A pathophysiologic study of the kidney tubule to optimize organ preservation solutions

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Background. Tissue damage at the time of organ transplantation has a negative impact on the subsequent success of the procedure, both in the immediate and longer term. Hypothermia is the principal element used to prolong organ viability ex vivo, but paradoxically also induces cellular edema through inhibition of energy-dependent adenosine triphosphatases (ATPases). This induces an electrolyte imbalance that leads to fluid influx and cell swelling. It is important, therefore, that improvements are made in the preservation of ischemic organs to reduce this injury.

Methods. This study has applied a novel in vitro system to model cold and warm ischemic-induced renal tubule swelling that characterizes tissue damage in ischemia/reperfusion injury. Biochemical blockade of ATPases in this system using strophanthidin modeled the effects of energy depletion and induced cell swelling. By measuring such tubule swelling and changes to tubular cell volume in isolated rabbit renal proximal tubules, an analysis was made that defined the basis on which an optimal preservation solution may be developed.

Results. The data show that our model could reproduce ischemically induced cell swelling and characterized the response at the cellular level of tubules to different components of preservation solutions. The data indicate that an isosmolar, phosphate-buffered, sucrose solution prevented tubule swelling more effectively than Euro-Collins, hyperosmolar citrate, or University of Wisconsin solutions that are in routine clinical use.

Conclusion. Future developments in organ preservation may significantly improve transplant outcomes. Our novel analysis forms the basis of future whole-organ studies that ultimately may allow us to propose an optimum platform for improved preservation solutions.

The process of organ procurement and implantation constitutes a severe physical stress on solid organs used in transplantation. Despite major advances in organ transplantation, surgical technique, transplant immunology, preservation techniques, and preservation solutions, every transplant organ faces two inevitable insults—ischemia and subsequent reperfusion. A period of cold ischemia extends from the time of organ procurement to the time when it is removed from cold storage just before implantation. This is followed by a shorter but potentially more hazardous period of warm ischemia during implantation. The final physical stress is that of reperfusion of the organ with recipient blood, the so-called reperfusion injury.

To minimize the effects of organ damage and ischemia/reperfusion (I/R) injury, but also to allow time for organ allocation, organ transportation and surgery, organ procurement requires both in situ flush with a cold preservation solution, and hypothermic storage at 0 to 4°C. During ischemia, active transport mechanisms involving Na⁺/K⁺ and Ca²⁺/Mg²⁺ adenosine triphosphatase (ATPase) are inhibited [1], which leads to a steady influx of Na⁺, Cl⁻, and Ca²⁺ into the cell with subsequent osmotic influx of water causing cellular swelling. Hypothermia reduces the rate of tissue damage by depressing the cellular metabolism, but although it forms the central component of any organ preservation strategy, hypothermia also directly contributes to cellular edema by direct depressant effects on ATPase-dependent active transport mechanisms [1].

Ischemia also results in lowered pH and an accumulation of toxic products of anaerobic respiration (e.g., lactate and hypoxanthine) that contribute to free radical damage upon reperfusion of the organ with recipient blood [2, 3]. It is well known that severe I/R injury leads to delayed graft function (DGF) posttransplant [4]. These authors have quoted a 5% incidence of DGF for liver, and a 20% to 30% DGF for renal transplantation, while United Kingdom Transplant reported a 19% incidence of DGF in UK adult cadaveric renal transplantation nationwide over 5 years to 2002 (UKT, Bristol, UK, personal communication). Such DGF has been linked to poorer
Table 1. Constituents of preservation solutions used in this study

<table>
<thead>
<tr>
<th>Components mmol/L</th>
<th>Euro-Collins</th>
<th>Hyperosmolar citrate</th>
<th>Phosphate-buffered sucrose 140</th>
<th>University of Wisconsin</th>
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<tr>
<td>Glucose</td>
<td>194</td>
<td></td>
<td>140</td>
<td></td>
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<tr>
<td>Mannitol</td>
<td></td>
<td></td>
<td>186</td>
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<td>Sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raffinose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobionic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HES %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate Na₂HPO₄</td>
<td></td>
<td>42.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄ · 2H₂O</td>
<td></td>
<td>26.7</td>
<td></td>
<td></td>
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<tr>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>KOH</td>
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<td>27</td>
</tr>
<tr>
<td>Citrate</td>
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<tr>
<td>Na⁺-3-citrate</td>
<td></td>
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<td>28</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄</td>
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<td></td>
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<tr>
<td>Glutathione</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Allopurinol</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Adenosine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin IU</td>
<td></td>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Penicillin G IU</td>
<td></td>
<td></td>
<td></td>
<td>200,000</td>
</tr>
<tr>
<td>Dexamethasone mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Osmolality mOsm.kg⁻¹</td>
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<td>400</td>
<td>300</td>
<td>325</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
<td>7.1</td>
<td>7.0</td>
<td>7.4</td>
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</table>

