A novel rodent model of severe renal ischemia reperfusion injury

Henry Whalena,b, Paul Shielsa, Marc Littlejohna and Marc Clancyb

abCollege of Medical, Veterinary and Life Sciences Institute of Cancer Sciences, University of Glasgow, Glasgow, UK; bDepartment of Renal Transplantation, South Glasgow University Hospital, Glasgow, UK

ABSTRACT
Renal ischemia reperfusion injury (IRI) is a major problem, currently without treatments in clinical use. This reflects the failure of animal models to mimic the severity of IRI observed in clinical practice. Most described models lack both the ability to inflict a permanent reduction in renal function and the sensitivity to demonstrate the protective efficacy of different therapies in vivo. To test novel cell-based therapies, we have developed a model of renal IRI in Fisher 344 rats. Animals were subjected to 120 min of unilateral warm ischemia, during which they underwent an intra-renal artery infusion of therapeutic agents or vehicle. At either 2 or 6 weeks post-surgery, animals underwent terminal glomerular filtration rate (GFR) studies by inulin clearance to most accurately quantify renal function. Harvested kidneys underwent histological analysis. Compared to sham operations, saline treated animals suffered a long-term reduction in GFR of ≈50%. Histology revealed short- and long-term disruption of renal architecture. Despite the injury severity, post-operative animal losses are ≈5%. This model produces a severe, consistent renal injury that closely replicates the pathological processes encountered in clinical medicine. Renal artery infusion mimics the route likely employed in clinical transplantation, where the renal artery is accessible. Inulin clearance characterizes GFR, allowing full assessment of therapeutic intervention. This model is useful for screening therapeutic agents prior to testing in a transplant model. This reduces animal numbers needed to test drugs for clinical transplantation and allows for refinement of dosing schedules.

Abbreviations: BUN: Blood Urea Nitrogen; CRH: Compensatory renal hypertrophy; CIC: Constant infusion clearance; ED: External diameter; GFR: Glomerular filtration rate; IRI: Ischemia reperfusion injury; SEM: Standard Error of the Mean.

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INTRODUCTION
Ischemia reperfusion injury (IRI) is a leading cause of acute kidney injury and represents a major clinical problem with high associated levels of morbidity and mortality.1–5 IRI to native kidneys results from pathological processes that cause organ hypo-perfusion such as trauma, dehydration and sepsis. Renal IRI also commonly occurs in patients undergoing aortic or cardiac surgery.6 Furthermore during kidney transplantation, IRI that results from allograft implantation has been shown to contribute to early organ dysfunction and poor long-term outcomes.7 Cell-based therapy has emerged as a potential treatment for patients at risk of renal IRI. It is postulated that such treatments may reduce renal damage by down regulating inflammatory pathways, promote tissue regeneration and consequently contribute to improved outcomes in the long-term.5,8–10 Unfortunately no such treatments are currently available for clinical use, with supportive therapy being the mainstay of treatment.11,12 Given the prevalence and morbidity associated with renal IRI, the development of such therapies should be considered a priority.13

While the mechanisms by which IRI occurs are relatively well understood, further insight into the efficacy of possible therapies might be provided by developing experimental models that mimic the severe IRI that is often seen in the clinical setting. In vitro models of renal IRI typically use renal epithelial cell lines or isolated perfused kidneys. Such models provide valuable information, but lack the physiological complexity of in vivo models and are thus limited in the data they provide.14 In vivo rodent models of renal IRI may broadly be divided into two types: bilateral or unilateral renal injury. Unilateral injury models may be further divided into models with or without a contralateral nephrectomy. Models using bilateral injury or unilateral injury with contralateral nephrectomy are the most
commonly studied, probably because blood analysis may demonstrate trends in renal function throughout the experimental time course.

