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Robust Rat and Mouse Models of Bilateral Renal Ischemia Reperfusion Injury

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Abstract. *Background/Aim: Acute and chronic kidney diseases are a major contributor to morbidity and mortality worldwide, with no specific treatments currently available for these. To enable understanding the pathophysiology of and testing novel treatments for acute and chronic kidney disease, a suitable in vivo model of kidney disease is essential. In this article, we describe two reliable rodent models (rats and mice) of efficacious kidney injury displaying acute to chronic kidney injury progression, which is also reversible through novel therapeutic strategies such as ischemic preconditioning (IPC). Materials and Methods: We utilized adult male Lewis rats and adult male wildtype (C57BL/6) mice, performed a midline laparotomy, and induced warm ischemia to both kidneys by bilateral clamping of both renal vascular pedicles for a set time, to mimic the hypoxic etiology of disease commonly found in kidney injury. Results: Bilateral ischemia reperfusion injury caused marked structural and functional kidney injury as exemplified by histology damage scores, serum creatinine levels, and kidney injury biomarker levels in both rodents. Furthermore, this effect displayed a dose-dependent response in the mouse model. Conclusion: These rodent models of bilateral kidney IRI are reliable, reproducible, and enable detailed mechanistic study of the underlying pathophysiology of both acute and chronic kidney disease. They have been carefully optimised for single operator use with a strong track record of training both surgically trained and surgically naïve operators.*

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Key Words: Kidney injury, ischemia reperfusion injury, mouse, rat.

Ischemia reperfusion injury (IRI) is a major contributor to acute kidney injury (AKI) following which, failed kidney recovery may progress to chronic kidney disease (CKD)(1), costing the National Health Service (NHS) £1.45 billion every year (2). Currently, there are no specific treatments for those with AKI and the mechanisms behind subsequent recovery *versus* progressive fibrosis remain poorly defined. As a clinically important cause of kidney injury, IRI is widely studied experimentally. A variety of techniques exist to model AKI, including toxin-induced nephropathy (*e.g.*, aristolochic acid, gentamicin, cisplatin, high dose folic acid, warfarin, and glycerol), and surgical models (*e.g.,* unilateral ureteral obstruction, renal mass reduction and renal vascular pedicle clamping) (3).

There are several surgical IRI models of kidney injury including unilateral IRI, bilateral IRI and unilateral IRI with contralateral nephrectomy, with specific advantages and disadvantages of each method. We advocate the use of a surgical model that involves the direct and timed clamping of both renal pedicles (bilateral IRI). This is because it more closely mimics the bilateral injury seen in human diseases and permits subsequent functional evaluation of kidney injury. Unilateral models rarely reflect the severity of injury, as remodeling and adaptive processes occur in the remaining kidney (4). This has led many

Table I. *Detailed protocol for surgical bilateral ischemia reperfusion injury in the mouse and rat model.*