prognosis [5, 6]. In a clinical study, one- and five-year graft survival was significantly reduced from 95% and 81% in early functioning grafts to 87% and 70% in delayed functioning grafts, respectively. In addition, a link was established between DGF and the incidence of acute rejection that negatively impacted upon graft half-life [7]. The effects of I/R injury and the subsequent tissue damage that manifests as DGF may render the organ more visible to the immune system of the recipient and promote activation of both innate and adaptive immunity against the donor organ [8, 9]. In particular, lipid peroxidation will lead not only to membrane damage and activation of arachidonic acid pathways with subsequent release of prostaglandins and leukotrienes, but may also increase tissue susceptibility to complement activation through the alternative pathway [10]. Such proinflammatory mediators will stimulate not only the release of cytokines and chemokines by damaged tissue, but also contribute to an acute-phase response that further promotes inflammation. Recent experimental studies have demonstrated that long-term outcomes may well be affected by tissue damage induced as a result of less than optimal ischemia and that modifications to preservation solutions can significantly improve organ function [11].

The core components of any preservation solution are impermeant agents that prevent fluid entry into cells, buffers that maintain pH, and ions (Table 1). Secondary components may confer additional benefits but are not essential for organ preservation. These include free radical scavengers, calcium antagonists, colloids, complement regulators, and antiplatelet agents. Solutions such as hyperosmolar citrate (HOC) also use a hypertonicity of 400 mOsm in a bid to prevent fluid entry into cells whose isotonic environment is approximately 285 to 300 mOsm.

Currently used buffer systems include phosphate, citrate, histidine, and bicarbonate. Preservation solutions, with the exception of University of Wisconsin (UW; pH 7.4), commonly have an acidic pH (6.9 to 7.1) in relation to plasma. This followed work which indicated that an acidic pH may actually protect against hypoxic injury [12–14]. The inclusion of high potassium in the preservation media has been thought to be important in preventing potassium loss during cold storage [15, 16]. It is also thought that a high potassium concentration helps to prevent the build up of intracellular calcium during ischemia. Euro-Collins (EC), hyperosmolar citrate (HOC), and UW solutions have a high potassium content and are thus classed as “intracellular” solutions. Phosphate-buffered sucrose (PBS140), developed by Andrews and Coffey [17] for morphologic preservation of kidney tubules, however, contains only sodium and no potassium and is thus classed as an “extracellular” solution.

The cellular edema caused by the influx of water is the primary pathophysiologic event that affects ischemic organs. In the kidney, it is the corticomedullary segments
of renal tubules that appear most vulnerable to I/R injury [18]. This compartmental susceptibility is possibly explained by the no-reflow phenomenon whereby severe edema constricts blood vessels and limits delivery of oxygen on reperfusion of affected tissues [19]. Tubule cell volume is actively regulated in vivo but this regulation is lost in the ischemic tissue since the process is energy dependent and ATP is almost totally depleted in an ischemic organ within 4 hours of ischemia [20]. The aims of this study were to develop a new in vitro system to model the effect of ischemia on tubule cell swelling, and to analyze the effect of preservation solutions and their core components in preventing this tissue damage. Following from this study, new recommendations are made for the basic constitution of an optimized preservation solution, specifically to address tubule cell edema. This forms the principal pathologic insult to whole organs, and prevention of such cell swelling could have significant benefit for transplant outcome if improved solutions are adopted by transplant centers.

METHODS

Animals and anesthesia

Specific pathogen-free (SPF), New Zealand White (NZW) rabbits 1.5 to 2.5 kg in weight were used throughout the experiments (Charles River, Surrey, UK). United Kingdom Home Office Guidelines (Scientific Procedures Act, 1986) were strictly adhered to throughout this work. Terminal anesthesia was induced by intravenous pentobarbitone 60 mg kg⁻¹ (Sagattal, RMB Animal Health Limited, Dagenham, UK).

Experimental plan

In brief, isolated rabbit kidneys were perfused in situ with a preservation fluid under test and maintained on ice for varied cold ischemic times. Following this, single proximal tubules were micro-dissected from the renal cortex and assessed for tubule swelling as a result of cold ischemia, and during a period of warm ischemia using the protocol described below.

