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REVIEW

Normothermic machine perfusion of the kidney: better conditioning and repair?

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Summary

Kidney transplantation is limited by hypothermic preservation techniques. Prolonged periods of cold ischaemia increase the risk of early graft dysfunction and reduce long-term survival. To extend the boundaries of transplantation and utilize kidneys from more marginal donors, improved methods of preservation are required. Normothermic perfusion restores energy levels in the kidney allowing renal function to be restored *ex vivo*. This has several advantages: cold ischaemic injury can be avoided or minimized, the kidney can be maintained in a stable state allowing close observation and assessment of viability and lastly, it provides the ideal opportunity to add therapies to directly manipulate and improve the condition of the kidney. This review explores the experimental and clinical evidence for *ex vivo* normothermic perfusion in kidney transplantation and its role in conditioning and repair.

Introduction

The reliance on more marginal donors in kidney transplant has emphasized the need to improve methods of preservation to optimize their use. Kidneys from donation after circulatory death (DCD) and extended criteria donors (ECD) are less likely to function as well as kidneys from healthier, younger, standard criteria or living donors due to additional ischaemic injury and donor comorbidities [1–3]. Nonetheless, they are an important resource that advantages patients with end-stage renal failure.

Kidney preservation techniques continue to rely on the basic principle of hypothermia to maintain cellular viability. Hypothermic temperatures act to inhibit the enzymatic processes [4]. This slows the depletion of adenosine triphosphate (ATP), inhibits degrading processes (phospholipid hydrolysis) and allows an organ to be stored without oxygen for a significant period of time. Nonetheless, with the depletion of ATP, there is build-up of toxic substances in the cell including adenosine, inosine and hypoxanthine. Anaerobic metabolism lowers the intracellular pH causing lysosomal instability with the activation of lytic enzymes causing cellular damage. The depletion of ATP also reduces a large number of cellular processes [5,6]. Inactivation of Na+/K+ ATPase pumps allows the accumulation of calcium, sodium and water within the cell causing cellular swelling [6].

Cold ischaemic time alone is an independent risk factor for graft failure and is directly associated with delayed graft function (DGF) [7]. The risk of graft failure after 3 months increases with every additional hour of cold ischaemia [8]. It also significantly increases allograft immunogenicity, provoking acute and chronic rejection.

Kidneys from ECD and DCD donors are less tolerant to hypothermia and therefore may benefit from improved methods of preservation [1,9,10].

Modifications to hypothermic preservation conditions have shown promise in reducing graft injury in the experimental setting. Oxygen can be administered to support a low level of aerobic respiration during hypothermic machine perfusion [11]. Therapeutic agents have also been added to the preservation solution during static cold storage [12]. However, the hypothermic conditions make it difficult to ensure the adequate uptake and synthesis of these therapies. Cellular signalling cascades can be altered during hypothermia, but the effector mechanisms are blocked until the organ is rewarmed [12]. Furthermore, without restoring normal cellular function, the injury process persists.

Normothermic preservation

The innovative vision of Le Gallois and Alex Carrel in maintaining an organ in a functional state is now a much more logical and feasible approach to organ preservation. Normothermic perfusion techniques are gradually being adopted in clinical transplantation for other solid organs [13–15]. Maintaining a kidney at a normothermic temperature has many advantages. Firstly, aerobic metabolism can be restored allowing the kidney to regain function, this can minimize or avoid the cold ischaemic insult. Secondly, the kidney can be maintained in a stable state allowing close observation and assessment of viability. Lastly, it provides the opportunity to add therapies to a functioning organ to directly manipulate and improve its condition.

Normothermic perfusion requires a number of essential components to ensure the adequate delivery of oxygen and nutrients to maintain cellular integrity and vascular processes during perfusion. The perfusate can be based on either a natural oxygen carrier such as blood or an artificial-based medium.

Normothermic perfusion – artificial preservation conditions

Brasile *et al.* [16] were the first to develop an acellular normothermic solution based on a perfluorocarbon (PFC) emulsion (Perflubron) and enriched tissue culture-like medium containing essential and nonessential amino acids, lipids and carbohydrates (Table 1). They have published numerous studies using canine, porcine and discarded human kidneys using this pressure-controlled device [17–20]. The exsanguinous metabolic support (EMS) system also included an oxygenator, pulsatile pump with controllers to maintain PaO_2, $PaCO_2$, pH and temperature.