In both of the commonly used models, warm ischemic times of between 30 and 60 min have been shown to cause acute renal dysfunction, but this is reported to resolve within a week of surgery. Longer periods of warm ischemia are associated with unacceptable numbers of post-procedure animal deaths. Using conventional rodent models, it therefore appears difficult to inflict a longstanding renal injury, as rodent kidneys appear to regenerate to function normally after non-fatal periods of renal ischemia. The benefit of testing the long-term effects of novel therapies in conventional rodent models is therefore questionable.

Unilateral injury models without contralateral nephrectomy leave animals with a healthy functioning kidney that serve as ‘protection’ against acute renal failure. Consequently, such models allow the study of prolonged ischemic times in the injured kidney, without excessive animal loses. Unfortunately unilateral injury models without nephrectomy do not allow functional elements of the ischemic injury to be studied using serial blood analysis, as renal function markers in such models are affected by the filtration of uninjured kidney.

Herein we described a novel rat model of renal IRI designed to address the problems described above. We employ a unilateral injury without contralateral nephrectomy model. To produce a high local dose of treatment, and to mimic the therapeutic route most likely employed in clinical transplantation, we inject therapy directly into the renal artery. Animals subsequently undergo inulin clearance studies as a secondary procedure to most accurately determine glomerular filtration rate (GFR) responses to injury ± intervention. Kidneys are later analyzed using histological methods to provide further insight into structural renal damage.

Methods
IRI procedure
Animals
We developed a model of renal IRI in Fisher 344 male rats, aged between 12 and 14 weeks, weighing 225–250 g.

Anesthesia
After placing the animal in an anesthetic chamber, induction was achieved using 5% inhaled isoflurane. Anesthesia was maintained with 5% isoflurane, given via facemask, mixed with 1 L of oxygen/min.

Position and temperature control
The rat was placed supine on a far infrared heating pad with the midline shaved and cleaned with chlorhexidine. The core temperature was measured regularly using an infrared thermometer and the heat mat was adjusted accordingly to maintain this between 36.5 and 37 °C.

Surgical procedure
A high midline incision was made. Bowel was wrapped in damp swabs and retractors were placed to enable access to the left renal pedicle. Division of the lateral peritoneal attachments mobilized the left colon, thereby exposing the renal vasculature. The renal artery was separated from the renal vein using blunt dissection with cotton tips and/or forceps. Full exposure of the renal artery from its origin on the aorta to the renal hilum necessitated tying of the suprarenal vein. Once exposed, an atraumatic vascular clamp was placed on the artery’s aortic origin, signifying the start of the ischemic period. A color change in the kidney confirmed correct placement of the clamp.

After 90 min had elapsed, an arteriotomy was made in the renal artery. Via this, the kidney was flushed with a 0.8 mL intra-arterial injection given via a 30G Ryecroft cannula. A thin rubber sloop was placed around the cannula and artery, preventing back-leak while an assistant gently injected the contents of the syringe. A color change in the cortex of the kidney confirmed infiltration of the kidney parenchyma with the desired agent or saline vehicle.

The artery was fully transected, then re-anastomosed in a standardized triangulated fashion, using 12 interrupted 10/0 nylon sutures. A frame clamp was used to secure the arterial ends during anastomosis and background material was placed to prevent damage to posterior structures. The arterial clamps were removed after exactly 120 min of ischemia and reperfusion of the kidney was confirmed visually.

Wound closure
The incisions were closed in two layers, using a continuous 4/0 polyglactin suture (Vicryl®).

Post-surgical care
The animal was injected with 2.5 mL of subcutaneous 0.9% saline post-operatively. Buprenorphine analgesia was injected subcutaneously, at 0.0045 mg per 100 g of body weight. This was repeated as necessary every 12 h for a maximum duration of 48 h post-surgery. The animal
was placed in a warming box at 38°C for 60 min. It was then returned to its cage and allowed free access to rodent chow and water. The animal was weighted daily.

**Quantifying renal injury**

**Determination of GFR with inulin clearance studies**

We have adapted a CIC model\(^2\(^3\)\) that allows the GFR of each kidney to be calculated individually. Animals may undergo inulin studies at any point after the IRI procedure – we have typically chosen time points of 2 or 6 weeks.

