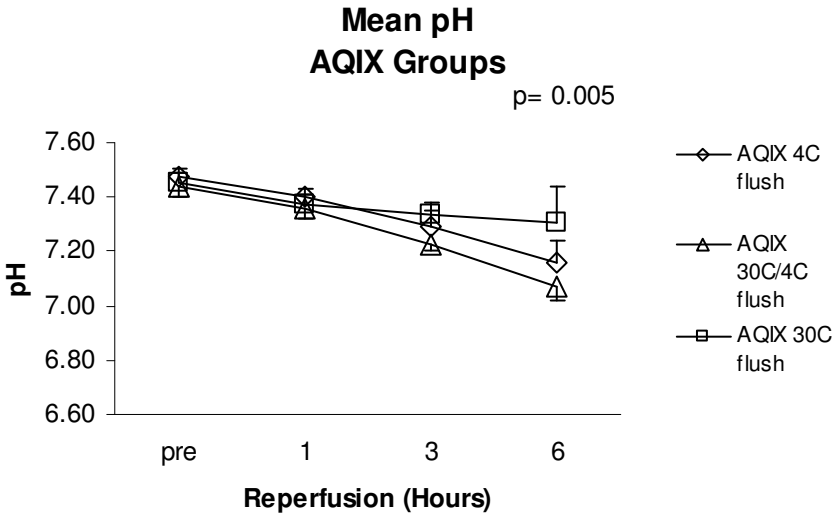


Figure 16: Blood pH during isolated organ perfusion after flushing with AQIX solution using 3 different temperature protocols.

	AQIX 4ºC		AQIX 30ºC/4ºC		AQIX 30ºC	
	Pre	6hr	Pre	6hr	Pre	6hr
pH	7.48 ± 0.62	7.16 ± 0.08	7.44 ± 0.04	7.07 ± 0.05	7.46 ± 0.03	7.30 ± 0.14*
Bicarbonate (mmol/L)	25.5 ± 3.30	12.78 ± 2.61	22.15± 1.72	10.32 ± 1.02	22.90 ± 1.98	17.82 ±5.92*
Base excess (mmol/L)	-1.52 ± 1.62	-17.23 ± 4.07	-4.17 ± 2.30	-21.17 ± 1.66	-3.04 ± 2.55	-7.98 ± 7.31*

* P < 0.05 between AQIX 30ºC and AQIX 4ºC/ AQIX 30ºC/4ºC groups

Table 11. Acid base balance Levels of pH, Bicarbonate and Base excess in the 3 AQIX groups pre and after 6 hours of reperfusion. (Kruskal-Wallis test).

Histology

There were no differences between pre and post-flush histology in the AQIX 4°C group (P = >0.05). Flushing with warm AQIX at 30°C followed by cold AQIX at 4°C significantly increased tubular dilation, epithelial flattening and glomerular shrinkage in the post-flush biopsies (P = 0.001). Flushing with AQIX at 30°C significantly increased glomerular shrinkage in post-flush biopsies (P = 0.001) (Table 13).

Cross-group comparison of the post-flush samples showed no significant differences between kidneys flushed with AQIX at 4°C and AQIX at 30°C followed by cold AQIX at 4°C (P = >0.05). There was significantly greater tubular dilation (P = 0.05) and epithelial flattening (P = 0.001) when AQIX at 30°C was compared to AQIX at 4°C, and a greater degree of glomerular shrinkage (P = 0.001) in kidneys flushed with AQIX at 30°C compared to the other two AQIX groups (Table12).

Figures 17-19. Histology of pre and post perfusion biopsies

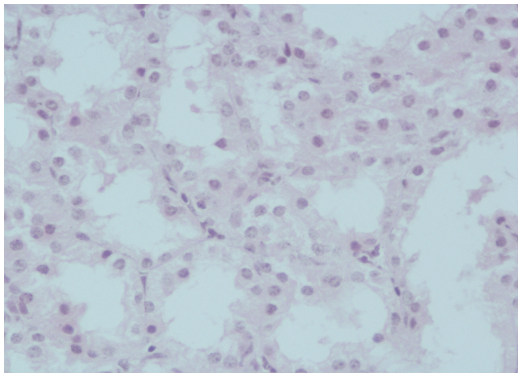


Figure 17a: Pre AQIX 4°C Flush, 4°C storage

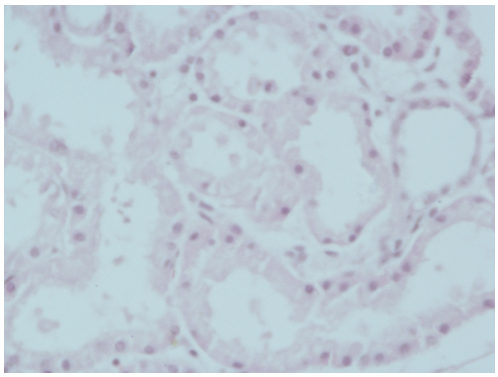


Figure 17b: 6hr AQIX 4°C Flush, 4°C storage

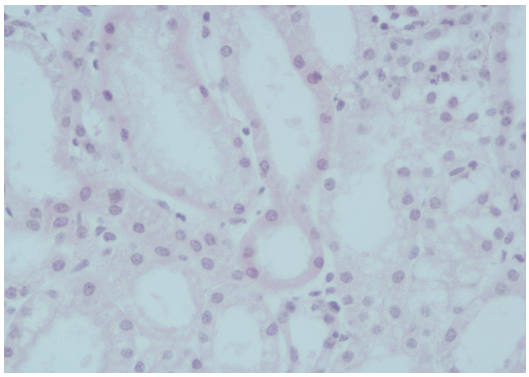


Figure 18a: Pre AQIX 30°C Flush, 4°C storage

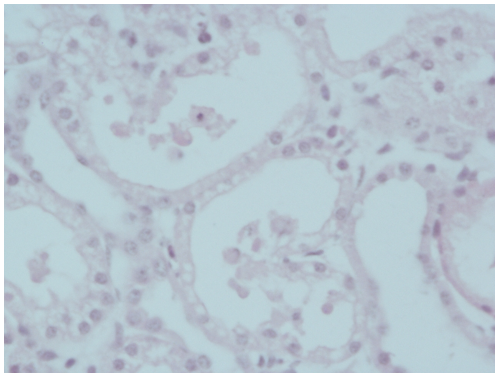


Figure 18b: 6hr AQIX 30°C Flush, 4°C storage

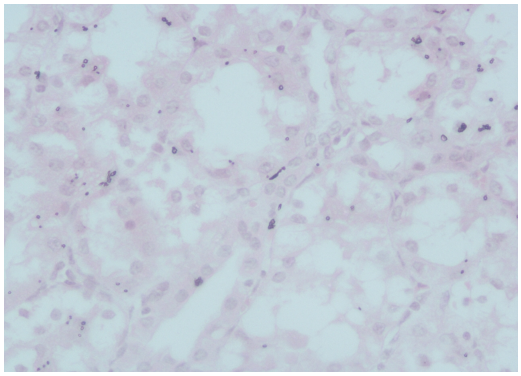


Figure 19a: Pre AQIX 30°C Flush, 4°C flush and 4°C storage

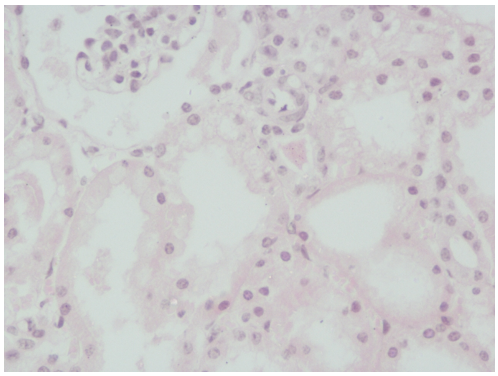


Figure 19b: 6hr AQIX 30°C Flush, 4°C flush and 4°C storage

Nikon Light-microscope x 400

Table 12. Pre and Post perfusion biopsy scores. Mean (SD).