PFCs are inert solutions that have a high capacity for dissolving oxygen [21]. The first-generation PFCs were relatively unstable solutions with variable half-lives and inability to be sterilized. When administered intravenously, they caused side effects such as anaphylaxis, hypotension, reduced platelet count and complement activation [22]. New more stable second- and third-generation PFCs are now being developed mainly as blood substitutes. Humphreys *et al.* [23] recently used a commercially made PFC (Oxygent) to provide oxygenation and reduce ischaemic injury to the kidney during warm ischaemia by retrograde infusion through the urinary collecting system. These new-generation PFCs could potentially be developed as normothermic preservation solutions. However, due to the complexity of manufacturing, the cost is considerable, which may limit their adaptation.

Historically, other artificial haemoglobin-based oxygen carriers have also been used as preservation mediums. A stroma-free haemoglobin-based solution caused toxic effects on the kidney [24]. However, a more stable pyridoxalated haemoglobin polyoxyethylene (PHP) solution has proved to be more successful. Brasile *et al.* have since replaced the PFC in their perfusion medium with this solution [25].

Other novel solutions such as Lifor, an artificial preservation medium containing a nonprotein oxygen carrier, nutrients and a growth factor, have also been used for preservation at more normothermic temperatures. Gage *et al.* [26] showed that porcine kidneys perfused with Lifor at room temperature had higher flow rates and lower levels of resistance compared with kidneys perfused with UW at room temperature or at 4 °C. There was no information on the outcome of the kidney; however, the higher perfusion flows suggested better preservation of the cellular structure. Lifor has also been found to be beneficial in reducing apoptotic cell death after cold ischaemic injury and was used in a recent study examining the effects of different temperatures during 6 h of oxygenated machine perfusion in a rat liver model [27]. Lifor at 21 °C improved cellular structure, increased bile production and lowered enzyme release; however, there was an increase in levels of lactate after perfusion which questions its efficiency as an oxygen carrier.

AQIX-RS-I® is nonphosphate-buffered solution designed to support cellular activity at a normothermic temperature. The composition of AQIX-RS-1® reflects physiological ionic concentrations, osmolarity and ion conductivity, to maintain the cell membrane and enzymatic processes. AQIX-RS-1® is capable of carrying oxygen in solution or when mixed with red blood cells. We have shown that porcine kidneys flushed with pre-oxygenated AQIX-RS-I at 32 °C adequately cleared the microcirculation with no detrimental effects [28]. It is also capable of maintaining cellular integrity under static storage conditions for up to 2 h at 32 °C [29].

The most newly reported acellular solution is Hemarina-M101. It is an extracellular haemoglobin derived from a marine invertebrate. It has been formulated into an oxygen carrier called Hemoxycarrier® mainly as a blood substitute. M101 has been used in cold storage solutions to deliver

Table 1. Ingredients used during *ex vivo* normothermic perfusion using the exsanguinous metabolic support (EMS) system.