Perfusion of kidneys

A 50 mL syringe reservoir was set at a height of 50 cm above the table and was filled with 40 mL of cold preservation solution (0 to 4°C) just prior to the flush. A wide bore polyvinyl tubing (2 mm internal diameter) was connected to the reservoir. The draining end was tapered for cannulation of the aorta or the renal artery. The tip of the catheter was also barbed with a scalpel blade to avoid the ligature slipping. A midline incision was made and a laparotomy performed. The lower abdominal aorta was ligated above the iliac bifurcation with a 3/0 silk tie (Ethicon Limited, Edinburgh, UK) followed by ligation of the aorta below the origin of the celiac trunk but above the origin of both renal arteries. An incision was made in the aorta above the lower tie, and with the flush solution running a catheter was introduced through this incision. The catheter was then secured with a 3/0 silk tie. Blood was drained by venting the inferior vena cava in the chest. The warm ischemia time of the whole procedure was less than 1 minute. After 25 mL flush solution had passed through, the kidneys were excised and placed in glass beakers containing 100 mL of the preservation fluid under test on ice. These were then stored in the refrigerator at 4°C for a period dictated by the protocol of the experiment.

Dissection of proximal tubules

After a defined period of storage (0, 24, 48, or 72 hours, according to the experimental protocol) the kidney was removed and the capsule was carefully stripped using fine forceps and dissection scissors. A thin (approximately 0.5 mm thick) cross-section slice was cut with a scalpel blade and placed in a Petri dish half filled with the preservation solution under test. The Petri dish was placed on a refrigerated metal dissecting surface of a purpose built dissecting table (Conair Refrigerator Circulator, Warrington, UK) under a stereoscopic zoom microscope (Nikon SMZ-2) mounted directly above the Petri dish. A servo-driven focusing mount controlled by a foot pedal left both hands free for dissection. At ×800 magnification various segments of nephron can be identified. They appear translucent-gold in color against the black background. Both proximal and distal tubules have a typical convoluted appearance and are readily identifiable within the outer cortex. The early S₁ and S₂ segments of the proximal tubules can be identified due to their convoluted nature and the attachment of the S₁ segment to the glomerulus by a narrow neck. In contrast, the proximal straight tubules have no convolutions and have a typical “ground glass” appearance. The cortical collecting duct is also straight and has a small diameter and an indistinct image. The thin loop of Henle is the smallest in diameter and is mostly confined to the medulla, as are the medullary collecting ducts, which are easily identified as tightly packed straight tubules in the medulla. The thick ascending limb of loop of Henle is difficult to differentiate from the cortical collecting ducts (CCD). However, it does not have the less defined appearance of CCD under high magnification. Cortical S₁ and S₂ segments of proximal tubules were used for these experiments. Two pairs of micro-fine forceps (Thackray, Leeds, UK) and a 23 G needle mounted on a 1 mL syringe were used for dissection. The cortical tissue was gently teased apart until a tubule was seen bridging the gap or hanging freely at the edge between two tissue pieces. Finally, the ends were cleanly cut by the sharp edge of the mounted needle acting as a
micro-blade. A transfer pipette was used to transfer the single dissected tubule to the perfusion bath. The transfer pipette consisted of a Pasteur pipette with the distal 1.5 to 2.0 cm bent at right angle. The bulb was controlled by an adjustable screw which allowed fluid to be drawn up and ejected. Before transferring the tubules, the inside of the pipette tip was coated with dilute albumin solution (5 mL of bovine 25% albumin solution in Tyrode’s buffer dissolved in 25 mL of saline) to prevent the tubules from sticking to the pipette glass.

The microperfusion rig

A standard inverted microscope (e.g., ×2.7, ×4, ×10, and ×20 objectives and a ×20 eyepiece) was used in these experiments. The perfusion bath (J. White, MD, Washington, USA) consisted of a circular 0.5 cm thick acrylic plate. A longitudinal 2.0 × 0.5 cm full thickness slot was cut in the center and formed the walls of the perfusion bath while a glass cover slip stuck to the base with melted wax formed the floor of the bath. The chamber held 1 cm depth of bathing fluid. There were multiple inlets into the chamber to allow experimental manipulations and monitoring (temperature, pH, etc.).

Experimental reperfusion protocol

Tubules were then set up on an inverted microscope and the tubule diameter was measured at 5-minute intervals using an eyepiece graticule (Graticules Ltd., Tonbridge, Kent, UK). The graticule was calibrated against a standard scale under various magnifications. This form of analysis was validated against later measurements using a photodiode array [21] and found to be accurate and reproducible. For the period of measurement, the tubules were initially bathed in oxygenated physiologic saline for 10 minutes, during which time the cell size equilibrated and reached a steady volume. The bathing fluid was then replaced by either physiologic saline or a test preservation solution with or without strophanthidin. This test period lasted for 35 minutes and was related to the warm ischemia of transplantation. Finally, the bathing fluid was again replaced by oxygenated physiologic saline, allowing the cells to recover. This second period in saline lasted for 25 minutes and paralleled reperfusion in transplantation while also acting as a second control period.