Pre Vs Pre Significance	**A - 4° Flush**	**B - 30°/4° Flush**	**C – 30° Flush**	**P value**
Tubular dilation	1.56 (0.82)	1.17 (0.41)	0.67 (0.82)	ns
Epithelial flattening	1.08 (0.86)	0.83 (0.75)	0.33 (0.52)	ns
Epithelial shredding	1.20 (0.45)	1.50 (0.55)	0.83 (0.41)	ns
Tubular debris	0.52 (0.50)	1.00 (0)	0.33 (0.52)	0.01
Vacuolation	0.00 (0)	0.17 (0.41)	0.00 (0)	ns
Condensed tubular nuclei	0.20 (0.45)	0.33 (0.52)	0.00 (0)	ns
RBC presence	0.12 (0.27)	0.00 (0)	0.00 (0)	ns
Glomerular shrinkage	0.84 (0.48)	0.00 (0)	0.17 (0.41)	0.01
Post Vs Post Significance	A	B	C	P value
Tubular dilation	1.67 (0.52)	2.67 (0.52)	3.00 (0)	0.05
Epithelial flattening	1.17 (0.75)	2.00 (0.63)	2.67 (0.52)	0.001
Epithelial shredding	1.50 (0.55)	1.67 (0.52)	1.00 (0)	ns
Tubular debris	1.17 (0.41)	1.33 (0.52)	0.5 (0.55)	ns
Vacuolation	0.33 (0.82)	0.33 (0.52)	0.17 (0.41)	ns
Condensed tubular nuclei	1.33 (1.03)	1.00 (0.89)	0.83 (0.41)	ns
RBC presence	0.83 (0.98)	1.17 (0.98)	0.17 (0.41)	ns
Glomerular shrinkage	0.50 (0.84)	0.67 (0.82)	1.83 (0.41)	0.001

Table 13. Pre versus post perfusion biopsy scores. Mean (SD).

Pre Vs post Significance	A pre	A post	**P Value**	B Pre	B post	**P value**	C pre	C post	**P value**
Tubular dilation	1.56 (0.82)	1.67 (0.52)	**Ns**	1.17 (0.41)	2.67 (0.52)	**0.05**	0.67 (0.82)	3.00 (0)	**Ns**
Epithelial flattening	1.08 (0.86)	1.17 (0.75)	**Ns**	0.83 (0.75)	2.00 (0.63)	**0.001**	0.33 (0.52)	2.67 (0.52)	**Ns**
Epithelial shredding	1.20 (0.45)	1.50 (0.55)	**Ns**	1.50 (0.55)	1.67 (0.52)	**Ns**	0.83 (0.41)	1.00 (0)	**Ns**
Tubular debris	0.52 (0.50)	1.17 (0.41)	**Ns**	1.00 (0)	1.33 (0.52)	**Ns**	0.33 (0.52)	0.50 (0.55)	**Ns**
Vacuolation	0.00 (0)	0.33 (0.82)	**Ns**	0.17 (0.41)	0.33 (0.52)	**Ns**	0.00 (0)	0.17 (0.41)	**Ns**
Condensed tubular nuclei	0.20 (0.45)	1.33 (1.03)	**Ns**	0.33 (0.52)	1.00 (0.89)	**Ns**	0.00 (0)	0.83 (0.41)	**Ns**
RBC presence	0.12 (0.27)	0.83 (0.98)	**Ns**	0.00 (0)	1.17 (0.98)	**Ns**	0.00 (0)	0.17 (0.41)	**Ns**
Glomerular shrinkage	0.84 (0.48)	0.50 (0.84)	**Ns**	0.00 (0)	0.67 (0.82)	**0.001**	0.17 (0.41)	1.83 (0.41)	**0.001**

Discussion

This paper suggests that the optimal condition to use AQIX as a flush solution is normothermically. The normothermically flushed kidneys demonstrated superior renal function and handling of acid base balance when assessed on an isolated organ perfusion system. This superior function was found despite more damage being observed histologically on post perfusion biopsies, highlighting the discrepancy that can be found between structure and function

As a hypothermic flush solution, AQIX demonstrated a poorer level of renal viability across all functional parameters. The rationale behind a normothermic followed by hypothermic flush (Group B) was to allow a rapid flush followed by rapid cooling to minimize ischaemic damage. Interestingly, this was not reflected in the perfusion experiments, as these kidneys demonstrated significant histological damage, and had the poorest acid-base control and lowest creatinine clearance. This may be due to deleterious effects of rapid cooling as observed by other authors. Otto and colleagues [112] observed a marked difference in endothelial damage after cold flushing with Ringer's solution at 2ºC compared to minor damage using a warmer flush at 15ºC in a porcine liver transplant model with 24 h of cold storage. Winchell and Halasz [191] found that rapid initial cooling and storage of rabbit kidneys at 0ºC markedly diminished function after 24 h of cold storage. When the rate of cooling was slowed by using a warmer flush (8ºC versus 2ºC), a twofold increase in creatinine clearance was observed.

The use of a warm flush is a rational attempt to achieve more rapid clearance of blood from the microcirculation. A warm preservation solution may reduce vasoconstriction and prevent stiffening of the cell membranes in both endothelial cells and the cellular components of the blood. This may then ameliorate the no-reflow phenomenon, which is an important mechanism in ischaemia-reperfusion injury

Unlike UW solution, AQIX contains no colloidal agent which would increase viscosity and could impair clearance of the microcirculation. The inclusion of calcium in AQIX, as1.25mmol/l $CaCl_2$, is thought to be protective as calcium loss during cold storage has been associated with loss of mitochondrial function, disruption of microtubules and the calcium paradox upon reperfusion [199,200].

AQIX is capable of carrying oxygen in solution or by incorporating red blood cells during machine preservation. Initial studies by the manufacturers of AQIX have focused on isolated tissues and small animal organs in a perfusion bath system using oxygenated AQIX at normothermic temperatures. For example they observed functional viability of rat jejunum for 9 days, rat colon for 5 days and rabbit uterus for 7 days [201].

In this paper the cold and warm ischaemic times were relatively short and further experiments are required to assess the benefits of a warm flush with extended ischaemic insults. Subsequent work is planned to compare AQIX flush under normothermic conditions to hypothermic flush using the more traditional hyperosmolar citrate and University of Wisconsin solutions.

Normothermic versus Hypothermic Ex-vivo flush using a novel phosphate-free preservation solution (AQIX) in porcine kidneys.

Abstract

Background

The initial flush of an organ is important to remove any cellular components from the microcirculation before storage. The aim of this study was to assess graft function after an *ex-vivo* warm flush with a novel non-phosphate buffered preservation solution AQIX® RS-I (AQIX) compared to a traditional cold flush.

Methods

Porcine kidneys were either warm-flushed with AQIX® RS-I at 30°C, or cold-flushed at 4°C with University of Wisconsin solution (UW) or hyperosmolar citrate (HOC) preservation solution at a pressure of 100mmHg (n= 6). Renal function was measured *ex-vivo* by perfusing the organs with autologous blood at 37°C on an isolated organ perfusion system.

Results

The AQIX group flushed significantly quicker than the cold flushed groups (22±1.8 vs UW 4.9±1.6 vs HOC 10±1.6 ml/min/100g;P=0.001), gained less weight (19±2.9 vs UW 21±7.7 vs HOC 30±3.4 %;P=0.02), and had superior acid-base homeostasis. Functional results, histological analysis and ADP: ATP levels were comparable between the groups.

Conclusion

Flushing kidneys with AQIX at 30°C cleared the renal microcirculation of blood more rapidly without any detrimental effects when compared to traditional cold flushing with UW or HOC at 4°C. Warm initial flushing has potential to be developed as part of normothermic renal preservation techniques.

Introduction

Hypothermic static storage of organs for transplantation yields good functional results and is a simple and cost-effective technique. As such it continues to be the preferred method of organ preservation in the UK [1, 197]. The initial flush of an organ is important to remove blood, any cellular components that may have clumped, and to perfuse the microcirculation, and hence the interstitium, with a solution that is designed to impair cellular swelling. The various preservation solutions currently in use are flushed cold, the aim being to slow metabolism down quickly in the face of a potentially damaging anaerobic environment. In keeping with renewed interest in normothermic preservation [11,161,202], some authors have assessed the role of a warm pre-flush and have reported beneficial results. For example, reduced hepato-cellular damage has been demonstrated in rat livers [105], and improved microperfusion in liver grafts by combining a warm flush with streptokinase [116].