DL- Alanine	0.12 g/l	Menadione (Na Bisulphate)	0.00003 g/l
L-Arginine HCl	0.14 g/l	Myo-Inositol	0.0001 g/l
DL-Aspartic acid	0.12 g/l	Niacinamide	0.00005 g/l
L-Cysteine HCl H_2O	0.00022 g/l	Nicotinic acid	0.00005 g/l
L-Cystine 2HCl	0.52 g/l	Para-Aminobenzoic acid	0.0001 g/l
DL-Glutamic acid	0.2672 g/l	D-Pantothenic acid Ca	0.00002 g/l
L-Glutamine	0.20 g/l	Polyoxyethylenesorbitan monoolate	0.04 g/l
Glycine	0.10 g/l	Pyridoxal HCl	0.00005 g/l
L-Histidine HCl H_2O	0.04376 g/l	Pyridoxine HCl	0.00005 g/l
L- Hydroxyproline	0.02 g/l	Retinol acetate	0.00028 g/l
DL-Isoleucine	0.08 g/l	Riboflavin	0.00002 g/l
DL-Leucine	0.24 g/l	Ribose	0.001 g/l
L-Lysine HCl	0.14 g/l	Thiamine HCl	0.00002 g/l
DL-Methionine	0.06 g/l	Thymine	0.0006 g/l
DL-Phenylalanine	0.10 g/l	Uracil	0.0006 g/l
L-Proline	0.08 g/l	Xanthine HCl	0.00069 g/l
DL-Serine	0.10 g/l	Calcium chloride $2H_2O$	0.265 g/l
DL-Threonine	0.12 g/l	Ferric nitrate $9H_2O$	0.00144 g/l
DL-Tryptophan	0.04 g/l	Magnesium sulphate (anhydrous)	1.2 g/l
L-Tyrosine 2Na	0.11532 g/l	Potassium chloride	0.40 g/l
DL-Valine	0.10 g/l	Sodium acetate (anhydrous)	0.10 g/l
Adenine hemisulphate	0.02 g/l	Sodium chloride	6.8 g/l
Adenosine triphosphate 2Na 2Na	0.002 g/l	Sodium phosphate monobasic (anh)	0.224 g/l
Adenylic acid	0.0004 g/l	D-Glucose	0.01 g/l
Alpha-tocopherol phosphate 2Na	0.00002 g/l	Insulin	0.01 g/l
Ascorbic acid	0.001 g/l	Bovine serum albumin	30 g/l
D-Biotin	0.00002 g/l	Sodium bicarbonate	4.4 g/l
Calciferol	0.0002 g/l	Pyruvate	0.22 g/l
Cholesterol	0.0024 g/l	Transferin	0.10 g/l
Choline chloride	0.001 g/l	Serum	10 ml
Deoxyribose	0.001 g/l	B-cyclodextrin	0.5 g/l
Folic acid	0.00002 g/l	Chondroitin sulphate B	0.004 g/l
Glutathione (reduced)	0.0001 g/l	Fibroblast growth factor	0.02 g/l
Guanine HCl	0.0006 g/l	Heparin	0.18 g/l
Hypoxanthine	0.0006 g/l		

oxygen with favourable results [30]. It has a high affinity for oxygen accompanied with an antioxidant activity. It functions over a wide range of temperatures (4–37 °C), and although not reported, it may be beneficial as a normothermic perfusion solution.

Blood-based solutions

Blood-based solutions were previously considered to have their limitations. Prolonged perfusion was associated with gross haemolysis, platelet activation and deterioration in the oxygen-carrying capacity of the red blood cells [31]. High intrarenal resistance and tissue oedema were a significant problem. However, much has been learnt from the development of atraumatic centrifugal pumps and equipment used in cardiac bypass surgery. These pumps reduce the risk of stress and haemolysis, and the membrane oxygenators enable filtration and highly efficient oxygenation [31]. They provide a more natural environment for the kidney compared with the artificial-based solutions. We first reported the development of a normothermic perfusion system in 2008 [32]. The isolated perfusion system utilizes adapted paediatric cardiopulmonary bypass technology. It is a pressure-controlled system including a centrifugal pump, membrane oxygenator and heater exchanger. The system was developed using porcine kidneys with a leucocyte-depleted red blood cell-based solution mixed with a crystalloid solution. Anti-inflammatory and antioxidant agents were added together with a vasodilator to enhance blood flow and protect the kidney. We have most recently described the adaptation of this system for clinical use, the first to be reported in man [15,33]. Kidneys were perfused with 1 unit of compatible packed red blood cells mixed with a similar priming solution with added protective agents (Table 2). The early results in a series of 18 ECD kidneys were promising with a DGF rate of 5.6% compared with 36% in similarly matched recipients receiving a kidney undergoing static cold storage [33].

Table 2. The components of the perfusate solution and supplements added during *ex vivo* normothermic perfusion.