	Pre-operative preparation of animal: Timing 3-5 min
1	24 h prior to the intended surgical procedure, each animal will have a thorough health check and transferred to individual cages. Note: Animals from an external animal facility require 7 days of acclimatization according to our local institutional policy prior to experimental use as per home office guidelines.
2	Each animal is given 200 micrograms of buprenorphine dissolved in 200 ml of water. This will continue until day 7 (for those animals being culled beyond day 7), after which normal drinking water is supplied. This is the same for both mice and rats.
	Anesthesia: Timing 4-5 min
3	Anesthesia is provided in the induction chamber using a combination of 5% isoflurane in oxygen at a rate of 2-3 ml/min.
4	Once anesthetized, animal is weighed and then transferred to the operating board.
5	Animal is placed in a supine position, immobilized with plain surgical tape.
6	Maintenance anesthesia is provided *via* tube mask covering the face the animal.
7	Flow of isoflurane and oxygen is titrated to maintain balanced anesthesia; for example, this is often provided by approximately 2% isoflurane delivered with approximately 1-2 ml/min oxygen.
8	Rat only: 0.3-0.4 ml of blood is taken from tail vein once the animal is fully anesthetized.
	Surgical Procedure: Timing dependent on time of ischemia (*e.g.*, 20-30 min for mouse and 45-50 min for rat)
9	The abdomen is prepared by the removal of excess fur from the abdomen with hair trimmers.
10	An incision is made in the midline using a scalpel and a plane is created between the skin and the muscle layer.
11	The rectus sheath is opened in the midline (through the linea alba) and rectus muscles on either side are retracted using an alms retractor.
12	Once safe access to peritoneal cavity is achieved, bowels are moved to the right side gently with sterile cotton buds. The left kidney renal vascular pedicle is identified, dissected out (with fine forceps with curved tips) and clamped under direct vision with a microvascular clamp.
13	Timing of the clamp is noted.
14	Bowels are then moved to the left side with the sterile cotton bud, the right kidney and its vascular pedicle is identified, dissected out and clamped. Time is noted again.
15	The kidneys are visually assessed for macroscopic ischemic injury which is evidenced by change in color from a normal pink to purplish brown.
16	Bowels are repositioned in the peritoneal cavity, Alms retractor removed, and a small saline soaked gauze piece is used to cover the incision to minimize the fluid losses and hypothermia.
17	Each clamp is removed after exactly the specified time of ischemia for each kidney according to the experiment (*e.g.*, 20 min for mice and 45 min for rats).
18	Kidneys are observed for reperfusion and a return of the pink color macroscopically.
	Closure process: Timing 4-5 min
18	Abdominal cavity is inspected for any immediate complications.
19	Rectus sheath and skin are closed with absorbable (4/0 vicryl) continuous sutures. The final knot in the skin is buried to prevent the animal attempting to chew on it.
20	The sham animals undergo the same procedure without the renal vascular pedicle clamping.
21	At the completion of the procedure, anesthesia is disconnected, and animal is recovered in their cage. The animal is observed closely until full recovery.
	Follow up: Timing 2-3 min per check (variable)
22	Animals are observed daily for general appearance, eating and drinking, wound inspection for infection or dehiscence and any signs of distress.
	Re-laparotomy and retrieval of tissue and blood: Timing 10-15 min
23	At the end of assigned time point for culling (0 h to 28 days), animals are again anesthetized using combination of isoflurane and oxygen, positioned on the operating board, and maintenance anesthesia is provided as mentioned above.
24	Midline wound is opened followed by opening of the pleura to gain access to the chest cavity. Total blood circulating volume is aspirated from the heart by direct puncture to cause exsanguination (and confirmation of permanent cessation of the circulation is made by surgical removal of cardiac tissue (as per project license).
25	Each kidney is carefully removed using dissecting scissors and placed in a receptacle for tissue processing and storage.
26	Anesthesia is disconnected and the animal carcass is then packed to be disposed of as per the animal unit guidelines and procedures.