The use of a warm flush is a rational attempt to achieve more rapid clearance of blood from the microcirculation. Warm preservation solution may reduce vasoconstriction and prevent stiffening of the cell membranes in both endothelial cells and the cellular components of the blood. This may then ameliorate the no-reflow phenomenon, which is an important mechanism in ischaemia-reperfusion injury.

We have previously published work using a novel normothermic preservation solution, AQIX, showing that it has the capability to maintain acid-base balance after warm flush and 2 hours of warm ischaemia [198].

AQIX has been designed as a universal normothermic organ preservation solution that would reflect physiological ionic concentrations, osmolarity and ion conductivity, thereby maintaining the ionized status of the cell membrane and the function of enzymatic and receptor moieties. The osmolarity of AQIX is similar to that of serum at 286 mOsmoles/l, and its ionic concentrations have been chosen to maintain it as an isosmotic solution. The ionic conductivity is also comparable to human serum, namely, 12.6 mS cm-1.

AQIX is a non-phosphate buffered solution that utilizes a combination of the bicarbonate system ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$) and Good's BES as its buffer which, because of its ideal pK_a value allows the pH to be maintained within a range of 7.2 – 7.46 over a temperature range of 4 – 37 ºC, thereby facilitating the transition from hypothermic to normothermic and vice versa.. A comparison of its constituents with traditional solutions is displayed in Table 14.

AQIX is capable of carrying oxygen in solution or by incorporating red blood cells during machine preservation. Initial studies using AQIX were focused on isolated, small animal tissues and organs preserved and perfused in an organ bath system using oxygenated AQIX at 15 – 37 ºC. Such studies demonstrated functional viability of rat jejunum for 9 days, rat colon for 5 days and rabbit uterus for 7 days [201]. The aim of the present study was to compare the effectiveness of initial perfusion with either warm AQIX solution or traditional cold perfusion using cold hyperosmolar citrate solution (HOC) or University of Wisconsin solution (UW).

Components	AQIX	HOC	UW
Sodium	110	84	25
Potassium	5.0	80	100
Citrate		54	
Magnesium	0.45	41	5
Sulphate		41	5
Sodium bicarbonate	25.0		
BES	5.0		
D-glucose	10.0		
Calcium chloride	1.25		
Glycerol	0.11		
L – Glutamate	0.30		
L – Glutamine	0.40		
L – Aspartate	0.02		
L – Carnitine	0.05		
Choline Chloride	0.01		
TPP (cocarboxylase)	40.0 nmol/l		
Insulin	28.0 mIU		
Potassium citrate		8.6g /l	
Sodium citrate		8.2g/l	
Mannitol		33.8g/l	
Magnesium sulphate		10.0g/l	
Poly (0-2 hydoxyethyl starch)			50 g/l
Lactobionic acid			105
Potassium hydroxide			100
Sodium hydroxide			27
Adenosine			5
Allopurinol			1
Potassium Dihydrogen phosphate			25
Magnesium sulphate			5
Raffinose			30
Glutathione			3
Ph	7.27	7.1	7.4
Osmolarity	286 mOsmol/l	486 mOsmol/l	320mOsmol/l

Table 14: Constituents of Preservation solutions. *All units in mmol/l unless specified

Methods

Kidney Recovery

Large white pigs (60–70kg) were sacrificed by electrocution followed by exsanguination and approximately 1 litre of blood was collected into a sterile receiver containing 25,000 units of heparin (Multiparin; CP Pharmaceuticals, Wrexham, UK). The kidneys were surgically removed with minimal warm ischaemic time and immediately flushed with 400 ml of preservation solution at a hydrostatic pressure of 100 cmH_20. The time taken for the total of 400 ml of solution to run into the kidney (the flush time) was recorded to the nearest minute along with the exact warm ischaemic interval. Perfusate flow rates were calculated and expressed as ml/min/100g of kidney. All kidneys were then preserved by cold storage on ice for 2 hours to allow transfer to the laboratory.

Three different preservation techniques were compared (n=6 kidneys per group): AQIX flush at 30°C; HOC flush at 4°C; UW flush at 4°C. For warm flushing, the AQIX solution was pre-warmed in a temperature regulated water-bath and maintained at 30°C during transport in an insulated organ retrieval carrier; temperature was monitored with a temperature probe.

Isolated Organ Perfusion

The Isolated Organ Perfusion system has been described in detail previously [22] but in brief consists of a centrifugal blood pump, a heat exchanger, a venous

reservoir and a membrane oxygenator. The circuit hardware includes a speed controller, a flow transducer, a pressure transducer and a temperature probe. Two infusion pumps and a urinometer were incorporated into the system.

The circuit was primed with 500 ml Ringer's solution containing mannitol 10mg (Baxter, Healthcare, Norfolk, UK), dexamethasone 10mg (Organon Labs Ltd, Cambridge, UK), cefuroxime 750mg (Britannia Pharmaceuticals Ltd, Surrey, UK) and 15ml 8.4% sodium bicarbonate (Fresenius Kabi, Warrington, UK). 500ml of heparinized whole blood was added to the circuit after priming and allowed to circulate at a temperature of 37ºC.

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

Experimental Protocol

Pairs of kidneys retrieved from the same animal were flushed by the same technique and then simultaneously perfused with autologous blood using two separate isolated organ perfusion circuits. The renal artery, vein and ureter were cannulated and any residual preservation solution was removed by flushing the kidneys with

90ml of plasma substitute (Gelofusine; B. Braun, Sheffield, UK) at 4 ºC. The kidneys were weighed immediately pre and post perfusion using a balance accurate to 1g; the weight change was recorded.

Kidneys (n=6 per group) were perfused for 6 hours at a mean arterial pressure of 55 mmHg. Renal blood flow (RBF), and mean arterial pressure (MAP) were continuously recorded and intra-renal vascular resistance calculated (MAP/RBF). The addition of 1000 µmol creatinine to the circuit enabled an accurate measurement of renal function to be made. Serum and urine samples were taken hourly for biochemical analysis and whole blood for haematology. Creatinine clearance (U_{cr} x U volume / P_{cr}) and fractional excretion of sodium [(urinary sodium x urine volume) / (glomerular filtration rate x plasma sodium) x 100] were calculated and serum creatinine levels recorded hourly. Blood gases were measured for the calculation of oxygen consumption in ml/min/g [(PaO_2 art – PaO_2 ven) x flow rate/weight] and acid-base balance. Arterial pH was measured prior to perfusing the kidneys, then at 1, 3 and 6 hourly intervals for assessment of acid-base homeostasis

Needle core biopsies were taken pre- and 6 hours post-perfusion, fixed in 4% formalin, dehydrated and embedded in paraffin wax. Sections were cut at 4µm then stained with haematoxylin and eosin for evaluation using light microscopy. Eight morphological parameters (tubular dilation, epithelial flattening, epithelial shredding, tubular debris, vaculolation, red blood cell presence, Condensed tubular nuclei and glomerular shrinkage) were each scored over 5 fields using a semi-quantitative scale (0 normal; 1 mild; 2 moderate; 3 severe).

Biopsies were also used to assess ADP: ATP ratios as a measurement of cellular viability both pre and post perfusion. This was performed using an adenylate nucleotide ratio assay kit (Cambrex Bio Science. Belgium), whose detection is based

on the bioluminescent measurement of ATP using the enzyme luciferase. This enzyme catalyses the formation of light from ATP and was measured using a luminometer.

Needle core biopsies were attached to cork blocks using tissue-tek medium, immediately submersed in liquid nitrogen and transferred to cryotubes for storage at -80°C. Six sections of 10µm thickness were cut from each core using a cryostat and placed in an Eppendorf tube pre-cooled to -20°C. 600µl of nucleotide releasing reagent was added to the sections and the sample vortexed and stored at 4°C. A 180µl aliquot of this sample was brought to room temperature (the remainder of the sample was kept at 4°C for replicate analysis). 20µl of nucleotide monitoring reagent was added to the aliquot and the sample placed on 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 then removed, 20µl of ADP converting reagent added and the sample placed back into the luminometer. A final reading (C) was taken after 5 minutes.