Perfusate	
Compatible cross-matched blood or recipient blood depleted of leucocytes and platelets	1 unit
Ringer's solution (Baxter Healthcare, Thetford, UK)	200–400 ml
Mannitol 10% (Sigma-Aldrich)	20–25 ml
Dexamethasone 4 mg (Organon Laboratories, Cambridge, UK)	2 ml
Sodium bicarbonate 8.4% (Fresenius Kabi, Cheshire, UK) to normalize pH	10–40 ml
Heparin 1000 iu/ml (CP Pharmaceuticals, Wrexham, UK)	2–4 ml
Augmentin 1.2 g	10 ml
Supplements	
Prostacyclin 0.5 mg (Folan, Glaxo-Wellcome, Middlesex, UK)	4 ml/h
Glucose 5% (Baxter Healthcare)	7 ml/h
Nutriflex® infusion (B Braun, Sheffield, UK) with the following added;	20 ml/h
Insulin (Novo Nordisk, Denmark)	100 units
Sodium bicarbonate 8.4% (Fresenius Kabi, Cheshire, UK)	25 ml
Multivitamins (Cerenvit®; Baxter Healthcare)	5 ml
Ringer's solution to replace urine output ml for ml	

Ideal perfusion

Providing a period of 'ideal' perfusion to ensure the kidney is kept in a protective environment is paramount. Nonetheless, rewarming an organ in a highly oxygenated environment after an ischaemic interval may increase the risk of damage. Changes in cellular structure and opening of the mitochondrial permeability transition pores can occur during this process [34]. Under normal circumstances when oxygenated blood is reintroduced to the organ immediately after transplantation, a complex inflammatory process arises causing further cellular injury [35]. Leucocytes play a pivotal role in this process. Endothelial cell damage caused by ischaemic injury stimulates a pro-inflammatory environment which activates and stimulates leucocytes. There is increased expression of intracellular adhesion molecules; intracellular adhesion molecule-1 (ICAM-1) and selectins which enhance leucocyte–endothelial interaction. Leucocytes migrate and infiltrate into the interstitium leading to microvascular congestion and the 'no-reflow' phenomenon. This also increases cytokine expression, production of oxygen free radicals and activates the complement system to sustain the injury response, causing cell death and tissue damage [35–37]. Platelets also have a damaging role in reperfusion injury [38]. Thrombus formation can clog the microvasculature and reduce circulation. They also mediate vasoconstriction and inflammatory processes causing tissue injury.

Ex vivo normothermic perfusion with a red blood cell-based solution in the absence of leucocytes and platelets limits infiltration and the inflammatory response to improve circulation and renal function [39,40]. Apoptosis and inflammatory mediators are also suppressed, reducing the likelihood of injury [39]. Artificial solutions are also designed in the same way to provide a protective environment avoiding the inflammatory response (Table 1).

Conditioning

Ex vivo normothermic perfusion can be used throughout the preservation interval or for shorter periods. The most practical approach to accommodate transportation is to combine normothermic and hypothermic techniques. Short periods of *ex vivo* normothermic perfusion have been carried out experimentally either in the middle of the preservation period or at the end after hypothermic preservation.

One of the early aims of kidney preservation was to extend the preservation period to a matter of days to facilitate cross-matching and antibody screening. van der Wijk *et al.* [41] were able to preserve canine kidneys for 144 h using a combination of hypothermic and normothermic preservation techniques. Half way through the hypothermic preservation period, kidneys were perfused at a normothermic temperature, with blood, for periods of 1–4 h before returning to hypothermic preservation. Three and 4 h of normothermic perfusion were deemed necessary to reverse the ischaemic damage. Rijkmans *et al.* [42] extended the preservation period to 6 days with a 3-h period of normothermic preservation in the middle of the preservation interval. This intermediate period of normothermic preservation restored energy metabolism, replenishing adenosine levels to effectively resuscitate and condition the organ to retain viability. The canine kidney can withstand a great deal of ischaemic injury compared with porcine or human kidneys, and these early studies describe extreme circumstances. Nonetheless, the concept warrants further investigation under more clinically applicable circumstances. This could lead to a system whereby marginal kidneys could be resuscitated at a centralized unit and then allocated to the most suitably matched recipient in another centre.