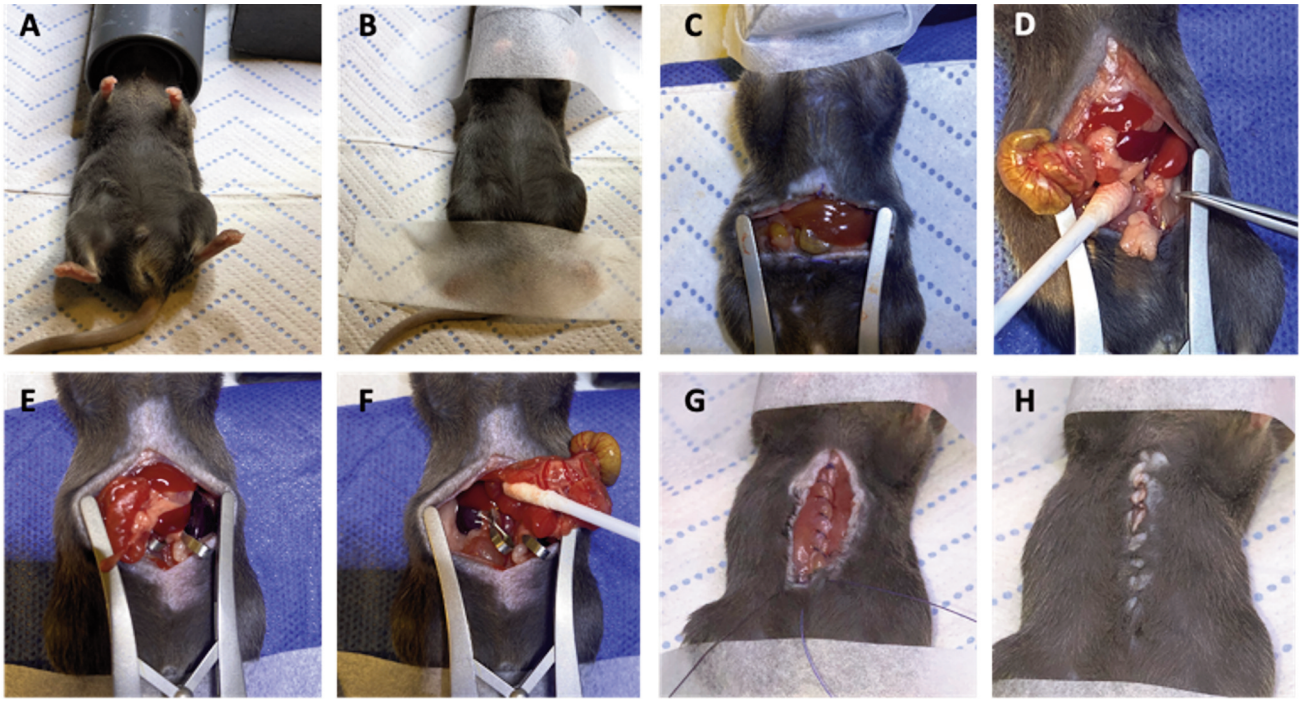

Figure 1. *Mouse model of ischemia reperfusion injury from maintenance of anesthesia to skin closure. A) Maintenance of anesthesia. B) Positioning and stabilizing the mouse. C) Exposure of the peritoneal cavity. D) The left kidney renal vascular pedicle is identified and dissected. E) Left kidney vascular pedicle clamped under direct vision with a microvascular clamp. F) Right kidney vascular pedicle clamped under direct vision with a microvascular clamp. G) Closure of the rectus sheath with continuous stitches. H) Closure of the skin. Note that sham animals undergo the same procedure without the renal vascular pedicle clamping.*

investigators to combine unilateral IRI with a second injury, such as nephrectomy or ureteric obstruction. The unilateral model with combined contralateral nephrectomy (used as a control) is a commonly utilized model. However, this requires very mild injury to ensure animal survival and is a non-functional model of modelling disease. In our opinion, sham animals provide ideal controls and a bilateral IRI model more accurately reflects the process of kidney injury seen in the clinical setting. Finally, our approach differs from other surgical IRI models utilizing flank incisions to gain access to the kidneys. We chose to use a midline laparotomy to gain access to the full abdominal cavity, as this allows excellent exposure of the operative field and easy access to both renal pedicles for accurate dissection and clamping.

Our overall aim was to characterize functional and structural kidney injury from the acute phase (AKI) through to its progression to CKD, mimicking human pathology, and secondly for this injury to be potentially reversible to enable testing of novel therapeutic strategies. In this study, we describe two rodent models (mouse and rat) of efficacious, recoverable and mild-moderate kidney injury that displays AKI secondary development of fibrosis compatible with progression to CKD.

Materials and Methods

Rodents and animal care. Eight-week-old Male adult Lewis Rats weighing approximately 180-240g (Charles River Laboratories, Bristol, UK) and Male adult C57BL/6 mice weighing >15 g (Charles River Laboratories) were purchased and maintained in an animal house with free access to water and pelleted food. All animal experiments were conducted according to the United Kingdom Use of Animals (Scientific Procedures) Act 1986, under license P6B0CD326. The animals were given a seven-day period of acclimatization to their new surroundings and were housed and handled according to the local institutional policies and procedures licensed by the Home Office. Ethical approval for all the protocols within the License was provided by the Animal Welfare and Ethical Review Body under the Establishment License held by Cardiff University. The ARRIVE guidelines were followed for all experiments involving use of animals.