Statistical analysis

Values are presented as mean ± SD. Continuous variables, such as serum creatinine were plotted against time and the area under the curve for individual perfusion experiments calculated using Microsoft ®Excel software (Reading, UK). Mean areas under the curve were then compared using the Kruskal-Wallis test and post testing was performed with Dunn's test for serum Creatinine, Creatinine clearance, renal blood flow and urine output. The Kruskal-Wallis test was also used for comparison of mean pressure, temperature, pH, oxygen consumption, weight gain and intra-renal

vascular resistance (mean values presented). A P value ≤ 0.05 was taken as statistically significant.

RESULTS

The mean (SD) warm ischaemic time during retrieval for all kidneys was 6.4 ± 1.0 minutes and there were no significant differences between groups (P = 0.335). Warm flushing with AQIX at 30ºC achieved higher perfusate flow rates than HOC or UW at 4^{o}C (22.0 ±1.8 vs. 10.0 ± 1.6 vs. 4.9 ±1.6 ml/min/100g respectively, P= 0.001). There were no significant differences in renal blood flow, intra-renal vascular resistance or oxygen consumption between the three groups (Table 15). Weight gain post-perfusion was greatest in the UW group and this was significantly higher than the warm AQIX group but not the HOC group (P = 0.02).

Parameters	HOC	UW	AQIX (30°C)	P value
WIT (mins)	7.1 ± 0.97	6.2 ± 1.47	6.2 ± 0.41	0.164
Flush (ml/min/100g)	10 ± 1.6	4.9 ± 1.6	22 ±1.8	0.001*
Arterial Pressure (mmHg)	54 ± 0.71	59 ± 2.6	57 ± 3.9	0.008*
Temperature (°C)	37.3 ± 0.4	37 ± 0.56	37.3 ± 0.34	0.200
Renal Blood Flow (ml/min/100g)	50 ± 10	56 ± 22	48.4 ± 19	0.77
Intra-renal Vascular Resistance (mmHg/ ml/min/100g)	0.52 ± 0.1	0.8 ± 0.43	0.5 ± 0.2	0.52
O_2 Consumption (ml/min/g)	31 ± 6.3	33.7 ± 15.1	25.3 ± 12.8	0.40
Weight Gain (%)	21 ± 7.7	30 ± 3.4	19 ± 2.9	0.02
pH	7.2 ± 0.1	7.2 ± 0.12	7.3 ± 0.1	0.09
Bicarbonate (mmol/l)	14.6 ± 2.9	15.3 ± 4.3	17.8 ± 5.9	0.70

Table 15: Comparison of flush rate and warm ischaemic time following initial flushing of porcine kidneys with HOC, UW or AQIX solution, and comparison of perfusion parameters after 6 hours normothermic perfusion on the IOPS (mean ± SD).

Acid Base Homeostasis

The arterial pH fell in all 3 groups over the 6 hour perfusion period with the steepest decline observed in the HOC group (pre-perfusion pH 7.49 ± 0.1 vs. 7.21 ± 0.1 after 6 hours perfusion; Figure 20). The warm AQIX group demonstrated superior acid-base homeostasis, with near-normal arterial pH at the end of the perfusion period (7.3 ± 0.1). Bicarbonate levels were also better in the warm AQIX group (falling from a mean of 22.9 mmol/l pre-perfusion to 17.8 mmol/l after 6 hours vs 25.4 to 14.6 mmol/l for HOC and 25.6 to 15.3 mmol/l for UW; Table 15).

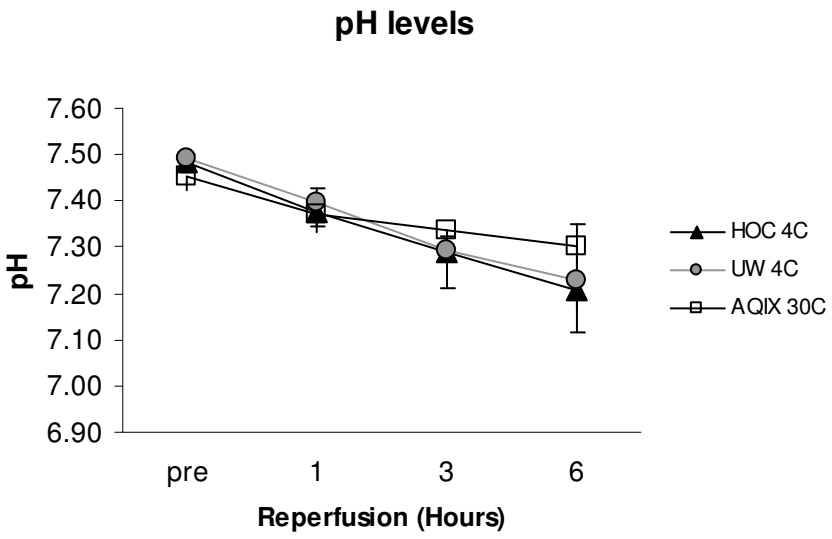


Figure 20: Blood pH during isolated organ perfusion after flushing with HOC, UW or warm (30°C) AQIX solution.

Functional parameters

Renal function during the 6 hour perfusion period was comparable between the three study groups, with no significant differences in serum creatinine levels, creatinine clearance, urine output or renal blood flow (Table 16).

	HOC	UW	AQIX (30° C)	P value
SCr (μmol/l.h)	2404 ± 596	2887 ± 865	2333 ±1066	0.50
CrCl (ml/min/100g.h)	14.4 ± 12	17.2 ± 9.8	11.6 ± 11	0.50
RBF (ml/min/100g.h)	226 ± 55	215 ± 69	172 ± 51	0.26
Urine flow (ml/hr)	536 ± 221	410 ± 189	309 ±189	0.33

Table 16. Renal function, renal blood flow and urine output after flushing with HOC, UW or warm (30°C) AQIX solution. Values are mean ±SD area under the curve for each parameter vs time. (Kruskal -Wallis test).

SCr serum creatinine; CrCl Creatinine clearance; RBF renal blood flow

ATP levels

The ADP: ATP ratio levels were highest in the pre-perfusion biopsies, reflecting the ischaemic damage sustained during the cold storage period. Levels were numerically highest in the UW group but there were no significant differences between the three groups (P = 0.55; Figure 21). The ADP: ATP ratio decreased after 6 hours of perfusion in all 3 groups, showing recovery of cellular function. The HOC group had a lower post-perfusion ADP: ATP ratio than the UW group (P <0.05), but there were no statistically significant differences between the warm AQIX group and either HOC or UW. The pre- to post-perfusion change in ADP: ATP ratio was numerically higher in the HOC group but did not reach statistical significance (P = 0.78).

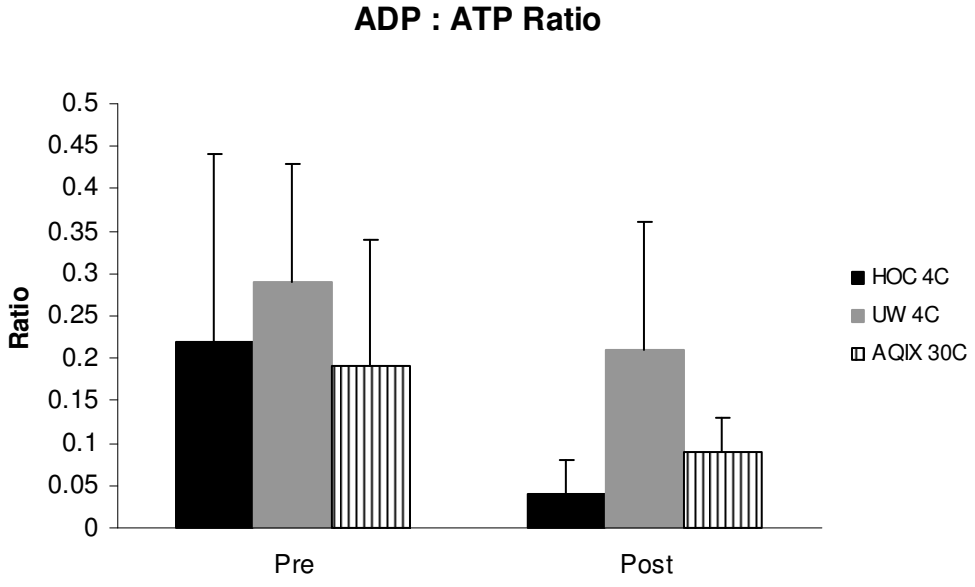


Figure 21: Renal cortical ADP: ATP Ratios pre- and post- 6 hours of isolated *ex-vivo* renal perfusion (mean ± SEM)

Histology

The HOC-flushed group showed most evidence of damage in pre-perfusion biopsies with significantly more tubular dilatation, epithelial flattening and tubular debris than the warm AQIX and the cold UW groups ($P < 0.05$; Table 17). Mild glomerular shrinkage was also evident with HOC. The warm AQIX group demonstrated a mild degree of tubular dilatation and epithelial shredding. The only pre-perfusion histological changes seen in the UW group was mild epithelial shredding.