Strophanthidin treatment of isolated tubules

Strophanthidin (5β, 20[22]-Cardenoline-19-one-3β, 5, 14-triol) is a 405 kD cardiac glycoside derivative of digitals and is related to digoxin and ouabain in its properties. Strophanthidin is a selective inhibitor of Na+/K+ ATPase, and competes with K+ for Na+/K+ ATPase receptors [22]. In cardiac myocytes, strophanthidin exerts a positive inotropic effect similar to that of digoxin. The rise in intracellular sodium with strophanthidin treatment is due to suppressed sodium extrusion via the Na+/K+ ATPase pump, and activated sodium influx via Na+/H+ exchange [23]. Specific sodium channel blockers (e.g., SUN1165), therefore, partly abolish the rise in intracellular sodium resulting from use of strophanthidin and digoxin [24]. Tubules prepared as described above were incubated with doses of strophanthidin as indicated. At concentrations greater than 10−3 mol/L, the solubility of strophanthidin was a limiting factor. For all test experiments a dose of 10−3 mol/L was applied.

Calculation of tubule cell volume

At 37°C fluids and solutes in the tubular lumen are pumped out across the basolateral membrane causing the tubular lumen to collapse. The outside diameter of a collapsed tubule (two rows of cells) can thus be used to calculate cell volume mathematically (ρLmm−1) with the equation \( \pi r^2 \times 1 \), or 3.1416 × (Tubule diameter/4)2 × 1000.

Statistics

Data were analyzed using Student t test or repeated measures analysis of variance as appropriate. \( P < 0.05 \) was considered to be significant. For each solution under test, tubules from 6 separate kidneys from 6 different animals were used for each time point of analysis (\( N = 6 \)).

RESULTS

Isolated tubule segments were prepared and exposed to different preservation solutions in order to examine cell swelling in a situation that paralleled ischemia. In all experiments tubule diameter was measured across the midpoint of the tubule segment (Fig. 1).

Evaluation of impermeants in organ preservation

Proximal tubules were prepared as per protocol and, following an initial equilibration period in physiologic saline, maintained in preservation solutions at 37°C that contained different impermeants (Table 2). Results were grouped into three categories: monosaccharides (Fig. 2A), disaccharides (Fig. 2B), and trisaccharides and anions (Fig. 2C). Impermeants currently used in preservation solutions were then compared as a group (Fig. 2D). This comparison showed that mannitol performed better in preventing ischemic induced cell swelling than either glucose, fructose or mannose (\( P < 0.001 \)). Although the disaccharides and trisaccharide prevented cell swelling to some degree in comparison to the monosaccharides, within this group sucrose and raffinose were most effective. Addition of anions in the form of gluconate or lactobionate (at concentrations given in Table 2) demonstrated that lactobionate was more effective as an
impermeant \((P < 0.001)\) than gluconate. By direct comparison of the effect of impermeants used in standard solutions (Fig. 2D), sucrose performed as well as raffinose (UW) with no significant difference between the two in this analysis \((P > 0.05)\), and significantly better than lactobionate, glucose (EC) or mannitol (HOC) \((P < 0.005)\). From these experiments it appeared that the disaccharide sucrose was as effective in the prevention of cell swelling as the trisaccharide raffinose and the anion lactobionate used in UW. Sucrose was significantly more effective an impermeant than glucose or mannitol, used in EC and HOC, respectively.

**The use of strophanthidin to mimic warm ischemic-induced cell swelling**

To investigate the effect of strophanthidin on cell volume a dose titration of this agent was applied to isolated rabbit proximal convoluted renal tubules and demonstrated increased cell swelling as a result of \(\text{Na}^+\)/\(\text{K}^+\) pump blockade in a dose and time-dependent manner.
Table 2. Test preservation solutions using various “impermeant” molecules

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Impermeant</th>
<th>Impermeant concentration mmol/L</th>
<th>Phosphate-buffered concentration mmol/L⁻¹</th>
<th>pH</th>
<th>Osmolality mOsm/L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBG</td>
<td>Glucose</td>
<td>140</td>
<td>Na₂HPO₄ 42.3</td>
<td>7.0</td>
<td>300</td>
</tr>
<tr>
<td>PBF</td>
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<td>300</td>
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<td>Mannitol</td>
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<td>42.3</td>
<td>7.0</td>
<td>300</td>
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<tr>
<td>PBMnse</td>
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<td>42.3</td>
<td>7.0</td>
<td>300</td>
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<tr>
<td>PBS140</td>
<td>Sucrose</td>
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<td>7.0</td>
<td>300</td>
</tr>
<tr>
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<td>9.26</td>
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</tbody>
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Abbreviations are: PBG, phosphate-buffered glucose; PBF, phosphate-buffered fructose; PBMmatl, phosphate buffered mannitol; PBMnse, phosphate-buffered mannose, PBS140, phosphate-buffered sucrose; PBT, phosphate-buffered trehalose; PBMlts, phosphate-buffered maltose; PBR, phosphate-buffered raffinose; PBL, phosphate-buffered lactobionate; PBGent, phosphate-buffered gluconate.