**Preparation of inulin solution**

Fluorescein Isothiocyanate Inulin (FITC-Inulin) (Sigma-Aldrich) solution (0.2%) was made by dissolving 40 mg of FITC Inulin in 20 mL of warmed 0.9% saline solution. One percent FITC Inulin was made by dissolving 10 mg FITC Inulin per mL of 0.9% physiological saline solution. The solutions were dialyzed with physiological saline overnight through a 1 kDa semipermeable membrane, removing any unbound fluorescein.

**Anesthesia**

Induction was with isoflurane as described previously. Anesthesia was maintained with an initial intraperitoneal injection of thiobutabarbitol sodium (Inactin\(^5\)\) using 12 mg per 100 g of body weight. Further maintenance doses of 2.4 mg per 100 g of body weight were given as required. This provided stable cardiovascular parameters.\(^2\(^4\)\)

**Surgical procedure**

Positioning and temperature control are as described above. The femoral artery and vein were fully dissected. After clamping the femoral vein proximally and ligating distally, microsurgical polyethylene tubing (0.96 mm external diameter (ED)) was used to cannulate the vein. The tubing was secured using 5/0 ligatures. A 0.5 mL bolus of 1% inulin solution was given before starting a constant infusion of 0.2% FITC inulin using a syringe pump at a rate of 3 mL/h.

A microsurgical catheter (0.8 mm ED) was then placed in the femoral artery. This was used to take blood samples during the experiment and provided continuous blood pressure monitoring to ensure hemodynamics stability. Between sampling, the line was locked with heparin-saline solution.

**Ureteric cannulation**

The abdomen was then re-opened along the line of the previous midline incision. The bowel was wrapped in damp swabs, the ureters were dissected and cannulated using a 7 cm length of microsurgical tubing (0.61 mm ED). Ligatures were applied to secure the catheters’ position.

Via these cannulas, urine was collected into separate tubes during each 30-min interval. In the middle of each 30-min collection period, an 80 μL blood sample was taken via the arterial line. Urinary volume for each 30-min period was measured using a Gilson’s pipette.

Serum and urinary fluorescence was calculated spectrofluorometrically using a fluorescence microplate reader (BIO-TEK flx-800) measuring emission and absorbance wavelengths of 495 nm and 430 nm, respectively. Forty microliters of 500 mM HEPES buffer was mixed with each 10 μL sample of serum or urine, since fluorescence of biofluids is known to vary across a pH range.\(^2\(^5\)\)

Steady state was reached in the plasma when the amount of inulin infused was equal to the amount filtered by the kidneys. This is defined as a <5% change in inulin plasma concentration over a 30-min period.\(^2\(^6\)\) Our data indicated that steady state is typically reached 60–90 min after the inulin infusion had commenced.

The experiment was terminated after six timed urinary/blood samples had been collected. The animal was killed and both kidneys, liver, spleen, heart and lungs were taken and stored in 10% formation, liquid nitrogen and RNA later.

All serum samples with the animal at steady state, along with the corresponding urine collections, were then used to calculate GFR using the following equation:

\[
GFR = \frac{V \times U}{P}
\]

where \(V\) = urine volume/min, \(U\) = urinary fluorescence and \(P\) = plasma fluorescence.

**Tips for achieving reliable GFR results**

Avoiding unnecessary blood loss is a key to ensure accurate GFR results. To achieve this, it is advisable to ligate all branches of the femoral vessels, and to ensure proximal and distal control of vessels before making an incision for cannulation. Bleeding points on the re-opened abdominal wall should be cauterized or oversewn. We also advise cauterization of small retroperitoneal vessels encountered during dissection of the ureter, as these may bleed profusely over the time course of the study if left uncontrolled.
Tips for successful microsurgical cannulation

Cannulation is easier if a precise hole is made in the front wall of the structure to be cannulated. A horizontal cut with a pair of pointed microsurgical scissors is most effective. This maneuver allows the structure to remain under tension, while the created defect gapes open, allowing easy access for the passage of the cannula (Figure 1). Fully dividing a structure causes proximal retraction, whereby cannulation becomes extremely problematic.