Post-perfusion, the only significant difference between the groups was the presence of more glomerular shrinkage in the warm AQIX group compared to both the HOC and UW groups ($P = 0.05$; Figures 22-24).

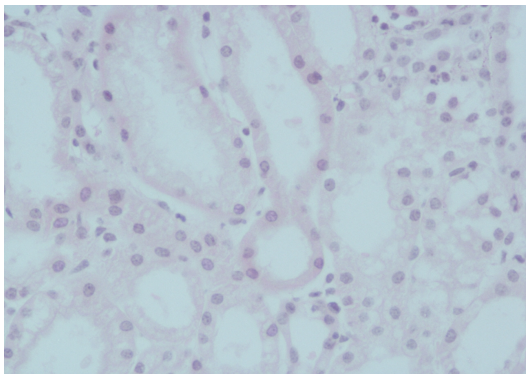


Figure 22a: Pre AQIX 30°C Flush, 4°C storage

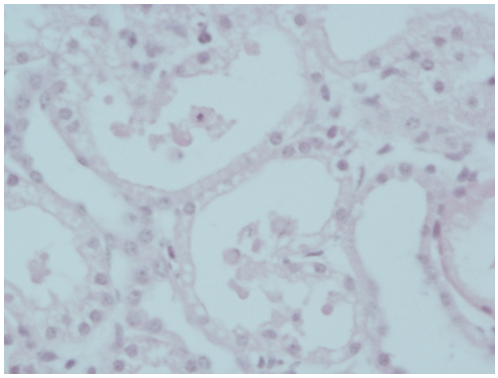


Figure 22b: 6hr AQIX 30°C Flush, 4°C storage

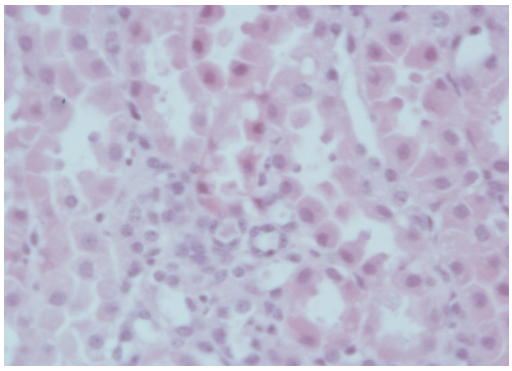


Figure 23a: Pre HOC 4°C flush /storage

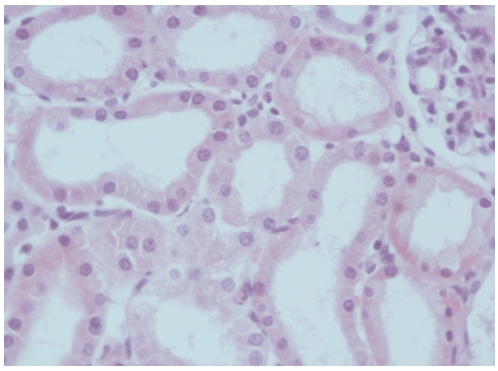


Figure 23b: 6hr HOC 4°C flush /storage

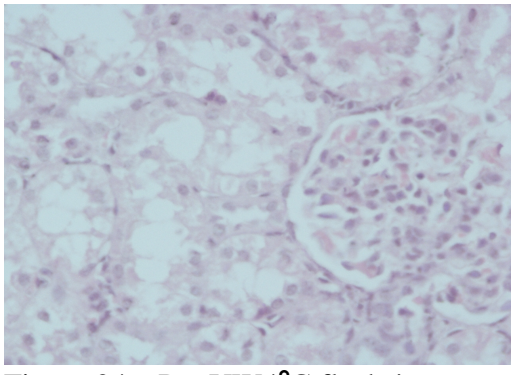


Figure 24a: Pre UW4°C flush /storage

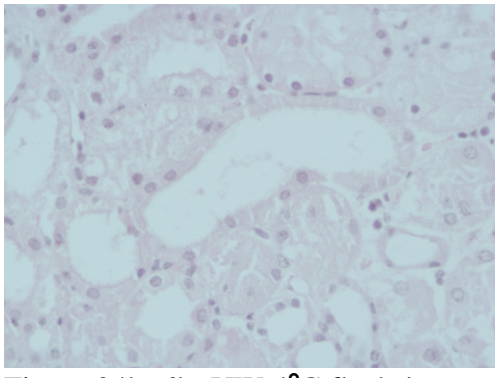


Figure 24b: 6hr UW 4°C flush /storage

Nikon Light-microscope x 400

Pre Vs Pre Significance	**HOC**	**UW**	**AQIX**	**P Value**
Tubular dilation	1.83 ± 0.41	0.17 ± 0.41	0.67 ± 0.82	0.05
Epithelial flattening	1.50 ± 0.84	0.33 ± 0.52	0.33 ± 0.52	0.001
Epithelial shredding	1.00 ± 0	1.00 ± 0	0.83 ± 0.41	Ns
Tubular debris	1.00 ± 0	0.33 ± 0.52	0.33 ± 0.52	0.05
Vacuolation	0.00 ± 0	0.00 ± 0	0.00 ± 0	Ns
Condensed tubular nuclei	0.33 ± 0.52	0.33 ± 0.52	0.00 ± 0	Ns
RBC presence	0.00 ± 0	0.17 ± 0.41	0.00 ± 0	Ns
Glomerular shrinkage	1.17 ± 0.41	0.00 ± 0	0.33 ± 0.82	Ns
Post Vs Post Significance	**HOC**	**UW**	**AQIX**	**P Value**
Tubular dilation	2.00 ± 0	2.67 ± 0.52	3.00 ± 0	Ns
Epithelial flattening	1.83 ± 0.75	1.50 ± 0.55	2.67 ± 0.52	Ns
Epithelial shredding	1.00 ± 0	1.00 ± 0	1.00 ± 0	Ns
Tubular debris	1.00 ± 0	0.67 ± 0.52	0.50 ± 0.55	Ns
Vacuolation	0.33 ± 0.52	0.17 ± 0.41	0.17 ± 0.41	Ns
Condensed tubular nuclei	0.67 ± 0.82	1.00 ± 0.63	0.83 ± 0.41	Ns
RBC presence	0.50 ± 0.55	0.50 ± 0.55	0.17 ± 0.41	Ns
Glomerular shrinkage	1.00 ± 0	0.33 ± 0.52	2.00 ± 0.63	0.05

Table 17. Pre-perfusion and Post perfusion biopsies (mean ± SD)

Score 0 = Normal, 1= Mild damage, 2= moderate damage, 3= severe damage

Discussion

This study shows that initial flushing with AQIX perfusate at 30 °C leads to a more rapid clearance of blood from porcine kidneys when compared to HOC or UW solutions at the more conventional temperature of 4°C. This improved rate of initial organ perfusion did not lead to an improvement in post-ischaemic renal function however, the additional period of normothermia with the use of AQIX did not have any deleterious effect on renal function. Histologically there were no significant differences between the groups after 6 hours of normothermic reperfusion. Furthermore, there were no differences in post-perfusion ADP: ATP ratios between the 30 °C AQIX group and the two cold flush groups.

Porcine kidneys that were of similar weight to adult human kidneys were used in this study, as they are known to have anatomical and physiological characteristics that more closely resemble the human situation than small animal models [203]. The isolated organ perfusion system was designed using state of the art cardio-pulmonary bypass technology and was successful in yielding reproducible results within each of the three study groups. Whilst this appears to be a valid model for studying post-ischaemic renal injury the system does not reproduce normal physiological conditions. Kidneys were perfused by non-pulsatile flow at a relatively low mean perfusion pressure. Another limitation of the study is that the kidneys were subjected to quite short periods of warm and cold ischaemia prior to reperfusion with blood. These ischaemic times are clinically relevant as they are similar to those to which human live donor kidneys are exposed, but future studies should include longer cold ischaemic times, which reflect the clinical deceased donor transplant situation.