Postfix after phosphate buffer indicates the sugar/anion molecules.

aSodium salt used, phosphate concentration reduced.

Fig. 2. Comparison of impermeants showed that of the monosacharrides (A), mannitol prevented ischemic induced cell swelling significantly better than either glucose, fructose or mannose (P < 0.001). Within the disaccharides and trisaccharides (B), sucrose and raffinose were most effective. Of the anions (C), lactobionate was more effective as an impermeant than gluconate (P < 0.001). By direct comparison of the effect of impermeants used in standard solutions (D), sucrose performed as well as raffinose [used in University of Wisconsin solution (UW)] with no significant difference between the two in this analysis (P > 0.05).

Such cell swelling was shown to be rapidly reversible by the flushed removal of strophanthidin (Fig. 3B) such that tubules returned to their normal diameter pre-strophanthidin. This protocol therefore appeared to mimic the warm ischemic-induced tubule cell swelling that was more severe after 24 hours cold ischemia. In vitro blocking of the Na⁺/K⁺ pump appeared to create a similar situation that was alleviated by removal of the agent. Therefore, strophanthidin was used to induce biochemical cell swelling of rabbit proximal convoluted renal tubules to model warm ischemia. This model was used to test the effectiveness of various preservation
solutions against the hypothesis that such solutions prevent cell swelling that occurs under ischemic conditions because of metabolic inhibition of the Na\(^+/\)K\(^+\) pump transporter.

### Assessment of impermeants in HOC and EC

The data showed that sucrose acted as the most effective impermeant during experimental ischemia. In order to establish the efficacy of the impermeants of the most commonly used renal preservation solutions EC and HOC, glucose and mannitol, respectively, were exchanged for sucrose. Strophanthidin blockade was applied in order to assess cell swelling during the warm ischemic phase (Fig. 4A to D). The data showed that cell swelling was significantly reduced in tubules preserved in EC containing sucrose instead of glucose following 0 (Fig. 4A, \(P < 0.001\)) or 24 hours (Fig. 4B, \(P < 0.001\)) of cold ischemia. However, improved preservation of HOC by using sucrose instead of mannitol was only evident after 24 hours of cold storage (Fig. 4C and D, \(P < 0.05\)). From our data it was apparent that sucrose provides superior protection from cell swelling over glucose or mannitol.

### Further assessment of modifications to HOC

Since HOC is the most commonly used kidney preservation solution in the United Kingdom, a further analysis of HOC was made. Osmolality has a critical impact upon fluid dynamics within cells and tissues. When the osmolality of HOC was reduced from 400 to 300 mOsm·kg\(^{-1}\) [isosmolar citrate (IOC)] our data showed minimal impact at time 0 (\(P > 0.05\)), but significant improvement (\(P < 0.05\)) after 24 hours cold ischemia as compared with tubules exposed to normally constituted HOC (Fig. 5A and B). The addition of MgSO\(_4\) to Collins original preservation solution was a beneficial development. To establish the requirement for MgSO\(_4\) in HOC a comparison was made using HOC in the experimental analysis of tubule cell edema (Fig. 5C and D). The data showed that there was no difference in cell swelling between these groups. In the third modification, sodium and potassium citrate buffer of HOC was replaced by a sodium and potassium phosphate, while maintaining the pH at 7.1 and the osmolality at 400 mOsm·kg\(^{-1}\). MgSO\(_4\) was also replaced with an equivalent amount of mannitol as it tends to form a precipitate with phosphate. The data showed that replacement of a citrate-based buffer in HOC with a phosphate buffer had no immediate effects on the ischemic-induced cell swelling at time 0 (Fig. 5E, \(P > 0.05\)), but after 24 hours cold ischemic time, significant improvement was noted (Fig. 5F, \(P < 0.05\)).

### Comparison of an optimized solution with commercially available preservation solutions

The data derived from the above experiments suggested that a phosphate-buffered, isosmolar, sucrose solution would combine the best characteristics for a renal preservation solution that would prevent proximal tubular cell swelling over prolonged periods of cold ischemia. Such a solution (PBS140) was applied to the strophanthidin-based isolated proximal tubule experiments, and compared against the ability of EC, HOC, and UW solutions to control cell volume during warm ischemia. The data showed that PBS140 was as good as UW in preventing cell swelling (Fig. 6A). The experiment was repeated on tubules rendered cold ischemic for 24, 48, and 72 hours in order to establish effects of solutions on increasingly severe ischemic injury. Here results showed that PBS140 was comparable if not better in reducing cell
swelling than any of the commercially available solutions, including UW (Fig. 6B to D).