Surgical techniques. See Table I, for further details. The animals underwent thorough health checks before transfer to individual cages 24 h prior to the intended surgical procedure. Analgesia was provided to individual animals 24 h prior to any surgical intervention until the time of tissue retrieval in the form of 200 mcg of buprenorphine (Sansoz, Camberley, UK, PC 05050650092042) dissolved in 500 ml of drinking water. Anesthesia was provided in the induction chamber using a combination of 5% isoflurane in oxygen at a rate of 2-3 ml/min. The animal was placed in a supine

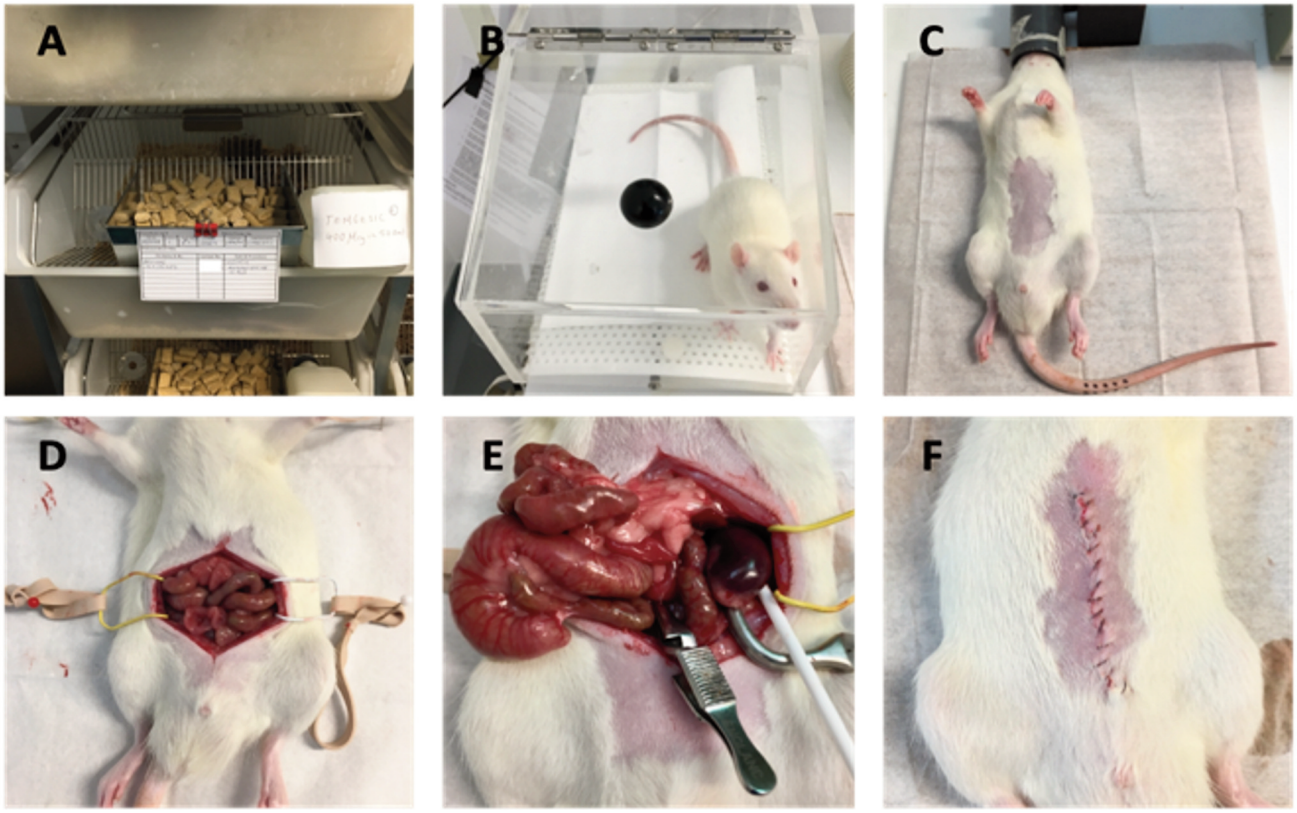

Figure 2. *Rat model of ischemia reperfusion injury demonstrating some aspects of the procedure. A) Rats were housed in conventional cages with readily available rat chow and water. B) Isoflurane anesthesia was delivered via an induction chamber. C) The central abdomen was shaved, and the rat positioned on a cork board covered in sterile drape. D) The abdomen was opened, and custom-made retractors were used to optimize visibility. E) The bowels were moved to the right side to allow visualization and clamping of the left renal pedicle. Ischemia was confirmed by the dark discoloration of the kidney. F) Following the procedure, the abdomen was closed using a two-layer technique.*

position, immobilized with plain surgical tape. Maintenance anesthesia was provided *via* tube mask covering the face by approximately 2% isoflurane delivered with approximately 1-2 ml/min oxygen. For rats, 0.3-0.4 ml of blood was taken from tail vein once the animal was fully anesthetized.