It is useful to have ligatures to secure a catheter loosely applied prior to cannula insertion. This allows simple tightening of the ligature as soon as the cannula is inserted, reducing the chance of the cannula inadvertently slipping out.

When the microsurgical tubing is cut to size, the use of a scalpel blade allows a smooth, angled but non-pointed end of the tube to be fashioned. The use of scissors leads to a ragged pointed tube that is difficult to insert, and will tear holes in vessel walls when advanced.

Histological methods of assessing renal injury

Hematoxylin and eosin staining and histopathology scoring

Formalin fixed specimens are paraffin embedded, sectioned at 5 μm and stained with hematoxylin and eosin for light microscopy analysis. We use an IRI scoring system that examines for tubular epithelial damage, tubular dilation and hyaline cast formation described by a previous study. Typically 10 non-overlapping cortical high power fields are examined and the mean scores are compared between treated and untreated kidneys.

Results

In more than 50 rats subjected to 120 min of unilateral warm renal ischemia, we have only experienced one post-procedure animal death. Furthermore, we have only failed to successfully anastomose the renal artery on one occasion. One hundred and twenty minutes of renal ischemia produces a permanent, severe and reproducible injury that results in long-term GFR deficit (see Figure 2).

Histological examination of kidney tissues as described above reveals significant disruption in renal architecture in those animals subjected to 120 min of warm ischemia (Figures 3–5).

Discussion

Cell-based therapy has emerged as a potential treatment for acute renal injury affecting either native or transplanted kidneys. Unfortunately no such treatments are in widespread clinical use. This is a particular problem in the field of transplantation where changing trends in organ donation have led clinicians to meet demand by utilizing organs from older and less fit deceased donors. Such kidneys may be subjected to longer periods of warm ischemia during retrieval and consequently are more susceptible to IRI during implantation. Developing strategies, including pharmacological interventions to improve the outcomes...
of these ‘extended criteria’ allografts is of the upmost importance.

The absence of interventions for the treatment of renal IRI reflects the lack of relevant animal models in which novel therapies may be easily studied. Many common rodent models used to induce renal dysfunction do not rely on warm ischemia as a mechanism of injury. These include models that utilize reduction of renal mass (5/6 nephrectomy), administration of nephrotoxins, or renal injury caused by ureteric obstruction.

However, warm renal ischemia and the injury that follows reperfusion are the most common causes of renal dysfunction encountered in clinical practice. Establishing authentic animal models that mimic the pathological process of renal IRI are of vital importance in evaluating the feasibility and efficacy of novel therapeutic interventions. Unfortunately, commonly used rodent models of renal IRI lack the capacity to

Figure 3. (a) Renal histology from sham operated rat at 2 weeks post-surgery. A glomerulus is marked with an arrow. (b) Histology from rat subjected to 120 min IRI, 2 weeks post-surgery. Note marked tubular dilation (green asterisk) with epithelial thinning and breaks (arrowheads). Also, present are hyaline casts (black dots). A glomeruli with an increased Bowman’s space is marked with an arrow. (c) Histology from rat subjected to 120 min IRI 6 weeks post-surgery. Hyaline casts are noted, but tubular dilation and epithelial thinning are less pronounced. (d) Renal histology from rat subjected to 120 min IRI, 6 weeks post-surgery. The animals were treated with intra-arterial stem-cell based therapy. Histological damage is less marked than with saline treated animals.

Figure 4. Renal histology shows that after 2 weeks recovery there are significantly more epithelial breaks in rats subjected to 120 min IRI when compared to sham controls ($p<0.0001$). Similarly more epithelial cell breaks are observed in the 6 week group than in sham operated animals. ($p<0.0001$; Error bars represent SEM.).
inflict a severe, long-standing renal injury without excessive post-operative animal deaths. To address this, Wang et al. investigated the use of prolonged warm ischemic times in order to create a severe and sustained renal injury model. They observed warm ischemic times of up to 90 min which were associated with long-term disruption of renal architecture, increased levels of apoptosis and renal fibrosis. However, they reported prolonged ischemia resulted in almost 80% animal deaths at 4 weeks, although renal function as determined by serum creatinine, had normalized by this time.