**Cell volume changes following cold and warm ischemia with current preservation solutions**

By analyzing pre- and post-warm ischemic cell volumes (pl.mm⁻¹) over 0, 24, 48, and 72 hours of cold ischemia, it was evident that at the earliest time points EC failed to prevent cell swelling that was further increased after warm ischemia (Fig. 7A). Similar analysis showed that using HOC (Fig. 7B) prevented the early cell swelling that was exhibited by tubules preserved in EC but did little to reduce warm ischemic cell swelling. After prolonged periods of cold ischemia a time dependent increase in cell volume was shown and susceptibility to warm ischemia was maintained in the presence of HOC. The use of UW (Fig. 7C) prevented to some degree the cold induced cell swelling but an increase in the response of tubules to warm ischemia was seen with increasing duration of cold ischemia. However, using PBS140 (Fig. 7D) effectively reduced the susceptibility of tubules to cold ischemia over 72 hours and reduced the subsequent cell volume response upon exposure to warm ischemia even after 72 hours of cold ischemia. In this analysis PBS140 demonstrated its capacity to prevent cell swelling more effectively than UW.

From these data it is evident that, following strophanthidin blockade, both PBS140 and UW show the least percentage increase in cell volume after CIT 0 (+10 and +8% respectively), when compared to the 30% increase of EC and 36% increase of HOC. It also appears that the starting cell volume after cold ischemia was lowest for PBS140 and highest for EC (Table 3). After a prolonged cold ischemic insult of 72 hours, PBS140 proved to be the most effective of the preservation solutions in preventing warm ischemic tubular edema. Here a minimal increase in cell volume of 7% was observed compared to 23%, 38%, and 25% for UW, EC, and HOC, respectively (Table 4).
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Fig. 5. Comparison of hyperosmolar citrate (HOC) against isomolar citrate (IOC) demonstrated only marginal protection when tissues had not endured any prolonged cold ischemia (A). After 24 hours of cold ischemia (B), a significant reduction in cell swelling was seen if an isomolar solution was used ($P < 0.05$). An analysis of the requirement for MgSO$_4$ in the solution showed that its exclusion had no effect on strophanthidin-induced cell swelling after 0 hours of cold ischemia ($C$, $P > 0.05$). Similarly, after 24 hours of cold ischemia ($D$), the exclusion of MgSO$_4$ in the solution had no effect on strophanthidin-induced cell swelling ($P > 0.05$). The exchange of the citrate buffer for a phosphate buffer ($E$) did not show an improvement after minimal cold ischemia, while replacing citrate with a phosphate buffer in HOC showed significant improvement if tubules had been rendered cold ischemic for 24 hours ($F$, $P < 0.05$).

**DISCUSSION**

This study analyzed the effect of preservation solutions and their core components on preventing cellular edema. An isolated renal proximal tubule system was developed that reproducibly demonstrated the edematous response of single tubules to cold and warm ischemia. Although this model examined only one aspect of ischemic injury, it is cellular edema that forms the principal lesion...
leading to pathologic features of ischemically damaged organs. For this reason the prime component of a preservation solution is its impermeant, whose function is the prevention of cell swelling. The nature of cell swelling in this model was believed to be due to abrogation of cell volume regulation. Maintenance of a constant cell volume is dependent on the rate of solute influx being equal to solute efflux. Biologic membranes are highly permeable to water and so ionic disturbance in the intracellular and extracellular fluid leads to osmotic movement of water across the plasma membrane. In the context of ischemic depletion of ATP, energy dependent solute transporters such as the Na\(^+\)/K\(^+\) ATPase are no longer able to regulate cell volume. An accumulation of intracellular Na\(^+\) is followed by influx of anions to maintain electroneutrality [25–27] and water follows osmotically into the cell. The use of strophanthidin in our system to block the Na\(^+\)/K\(^+\) pump modeled the physiologic response of tubules to starvation of ATP with regard to cell volume control. The cell swelling that was detected was reversible once strophanthidin was removed from the perfusion solution, as tubules attempted to regain cell volume control. The isolated tubule experiments therefore putatively reflected cellular edema, and since the experiments were conducted at 37\(^{\circ}\)C following periods of cold preservation, the model system allowed an analysis of tubule responses to both cold and warm ischemia.