Extended warm times could also be added to the model in order to study the effects of initial warm flushing in deceased donor kidneys.

AQIX was designed as a universal organ preservation and perfusate solution that would reflect physiological serum ionic concentrations, osmolarity and ion conductivity, thereby maintaining the ionized status of the cell membrane and the function of enzymatic and receptor moieties under *ex vivo* conditions. Acid-Base homeostasis is a key function of the kidney that is necessary for the maintenance of optimum enzyme function. In this study, the HOC and UW flushed groups developed profound acidosis in comparison with the warm-flushed AQIX group which maintained an almost normal pH throughout the 6- hour perfusion period. AQIX utilizes the NaHCO3/pCO2 buffer system in combination with Good's buffer BES rather than the traditional phosphate-buffered solutions which are known to inhibit glycolysis and oxidative phosphorylation [204]. Inorganic phosphate has also been associated with the release of reactive oxygen species [205]. BES maintains a stable pH and has been shown to give superior metabolic effects in hypothermic preservation of porcine hearts with higher ATP levels, ATP: ADP ratios, and conservation of phosphofructokinase compared to other buffering agents when included in a modified UW solution [206].

The AQIX group displayed a significantly lower percentage weight gain compared to the UW group. Weight gain is an important index of the cellular oedema, which develops during anaerobic cold storage and leads to functional impairment. The degree of cellular swelling is related to the length of storage and the composition of the preservation solution used [207,208,209] and relationships between cell swelling, histological changes and cell death have also been observed [210, 211].

In metabolically active cells, ATP levels are maintained constantly, but ischaemic cell death is associated with severe ATP depletion and the ratio of ADP: ATP can therefore be used as an indicator of cell viability. ATP depletion is also important as the breakdown products of its degradation, such as hypoxanthine, result in free radical production and subsequent reperfusion injury [212]. Despite reducing cellular metabolism, hypothermic organ preservation still results in depletion of ATP levels and a corresponding increase in ADP levels. This was apparent in our study where the ADP: ATP ratios were relatively high after only two hours of cold storage. Following normothermic reperfusion, this ratio decreased in all three groups suggesting good recovery of cellular function. This effect was more marked in the HOC and AQIX groups compared to UW. Some authors have assessed mechanisms of reducing ATP depletion by the use of a period of re-warming during storage [213], or oxygen persufflation [214]. Others have modified the preservation solution with substrates that promote ATP regeneration such as inorganic phosphate [215] and histidine-lactobionate-raffinose-based solution [216]. It is clear from the results presented here that a period of normothermic machine preservation using autologous blood has potential for the resuscitation of high energy phosphate metabolites in ischaemically damaged kidneys [165].

The use of an initial warm flush prior to cold preservation has previously shown improved survival of rat livers flushed with warm UW compared to cold UW, possibly because warm UW has a significantly lower viscosity [114]. Other authors have demonstrated improved function by combining a warm pre-flush with streptokinase in rat donation after cardiac death (DCD) donor livers and kidneys [117, 118] rather than warm pre-flush alone.

The three strategies used in this study all have potential advantages and disadvantages. HOC solution has a low viscosity, which would facilitate flushing, but may be less cytoprotective than UW solution. UW is much more viscous and so flows more slowly, but is the gold-standard hypothermic preservation solution for a number of organs. Warm AQIX flushes kidneys at the quickest rate as a result of its low viscosity and its use at 30°C, but this temperature increases the first warm ischaemic time. The comparable post-ischaemic renal function in the three groups suggests that these positive and negative influences have balanced each other out to some extent. Further studies are needed to assess the influence of prolonged cold ischaemic storage to mimic the clinical situation.

The findings of this study suggest that an initial warm flush with AQIX solution clears the renal microcirculation more effectively than cold flushing with conventional preservation solutions. An initial warm flush is a new paradigm in organ preservation but further work is required before this technique can be considered for introduction to clinical practice.

Static normothermic preservation of porcine kidneys using a novel non -phosphate buffered preservation solution.

Objective

Throughout the UK, hypothermic static storage of organs prior to reperfusion and transplantation is the preferred method of preservation. The rationale behind this is the simplicity, cost-effectiveness, portability and proven results using such methods[1,11]. Whilst the cold environment slows metabolism, anaerobic metabolism continues to occur, albeit at a slower rate, and the end-products contribute to the generation of oxygen free radicals and hence ischaemia-reperfusion injury. It is known that cellular oedema and functional impairment develop during anaerobic cold storage, the extent of which is related to the length of storage and the composition of the preservation solution used [105,116,207].Cold storage does not allow assessment of viability markers as it renders metabolic studies difficult to interpret. Thus some marginal organs may be discarded unnecessarily, or transplanted and have primary non-function.

The expanding mismatch between supply and demand for renal allografts has prompted development of preservation techniques to try and help expand the donor pool and improve allograft function. Normothermic preservation is not a new concept but has seen a recent revival in renewed interest [208,209,161] The potential beneficial roles include maintaining an organ at physiological conditions thus avoiding the harmful effects of an anaerobic environment, viability assessment allowing use of marginal organs and possible ex-vivo manipulation.

The aim of this study was to assess the viability and function of porcine kidneys under normothermic conditions using a novel non-phosphate buffered normothermic preservation solution AQIX®RS-I.

Kidney Retrieval

Large white pigs (60–70kg) were sacrificed by electrocution followed by exsanguination and approximately 1 litre of blood was collected into a sterile receiver containing 25,000 units of heparin (Multiparin; CP Pharmaceuticals, Wrexham, UK). The kidneys were surgically removed and warm flushed at 30°C with 250 mls of AQIX®RS-I at a hydrostatic pressure of 100 cmH_20 after 5-10 minutes warm ischaemic time. Flush times were measured using a stopwatch and both warm ischaemic time and flush time recorded. Perfusate flow rates were calculated and expressed as ml/min/100g of kidney. The kidneys were then stored statically in AQIX solution either on ice at 4°C or at 30°C for 2 hours (n= 6 per group) during transportation back to the laboratory. The AQIX solution was pre-warmed in a temperature regulated water-bath prior to flushing, and maintained at 30°C during transport in an insulated organ retrieval carrier with warmed solution bags, the temperature monitored with a temperature probe.

Experimental Protocol

The renal artery, vein and ureter were dissected and cannulated. The kidneys were weighed using a balance accurate to 1g, both immediately pre and post perfusion and weight change recorded. Kidneys were then perfused with autologous blood at 37°C–39°C, on an isolated organ perfusion system for 6 hours as an *ex-vivo* method of transplantation. Creatinine was added to the system to give an initial circulating serum concentration of 1000µmol/l thus allowing an accurate measurement of renal function to be made.

The kidneys were perfused at a set mean arterial pressure, with renal blood flow (RBF), and mean arterial pressure (MAP) being continuously recorded and intra-renal vascular resistance calculated (MAP/RBF). Serum and urine samples were taken hourly for biochemical analysis and whole blood for haematology. Creatinine clearance (U_{cr} x U volume / P_{cr}) and fractional excretion of sodium (U_t x U flow) / (GFR x P_t) x 100 were calculated and serum creatinine levels recorded hourly. Blood gases were measured for the calculation of oxygen consumption in ml/min/g (PaO_2 art – PaO_2 ven) x flow rate/weight) and acid-base homeostasis measured. Statistical analysis was carried out using the Mann-Whitney test with a P value of <0.05 taken as significant.

Biopsies were used to assess ADP: ATP ratios as a measurement of cellular viability both pre and post perfusion. This was performed using an adenylate nucleotide ratio assay kit (Cambrex Bio Science. Belgium), whose detection is based on the bioluminescent measurement of ATP using the enzyme luciferase. This enzyme catalyses the formation of light from ATP and was measured using a luminometer.