The abdomen was prepared by the removal of excess fur from the abdomen with hair trimmers. Under aseptic conditions, a midline laparotomy was performed. Once safe access to peritoneal cavity was achieved, bowels are moved to the right side gently with sterile cotton buds. The left kidney renal vascular pedicle was identified, dissected out (with fine forceps with curved tips) and clamped under direct vision with a microvascular clamp. The bowels were then moved to the left and the same process was done for the right kidney renal vascular pedicle. Care was taken to avoid any damage to surrounding structures. If IPC was utilized, this can be performed according to our previous methods (5). The kidneys were visually assessed for macroscopic ischemic injury which is evidenced by change in color from a normal pink to purplish brown. Each clamp was removed after exactly the specified time of ischemia for each kidney according to the experiment (*e.g.*, 20 min for mice and 45 min for rats). The kidneys were visually assessed again for return of color to pink. Rectus sheath and skin were closed with absorbable (4/0 vicryl) continuous sutures. Sham controls underwent the same operation without the renal pedicle clamping. At the completion of the procedure, anesthesia was disconnected, and the animal was observed closely until full recovery.

At the end of assigned time point for culling (1 to 28 days), animals were again anesthetized using combination of isoflurane and oxygen, positioned on the operating board, and maintenance anesthesia was delivered as mentioned above. Midline wound was opened followed by opening of the pleura to gain access to the chest cavity. Total blood circulating volume was aspirated from the heart by direct puncture to cause exsanguination (and confirmation of permanent cessation of the circulation was made by surgical excision of the heart. Each kidney was carefully removed using dissecting scissors and placed in a receptacle for tissue processing and storage. Anesthesia was disconnected and the animal carcass was then packed to be disposed of as per the animal unit (Joint Biological Services Unit, Cardiff University) guidelines and procedures.

Histology. Kidney tissue was embedded in paraffin and sectioned for Hematoxylin and eosin (H&E) staining. Scoring was carried out in a blinded fashion using the Endothelial, Glomerular, Tubular, and Interstitial (EGTI) scoring system, developed specifically for animal research on kidney tissue in the context of injury (5, 6).