The usefulness of the isolated tubule experiments was validated by the experimental data in this study that showed that UW solution was more effective in preventing cellular edema than EC or HOC. UW solution is regarded as the “gold standard” in organ preservation solutions so these observations widely reflected other work using whole organs [28], but this study provided a novel physiologic analysis of the edema that forms the principal source of preservation injury. The isolated perfused tubule system permitted a direct investigation of the effects of alterations to preservation solutions that might predict outcomes in whole organ transplants, and

Fig. 6. By using the strophanthidin-based model, a comparison (A) of the commercially available solutions with a phosphate buffered sucrose solution (PBS140) showed that cell swelling was minimized by either University of Wisconsin (UW) or PBS140 compared to Euro-Collins (EC) and hyperosmolar citrate (HOC) at time 0 \(P < 0.01\). The effect of 24 hours of cold ischemia (B) showed that cell swelling was most effectively prevented during a warm ischemic episode if PBS140 was used \(P < 0.01\). After 48 hours of cold ischemia (C), our data show prolonged protection with PBS140 comparable with UW. Prolonged protection with PBS140 comparable with UW was also seen at 72 hours of cold ischemia (D).
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Fig. 7. Tubule cell volume after 0 to 72 hours of CIT and subsequent increases following warm ischemia are shown for tubules preserved in EC (A), HOC (B), UW (C), and PBS140 (D). The data showed that PBS140 minimized tubule volume changes after both cold and warm ischemic periods, providing evidence of reduced edema compared to the other solutions.

Table 3. Percentage change in cell volume during pump blockade with strophanthidin and upon reversal of pump blockade compared to the first control period in physiologic saline (0 hour cold ischemic time)

<table>
<thead>
<tr>
<th>Test solutions</th>
<th>Physiologic saline</th>
<th>Test solution</th>
<th>Cell volume</th>
<th>% change</th>
<th>Physiologic saline</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS140</td>
<td>221 ± 16</td>
<td>196 ± 18</td>
<td>−11</td>
<td>243 ± 22</td>
<td>+10</td>
<td></td>
</tr>
<tr>
<td>UW</td>
<td>225 ± 16</td>
<td>185 ± 17</td>
<td>−18</td>
<td>244 ± 30</td>
<td>+8</td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>282 ± 35</td>
<td>455 ± 18</td>
<td>+62</td>
<td>367 ± 46</td>
<td>+30</td>
<td></td>
</tr>
<tr>
<td>HOC</td>
<td>244 ± 14</td>
<td>257 ± 15</td>
<td>+5</td>
<td>332 ± 16</td>
<td>+36</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations are: PBS140, phosphate-buffered sucrose; UW, University of Wisconsin; EC, Euro-Collins; HOC, hyperosmolar citrate.

Table 4. Percentage change in cell volume during pump blockade with strophanthidin and upon reversal of pump blockade compared to the first control period in physiological saline (72 hours cold ischemic time)

<table>
<thead>
<tr>
<th>Test solutions</th>
<th>Physiologic saline</th>
<th>Test solution</th>
<th>Cell volume</th>
<th>% change</th>
<th>Physiologic saline</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS140</td>
<td>323 ± 37</td>
<td>254 ± 22</td>
<td>−21</td>
<td>344 ± 45</td>
<td>+7</td>
<td></td>
</tr>
<tr>
<td>UW</td>
<td>325 ± 27</td>
<td>269 ± 16</td>
<td>−17</td>
<td>398 ± 29</td>
<td>+23</td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>291 ± 16</td>
<td>468 ± 28</td>
<td>+61</td>
<td>402 ± 18</td>
<td>+38</td>
<td></td>
</tr>
<tr>
<td>HOC</td>
<td>427 ± 45</td>
<td>465 ± 42</td>
<td>+9</td>
<td>534 ± 48</td>
<td>+25</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations are: PBS140, phosphate-buffered sucrose; UW, University of Wisconsin; EC, Euro-Collins; HOC, hyperosmolar citrate.
provided an elegant method to potentially design the optimal platform for a preservation solution to reduce cellular edema.

For a variety of reasons, transplant centers worldwide have adopted alternatives to UW for renal preservation that are accepted as less than optimal because of the “gold standard” status ascribed to UW. By implication, therefore, a higher degree of tissue injury possibly occurs at the time of transplantation where alternatives to UW are used. Delayed graft function and acute tubular necrosis due to I/R injury have been linked to subsequent acute rejection episodes [4, 5], the incidence of which is a major predictor of chronic allograft nephropathy—itself the most common cause of allograft failure. It is clear therefore that improvements in preservation solutions may have significant benefits for clinical transplantation worldwide.

The specific analysis of the core components of preservation solutions in this study highlighted not only the differences between various solutions in current clinical use, but also could be used to establish the constituents of an optimized solution for improved organ preservation. This study confirmed the beneficial effects of impermeants in preventing cell swelling, and appeared to show that sucrose could be as effective as the raffinose that is used in UW. Indeed, cell swelling was reduced in the presence of EC or HOC if their impermeants were changed to sucrose from mannitol or glucose, respectively, demonstrating that tubule cell impermeability to monosaccharides is at best only partial, and is not sustained through prolonged ischemia. The data in this study, therefore, showed that the renal tubules were more impermeant to sucrose. Unlike glucose, sucrose is not transported across cell membranes, nor broken down by succrase activity in the nephron, and so remains in the extracellular space under both hypothermic and normothermic conditions. Therefore, sucrose is a more suitable impermeant agent for use in organ preservation than glucose, which once transported into cells by the Na+/glucose transporter may be used as a substrate for anaerobic metabolism and further contribute to the accumulation of toxic metabolites during ischemia. In addition, our observations also suggested that tubular cell membranes may become more permeable to mannitol over prolonged periods of cold ischemia. The data demonstrated that the benefits in using sucrose were even more apparent the longer the period of cold ischemia.