Needle core biopsies were attached to cork blocks using tissue-tek medium, immediately submersed in liquid nitrogen and transferred to cryotubes for storage at -80°C. Six sections of 10µm thickness were cut from each core using a cryostat and placed in an Eppendorf tube pre-cooled to -20°C. 600µl of nucleotide releasing reagent was added to the sections and the sample vortexed and stored at 4°C. A 180µl aliquot of this sample was brought to room temperature (the remainder of the sample was kept at 4°C for replicate analysis). 20µl of nucleotide monitoring reagent was added to the aliquot and the sample placed on 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 then removed, 20µl of ADP converting reagent added and the sample placed back into the luminometer. A final reading (C) was taken after 5 minutes.

Isolated Organ Perfusion

The IOPS was designed using commercially available clinical grade cardio-pulmonary technology (Medtronic, Watford, UK) and consisted of a centrifugal blood pump (550 Bio-pump), a heat exchanger (Grant, GD120, Cambridge, UK), a 5L venous reservoir (Medtronic) and a minimax plus membrane oxygenator (Medtronic). The circuit hardware included a speed controller, a TX50P flow transducer, a pressure transducer and a temperature probe (Cole-Parmer, London, UK). Two PC- 2 Gemini infusion pumps (Alaris, Basingstoke, UK) and a urinometer (Bard, Crawley, UK) were incorporated into the system.

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

and 12ml 8.4% sodium bicarbonate (Fresenius Kabi, Warrington, UK). 500ml of heparinized whole blood was added to the circuit after priming and allowed to circulate at a temperature of 37ºC.

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

Results

The warm flushed/hypothermically stored group demonstrated overall superior renal function, with significantly better oxygen consumption (p=0.0015), renal blood flow (p=0.0043), percentage creatinine fall (p=0.0043) (Figures 25), and lower renal vascular resistance (p=0.015) after 6 hours perfusion (Table 18). Total urine output was greatest in the cold stored group (692 mls ± 230 vs 257 mls ± 118, p=0.0043), and the percentage weight gain was lower in this group (12% ± 9 vs 30.3% ± 9, p=0.017).

However there was no statistical significant difference when comparing acid-base balance (pH of 7.37 cold stored vs 7.3 warm stored p=0.24) (Figure 26) after 6 hours of perfusion. Furthermore renal function was still evident at 6 hours perfusion

despite a warm time of over 2 hours. Whilst not reaching significance, both groups displayed improvement during the 6 hour perfusion period with increasing oxygen consumption (37.3 ± 9.6 ml/min/g at 1 hour, 47.3 ± 12.1 ml/min/g at 6 hours, p=0.23 for kidneys at 4 ºC cold storage, and 23.8 ± 5.2 ml/min/g at 1 hour, 28.7 ± 6.5 ml/min/g at 6 hours, p=0.34 for 30 ºC warm storage), renal blood flow (63.6 ± 15.1 ml/min/100g at 1 hour, 79.3 ± 18 ml/min/100g at 6 hours p=0.26 for 4 ºC cold storage, and 40.3 ± 7.3 at 1 hour, 48 ± 11.3 at 6 hours, p=0.47 at 30 ºC storage) and reduced renal vascular resistance (0.19 ± 0.05 mmHg/ml/min/100g at 1 hour, 0.2 ± 0.04 mmHg/ml/min/100g at 6 hours, p=0.57 at 4 ºC storage, 0.40 ± 0.17 mmHg/ml/min/100g at 1 hour, 0.34 ± 0.16 mmHg/ml/min/100g at 6hours, p= 0.9 at 30 ºC storage).

	AQIX 4ºC Storage		AQIX 30ºC Storage	
Functional parameters	**1 Hour**	**6 Hours**	**1 Hour**	**6 Hours**
pH	7.4 ± 0.03	7.37 ± 0.15	7.37 ±0.03	7.3 ± 0.09
Bicarbonate mmol/L	22.7 ± 1.6	21.8 ± 6.8	20 ±1.1	17.6 ± 4.2
O_2 consumption ml/min/g	37.3 ± 9.6	47.3 ± 12.1	23.8 ± 5.2	28.7 ± 6.5
% Creatinine fall	52 ± 11.1	84 ± 10	33 ± 4.3	58 ± 7.9
GFR ml/min/100g	6 ± 4.7	1.1 ± 0.9	0.9 ± 0.7	0.5 ± 0.29
RBF ml/min/100g	63.6 ± 15.1	79.3 ± 179	40.3 ± 7.3	48 ± 11.3
RVR mmHg/ml/min/100g	0.19 ± 0.05	0.2 ± 0.04	0.40 ± 0.17	0.34 ± 0.16

Table 18. Functional results and acid-base control during 6 hours perfusion after flushing with warm (30°C) AQIX solution followed by cold (4°C) or warm (30°C) storage (n=6 per group).

Values are mean ± SD.

GFR glomerular filtration rate; RBF renal blood flow; RVR renal vascular resistance

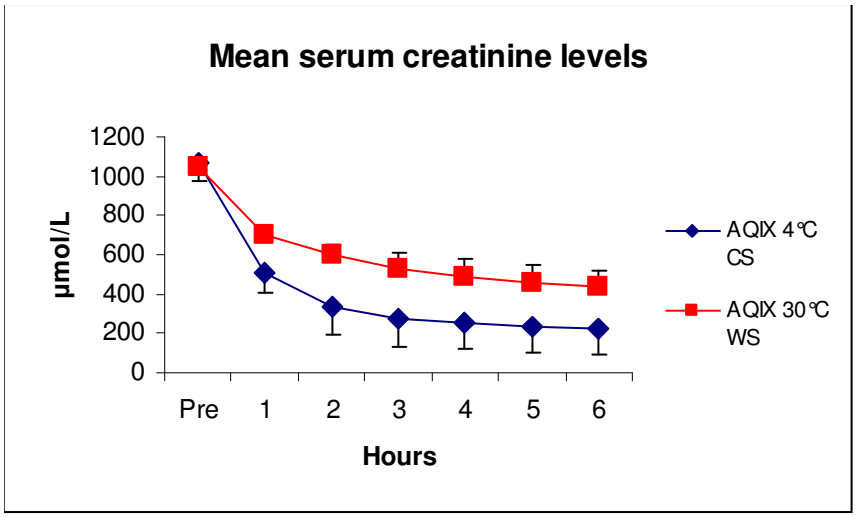


Figure 25. Serum creatinine levels during 6 hours perfusion for kidneys flushed with AQIX at 30°C then statically stored at either 4 °C or 30 °C.

Values are the mean ± SD.

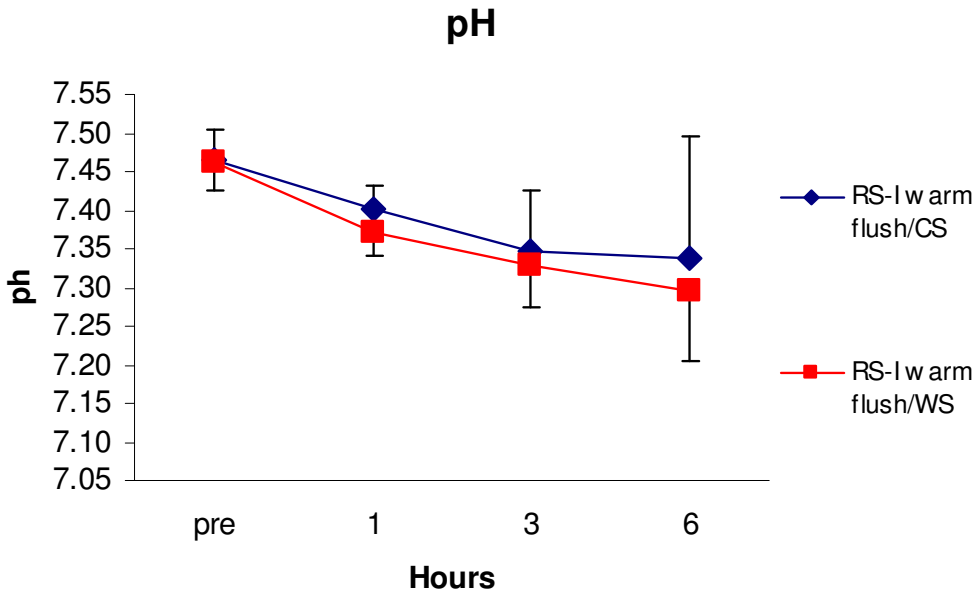


Figure 26: Blood pH during isolated organ perfusion after flushing with warm (30°C) AQIX solution followed by either cold (4°C) or warm (30°C) static storage.