Rodent kidneys subjected to prolonged periods of warm ischemia have been reported to display abnormal histology after several days’ recovery, while the serum creatinine/blood urea nitrogen (BUN) was found to be normal. This paradox may be explained by the relative insensitivity creatinine and BUN at quantifying renal function in rodents. While convenient, these surrogate markers of glomerular filtration do not become significantly elevated until 50–75% of kidney function is lost. In addition, creatinine secretion varies in acute renal failure. Creatinine clearance studies are reported to be more accurate than serum analysis alone, but necessitate the timed collection of urine in conjunction with serum analysis. This makes creatinine clearance an impractical tool for estimating renal function in post-operative rodents. Furthermore, such studies have been shown to overestimate GFR by as much as 10%.

With blood analysis as it was unable to accurately determine both short and long-term renal function in IRI experiments, any conclusion regarding the efficacy of therapies based on such markers is questionable. In order to accurately quantify the effect of novel treatments, other methods of demonstrating renal function should be employed.

Glomerular filtration rate (GFR) is accepted as the best overall measure of kidney function. Many different experimental approaches have been used to calculate GFR, with constant infusion clearance (CIC) or bolus clearance techniques, widely accepted methods. Both approaches are based on the clearance of exogenous renal markers, with inulin clearance regarded as the ‘gold standard.’ Inulin is an ideal GFR reporter since it is freely filtered at the glomerulus, but not secreted nor reabsorbed by tubular epithelial cells. Using a CIC approach, a continuous inulin infusion achieves a constant plasma inulin concentration, and timed urinary and blood collection are used to determine GFR. However, a number of different CIC protocols are described in rodent experimentation, without a single recognized optimized procedure enjoying widespread use.

The model we describe goes some way to addressing the problems encountered by Wang et al., and additionally has several advantages over commonly used in vivo models. We are able to inflict a reproducible and severe renal IRI without excessive post-operative mortality, and by cannulating both ureters in a terminal procedure, are able to accurately calculate the GFR of each kidney by inulin clearance. This is the most sensitive method of characterizing the injury that results from a given ischemic time and also of assessing the reno-protective effect of any therapeutic intervention.

Given the severity of the ischemic insult inflicted, a major advantage of our model is the low number of post-operative animal deaths we observed. This probably reflects the relative lack of metabolic disturbance that results from the filtration of the uninjured right kidney. It may be argued that this produces an artificial setting, leaving an injured kidney to recover in a non-uremic environment. However, a non-uremic state after renal IRI has been reported to increase fibrosis in injured kidneys, hindering long-term renal recovery by arresting tubular epithelial cells in G2/M phase of the cell cycle. We are not aware of any studies that show such a model to be unreliable.

Furthermore, we believe this is the first description of therapy given directly into the renal artery in an in vivo animal model. Intra-renal artery infusion is a more technically difficult procedure than either intravenous injection or aortic injection via carotid cannulation, but with practice it is possible to achieve arterial anastomotic patency rates approaching 100%. By using this
route, we are able to minimize systemic drug distribution and provide a high local concentration of therapy to the damaged kidney. Importantly, this method mimics the therapeutic route likely to be employed in clinical transplantation, where the renal artery is easily accessible for drug administration prior to implantation. This route could easily be utilized in a rodent transplant model, however results obtained using our model are less variable as there is no need for venous and ureteric anastomosis. Hence, we are able to screen potential therapies’ efficacy prior to their use in a more technically complex transplant model. This reduces animal numbers needed to test potential drugs for clinical transplantation and allows refinement of dosing schedules.

Disclosure statement

The authors report no conflicts of interest.

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