Cell swelling was also reduced after a prolonged cold and warm ischemic period if an isosmolar solution was used instead of a hyperosmolar solution. In a previous study, a comparison of hyperosmolar and isosmolar forms of HOC showed better preservation of isolated perfused rat kidneys using isosmolar solution [29]. In the current study of isolated tubules, on return to physiologic saline, an irreversible increase in tubule diameter was seen in the 0 hour HOC group. A similar effect has been observed during the investigation of cell volume regulation during hypertonic shock [30]. During volume regulation in hypertonic medium, intracellular Na+ and Cl− concentrations increase with no concurrent alteration in K+ concentration. This imbalance drives an influx of water upon rapid exchange of medium. The data in this study would suggest that cell volume after exposure to HOC remains at this increased level, though the reason for this is unclear. However, this could have important implications for the clinical use of hypertonic preservation solutions such as HOC and EC; cell swelling triggered by return to isotonic conditions from hypertonic storage could be responsible for some post-ischemic tissue damage.

This study also investigated the effect of exchanging the buffer from citrate (HOC) to phosphate (HOPBM). After 0 hours of preservation there was no significant difference in tubule diameter. However, after 72 hours of cold ischemia, tubule diameter in the HOC group was greater than the HOPBM group. This parameter was not significantly different when comparing the IOC group at 72 hours. Taken together, these findings suggest that citrate is more permeable than phosphate, at least under hypertonic conditions.

The direct comparison of solutions over 72 hours indicated that cell volume during warm ischemia was essentially uncontrolled in tubules preserved in EC, and worsened with prolonged CIT in HOC. Upon reperfusion with saline at the end of the experiment to remove the preservation fluid under test, regulatory volume decrease (RVD) was repeatedly seen in the tubules exposed to EC as they presumably recovered from the more severe warm ischemic swelling induced in the presence of glucose. Such RVD was not seen with the other solutions that did not induce the same degree of swelling. However, the data shows that tubule diameter was lowest in the PBS140 group at the end of the experimental protocol after 24, 48 or 72 hours of CIT, even when compared to the performance of UW.

Our data clearly demonstrate the shortcomings of HOC, as well as solutions that used a less effective impermeant, such as EC. These solutions have been used widely in clinical transplantation but clearly there may have been increased posttransplant complications as a result. Direct comparisons with the common preservation solutions have been made in clinical and experimental transplant studies and these have revealed that UW is better than either EC or HOC.

However, the experimental analyses presented here would suggest that an isosmolar, phosphate-buffered sucrose solution such as PBS140 could form the basic platform for an optimized renal preservation solution. Earlier studies have hinted that this may be the case [31–33], but this study is the first to definitively examine at the tubular
level, the physiologic response of renal tissue to preservation solutions. The data here suggest that phosphate-buffered sucrose solutions have the potential to offer better conditions for organ preservation than UW. Indeed, in a previous trial of PBS140 in clinical renal transplantation, improved results posttransplant were seen [34]. The data presented here revealed that PBS140 prevented cell swelling more effectively than UW in this experimental setting, and that warm ischemic cell swelling after 72 hours of cold ischemia was reduced threefold by PBS140, compared to UW-preserved tissue.

These observations could therefore have significant implications for the clinical management of ischemic damage. If PBS140 could reduce cell swelling more effectively than UW, then by implication less tissue damage occurs in PBS140 preserved tissues. This can be interpreted in two ways: first, less tissue damage occurs in short (<24 hours) cold ischemic times, and second, tissues remain more viable for longer ischemic times. In addition, PBS140, being a relatively simple solution, overcomes the shortcomings of using UW. PBS140 has a lower viscosity, making its use easier and permitting more rapid and efficient perfusion of the donor organ. These factors all have the potential to significantly improve transplant outcomes by reducing the initial tissue damage at the time of transplantation.

This study, therefore, dissected the core components of clinical preservation solutions and has proposed the basis of an optimized preservation solution that may perform better than all other solutions currently in common clinical practice. Further studies in whole organ and in transplant systems are already underway. Any possible benefit in switching from EC or HOC-based solutions would probably be seen in reduced rates of acute tubular nephropathy or in the incidence of DGF, but longer term analysis of transplant recipients is required to demonstrate the effectiveness of better organ preservation on transplant outcome. The potential exists to reduce the non-specific tissue injury that occurs in all donor organs and to extend the possible pool of available organs.

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