The ADP: ATP ratio levels were highest in the pre-perfusion biopsies, reflecting the ischaemic damage sustained during the storage period. There was no significant difference in the pre-perfusion ADP: ATP ratio (p=0.54) between the two groups (Figure 27). After 6 hours perfusion, the ratio had improved in both groups showing recovery of cellular function, with no significant difference observed between the groups (p=0.71).

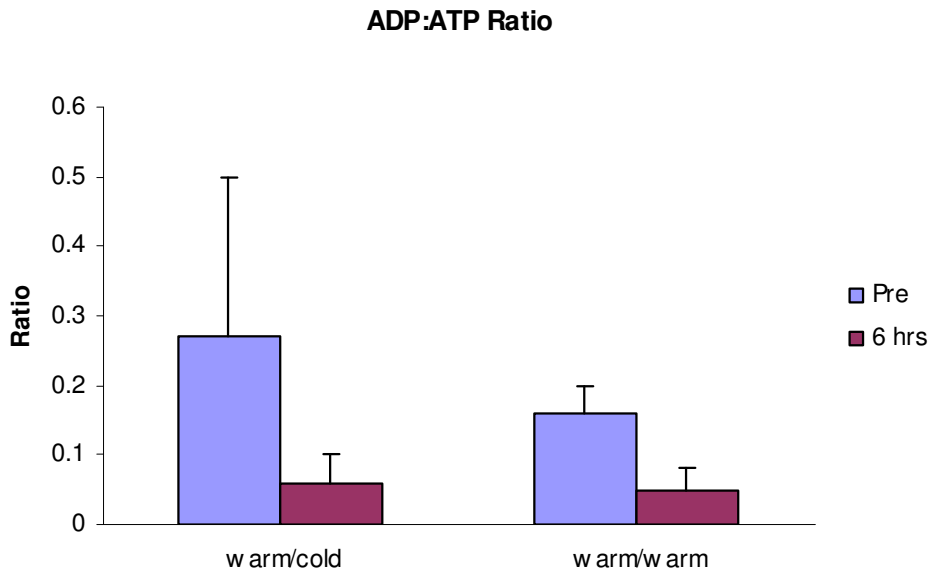


Figure 27: Renal cortical ADP: ATP Ratios pre- and post- 6 hours of isolated *ex-vivo* renal perfusion (mean ± SEM)

Discussion

This study has revealed promising results for AQIX®RS-I as a preservation solution. Renal allografts functioned well after a warm flush and hypothermic storage. The rationale behind the warm flush is that it should in theory achieve a more rapid flush of erythrocytes from the microcirculation without causing vasoconstriction. Without the vasoconstrictive effect, it is likely that there is a better clearance from the microcirculation. The initial flush is important to remove blood, cellular components that may have clumped, and perfuse the microcirculation with a composition that is designed to impair cellular swelling. Reduced hepato-cellular damage has been demonstrated in warm-flushed rat livers [11], and improved microperfusion in liver grafts by combining a warm flush with streptokinase [202].

Most encouraging was the maintenance of acid-base homeostasis, a key function of the kidney and necessary for the maintenance of conditions conducive for enzyme action. pH was normal in the cold stored group after six hours assessment and near normal in the warm stored group, which had undergone 2 hours of un-impaired anaerobic metabolism. Furthermore the recovery of cellular function as demonstrated by the improvement in the ADP: ATP ratio highlights the potential of AQIX as a preservation solution.

Porcine kidneys that were of similar weight to adult human kidneys were used in this study, as they are known to have anatomical and physiological characteristics that more closely resemble the human situation than small animal models [203]. The

isolated organ perfusion system was successful in yielding reproducible results making it a valid model for studying post-ischaemic renal injury as previously demonstrated in other papers[22,167].

There are limitations of the study such that the kidneys were subjected to quite short periods of warm and cold ischaemia prior to reperfusion with blood. Future studies should include longer cold ischaemic times, which reflect the clinical transplant situation. Extended warm times could also be added to the model in order to study the effects of initial warm flushing in non heart-beating kidneys.The IOPS system was reliable, but arguably does not reproduce normal physiological conditions such as that of an auto-transplant model.

Whilst overall those kidneys stored normothermically did not function as well as hypothermic storage, renal viability was maintained after 6 hours perfusion. To fully assess the potential benefit of normothermic preservation, warm machine perfusion during transport back to the laboratory prior to viability assessment should be utilized. The logistics of this are somewhat complex, making a period of cold preservation a likely pre-requisite prior to viability assessment. The above results are encouraging both for the use of a warm flush, and of AQIX ® RS-I as a preservation solution.

Summary and Conclusions

New insights and developments in organ preservation are potentially important factors to improve organ function and to increase the donor organ pool which may thereby help alleviate current pressure on the waiting list for transplantation. The aims of this work were to present an overview of organ preservation, the current methods used, and the relative benefits of the various methods and solutions used. The experimental chapters assessed the role of the organ flush, and compared a new preservation solution, AQIX RS-I, with traditionally used solutions.

The first experimental chapter assessed the efficiency of organ cooling using the commonly used preservation solutions, UW and hyperosmolar citrate. In this experiment, porcine kidneys were harvested and flushed at 4° C with continuous evaluation of temperature change at two depths. The viscosity of UW resulted in a slower fush and hence slower rate of cooling, but this was not statistically significant. Furthermore less volume of UW was required to cool kidneys < 10° C, and UW-flushed kidneys gained significantly less weight and had less histological injury than HOC-flushed kidneys. UW thus appeared to be more efficient at cooling with less injury observed. Kidneys were then harvested under the same conditions and perfused *ex-vivo* using the IOPS machine to compare functional and histological parameters, with equivocal results observed.

The second experiment also assessed the rate of organ cooling using a new solution, AQIX RS-I, with kidneys flushed with a combination of warm AQIX followed cold AQIX solution. These results were compared to UW and HOC cold-flushed kidneys.

The theory of the initial warm flush was to reduce vasoconstriction and hence give a more rapid flush. Subsequently this should allow a more efficient cold flush to the microcirculation and cool the kidneys faster. The results revealed that the combination of a warm and cold AQIX flush did give a faster rate of flush, but this did not translate into a faster cooling rate.

The third experiment compared different flush regimens using AQIX RS-I to assess the optimal flush conditions for its use. Three different flush conditions were compared (4°C vs 30 °C followed by 4 °C vs 30 °C) followed by storage on ice. Functional assessment was made on the IOPS machine. Initial Flushing with warm AQIX at 30 °C followed by storage on ice produced the best functional results.

The fourth experiment compared the best AQIX flush (30 °C followed by ice storage) with a traditional 4 °C flush using UW or HOC. The warm flushed AQIX group flushed significantly faster and had superior acid-base homeostasis. Functional results were equivocal between the groups.

The final experiment compared the best AQIX flush (30 °C followed by ice storage) with a warm (30 °C) flush followed by warm static storage prior to perfusion on the IOPS. These warm-stored kidneys thus had more then 2 hours warm ischaemic time and no cold preservation. Despite this they displayed a near normal pH after 6 hours of perfusion, and showed recovery of cellular function as demonstrated by the improvement in ADP: ATP ratio.

The IOPS produced reproducible results, and porcine kidneys of similar weight to human kidneys were used as they are known to give more clinically relevant results than small animal models.

Future Directions

Limitations of experiments presented are the relatively short periods of warm and cold ischaemia. Extended warm and cold times could be used in future experiments to reflect the clinical deceased donor. Whilst the IOPS is a valid model for studying post-ischaemic renal injury, the system does not reproduce normal physiological conditions and hence the gold standard for future experiments would be to use an auto transplant model.

Future experiments should also include groups of kidneys using warm UW and warm HOC to compare to the warm AQIX flush. This would help determine if it were the effects of the warmer temperature, or the constituents of the preservation solution that had the greatest effect.

Finally to fully assess the potential benefit of normothermic preservation, cold ischaemia would need to be eliminated. This would entail the use of warm machine perfusion during transport back to the laboratory. The logistics of this are somewhat complex, making a period of cold preservation a likely pre-requisite prior to viability assessment.

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