Our own group and that of Brasile *et al.* have shown that normothermic perfusion can be carried out effectively at the end of the preservation interval. Stubenitsky *et al.* warmed canine kidneys for 3 h using their artificial EMS system after warm and cold ischaemic injury. It suppressed the level of reperfusion injury to reduce rates of DGF and PNF [25].

Our described *ex vivo* normothermic perfusion system used periods of 1 or 2 h of normothermic perfusion to restore function and replenish ATP after warm and cold is-

chaemic injury in porcine kidneys [32,43,44]. The kidneys had less tubular injury, improved blood flow and oxygenation and were metabolically more stable than the cold-stored kidneys [32]. In an autotransplant model, 2 h of normothermic perfusion after 30 min of warm ischaemia and 20 h of hypothermic machine perfusion proved to be a feasible and safe method of preservation [44]. There were no complications associated with the technique and some indication of improved recovery of renal function compared with hypothermic machine perfusion alone. Clinically, the technique has again proved to be feasible and safe with no adverse effects such as arterial or venous thrombosis, ureteric complications or infection [15,33]. Perfusion was carried out for approximately 60 min in the operating theatre during the anaesthetic time and whilst the transplant bed was being prepared for engraftment. This short period of *ex vivo* normothermic perfusion is sufficient to restore renal function and replenish ATP without delaying the transplant procedure.

Prolonged periods of normothermic perfusion have also been successful. Brasile *et al.* [17] were able to maintain viability for up to 24 h with their artificial-based solution. At this time, there are no reports of kidneys being maintained for such a prolonged period using blood-based solutions.

Repair

Ex vivo normothermic perfusion has the ability to recover an organ after significant ischaemic injury. Although not trialled clinically in DCD kidneys, if cold ischaemia was to be limited or avoided, it may be possible to recover kidneys from extreme periods of warm ischaemia. Brasile *et al.* [19] eloquently described the resuscitation and repair of canine kidneys after 120 min of warm ischaemia. If this could be applied clinically, it would encourage uncontrolled DCD programmes increasing thus the utilization of Maastricht category 1 and 2 donors.

The mechanistic effects of *ex vivo* normothermic perfusion have not been thoroughly investigated. However, in addition to replenishing ATP, our studies with porcine kidneys have shown that repair mechanisms are upregulated [43]. We found that the stress protein heat-shock protein 70 (HSP 70) was expressed after 1 h of perfusion. HSP 70 reduces stress-induced denaturation and aggregation of intracellular proteins and is thought to be a mediator of tolerance [45].

The expression of protective proteins can also be enhanced by modulating the perfusion conditions. Brasile *et al.* [46] used the haem analog cobalt protoporphyrin during normothermic perfusion to upregulate the protective gene haemoxygenase-1 (HO-1). HO-1 is one of the three isoforms that catalyse the conversion of haem to biliverdin, free iron and carbon monoxide. These compounds reduce free radical injury and have anti-inflammatory actions. Carbon monoxide is also a potent vasodilator promoting blood flow and oxygenation [47]. They demonstrated that gene expression could be accomplished during 6 h of normothermic perfusion, supporting new protein synthesis. When administered under hypothermic conditions, the same effect was not evident.

Therapies

Restoring normal cellular function provides the ideal opportunity to deliver therapies directly to the kidney. This would avoid treating the donor or recipient and ensure that a predetermined dose can be given in a controlled manner directly to the kidney. Furthermore, the effect of the therapy can be closely monitored. Therapies that target multiple injury pathways or promote vasodilation to enhance blood flow and oxygenation have been particularly advantageous during *ex vivo* normothermic perfusion. Gaseous molecules such as nitric oxide donors or carbon monoxide in the form of soluble carbon monoxide-releasing molecules (CORMs) can enhance renal blood flow via the activation of soluble haem-containing guanylate cyclase to produce guanosine 3′, 5′-cyclic monophosphate (cGMP) in vascular smooth muscle, causing relaxation [47]. The administration of these molecules during 2 h of *ex vivo* normothermic perfusion was protective against reperfusion injury in porcine kidneys [48]. Renal blood flow was enhanced and renal function improved. This form of drug delivery provides enormous scope in the treatment of reperfusion injury, rejection or to prolong graft survival. Many therapies that have been used to treat the donor or recipient in the experimental setting in an attempt to reduce the severity of ischaemia reperfusion injury could be applied in this way.

Gene therapy

Delivering genes to block or stimulate protective pathways targeting acute and chronic rejection or ischaemia reperfusion injury is an attractive therapy in transplantation [49]. To ensure delivery and replication, genes are administered with a vector, either viral, nonviral or cell based. Viral vectors are more efficient; however, their toxicity and ability to stimulate an immune response make them difficult to administer directly to the patient [49,50]. Gene delivery during *ex vivo* normothermic perfusion could avoid this problem and ensure that the kidney alone is targeted.

There is little evidence of this application in the kidney. However, Brasile *et al.* [51] demonstrated that the kidney could be effectively transfected during 24 h of *ex vivo* normothermic perfusion. They administered the recombinant adenovirus, Ad5, CMV5 GFP encoded with green fluorescence protein and found effective transfection and synthesis

occurred over 24 h of perfusion. The reporter gene GFP localized in the intima of the blood vessels demonstrating the ability to deliver and target the vascular endothelium.

It may also be possible to manipulate the kidney in other ways. RNA interference using a 21-nucleotide small interfering RNA (siRNA) is another approach to modulating and protecting the kidney [52,53]. The introduction of a double-stranded RNA elicits the selective degradation of homologous mRNA transcripts. siRNAs are capable of blocking gene expression in mammalian cells and have proved to be a potent and specific method of gene silencing [52]. We have demonstrated siRNAs ability to reduce caspase 3 expression and reduce some of the effects of reperfusion injury in the isolated kidney when added to the cold preservation solution [54]. The effect maybe greater when administered under normothermic conditions, and it is likely that prolonged periods of *ex vivo* normothermic perfusion are needed to ensure that the kidney is modified before reperfusion. Nonetheless, shorter periods of perfusion may be used to deliver the genes and target the later events in the reperfusion and immune cascade.

Stem cell therapies

Mesenchymal stromal cells (MSCs) can be derived from most tissues, but at present, bone marrow MSCs are the best characterized. MSCs have the ability to migrate to the site of injury and differentiate into various mesenchymal tissues to modulate tissue regeneration and repair [55]. They also exert paracrine and endocrine properties secreting growth factors and cytokines that have mitogenic, anti-apoptotic, anti-inflammatory and antifibrotic properties [56,57]. They have been beneficial for acute repair of the kidney in various experimental models, improving renal function, reducing tubular injury and prolonging survival [57–59]. Although they can home in on damaged tissue, there remains difficulty in targeting the specific organ with systemic administration. Again, *ex vivo* normothermic perfusion can facilitate their delivery directly to the kidney. Brasile *et al.* [60] used fibroblast growth factors 1 and 2 to upregulate cellular processes to modulate recovery and repair after substantial warm ischaemic injury in canine and several discarded human kidneys. These growth factors have a role in regulating the metabolic rate, transport processes and also have a mitogenic effect on epithelial cells. The perfusion conditions supported cellular metabolism with evidence of protein synthesis again after 24 h of *ex vivo* normothermic perfusion [20,60].

Conclusion

Ex vivo normothermic perfusion has many advantages compared with hypothermic techniques. Its application in clinical practice is in its infancy; however, the early results are positive. *Ex vivo* normothermic perfusion offers many opportunities to advance kidney transplantation. Avoiding or minimizing the cold ischaemic insult using prolonged or short periods of *ex vivo* normothermic perfusion may allow us to extend the boundaries of kidney transplantation. Kidneys with greater ischaemic insults could be successfully utilized leading to the adoption of more uncontrolled DCD programmes. Marginal kidneys could be treated with protective agents during perfusion to reduce the impact of ischaemia reperfusion injury or the immune response. Gene-based therapies could be used to manipulate the kidney to suppress injurious processes or upregulate protective pathways. Stem cells could also be used to stimulate regeneration and repair. *Ex vivo* normothermic perfusion has the potential to open up many new avenues in kidney transplantation.

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