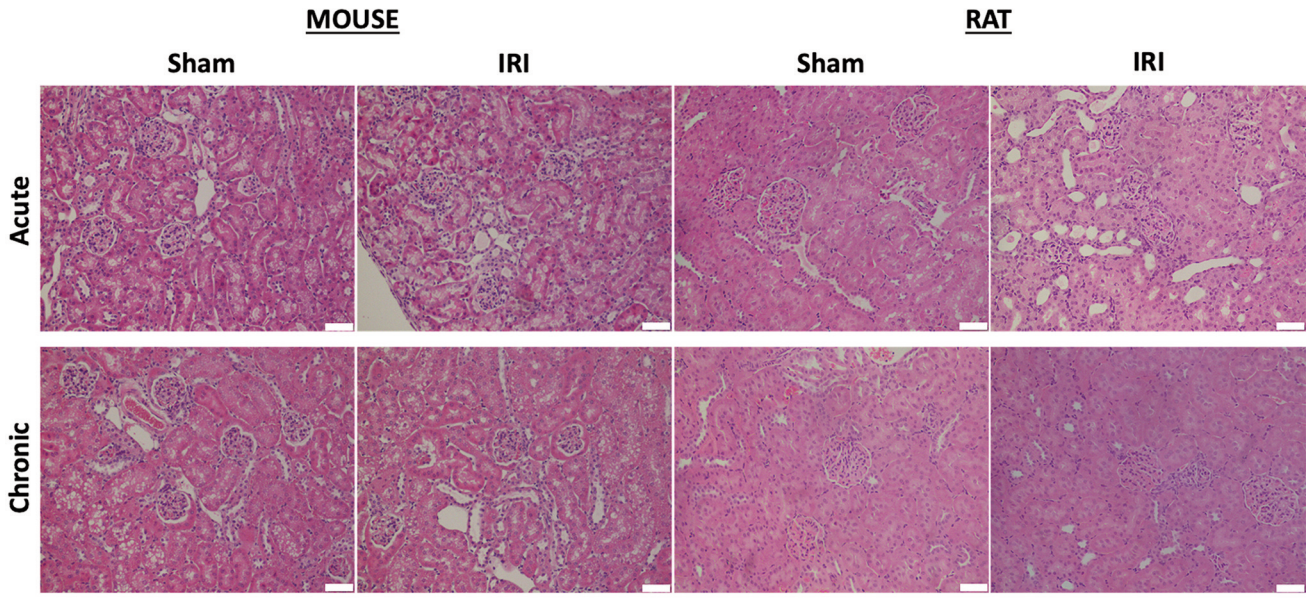

Figure 3. *Histological analysis of mouse and rat renal cortex in both sham (control) and IRI models. Kidney tissue was embedded in paraffin and sectioned for hematoxylin and eosin (H&E) staining. Sections from the mouse and rat were examined using a Leica DMLA Light Microscope. Representative photographs were taken using an Olympus DP27 Microscope Digital Camera. Representative renal cortex mouse and rat histology in ischemia reperfusion injury (IRI) (20 min in the mouse 45 min in the rat) and sham animals, 48 h (Acute) and 28 days (Chronic) after injury. H&E micrographs (×200) show normal appearance in sham (A, C, E and G), and moderate to significant damage in both mouse and rat IRI models (B, D, F and H). Scale bar=50 mm.*

Renal function. Serum creatinine was measured in blood samples of rats taken pre-operatively (at 0 h) and in samples from rats and mice at time of retrieval using the Jaffe reaction.

RT-qPCR. Whole kidney tissue was stored in RNA later solution immediately at time of retrieval and frozen at −80˚C. Total RNA was extracted using TRIzol reagent (Cat. No. 15596018; Life Technologies, Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to manufacturer's instructions. RNA quality was assessed using the Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Santa Clara, CA, USA) and quantified prior to RT-qPCR. cDNA was generated using a High-Capacity Reverse Transcription Kit (Cat. No. 4368814; Life Technologies) with random primers. RT-qPCR was performed on a ViiA7 Fast Real-Time PCR System (Life Technologies). Primer sequences: NGAL (forward: 5'-GGGCTGTCCGATGAACTGAA-3'; reverse: 5'-CATTGGTCGGTGGGAACAGA-3') and KIM-1 (forward: 5'CGGCTAACCAGAGTGACTTGT-3'; reverse: 5'-TACAGAGCCTGGAAGAAGCAG-3') were quantified using POWER SYBR GREEN PCR Master Mix (Cat. No. 4368706; Life Technologies) with gene-specific primers, normalized to GAPDH (forward: 5'-CCTCTGACTTCAACAGCGACAC- 3'; reverse: 5'-TGTCATACCAGGAAATGAGCTTGA-3'), as previously described (5, 6).

Statistical analysis. Statistical analyses were performed using GraphPad Prism v10 software (San Diego, CA, USA). Data are expressed as mean±SD or median (and range) and assessed for statistical significance using unpaired Student's *t*-test or Mann-Whitney *U*-test.

Results

This procedure has been adapted in both male and female rat and mouse strains. We have successfully used rats aged 10-20 weeks weighing 150-400 g, and mice aged 10 weeks – 1 year weighing over 15 g.

The model of IRI is a stepwise process (Figure 1 and Figure 2) that enables the assessment of induced kidney disease. Animals were randomly allocated by random number generator on the day of the experiment into either sham or IRI groups until each experimental group was complete (n=5 per group), data analysis was performed by the operator. Animals were kept in the same living and maintenance facility to reduce the influence of external confounders. All experiments were performed blinded to group allocation until the point of data analysis.

The effect of IRI was dose-dependent as shown in the mouse model, where increasing durations of renal pedicle clamping increases the kidney injury response according to the established biomarkers of kidney disease: serum creatinine, neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1). In addition, quantitative histological analysis was included to validate the effects of acute injury seen at 48 h (Figure 3, Figure 4, Figure 5). Mouse and rat IRI models both showed increases in creatinine, KIM-

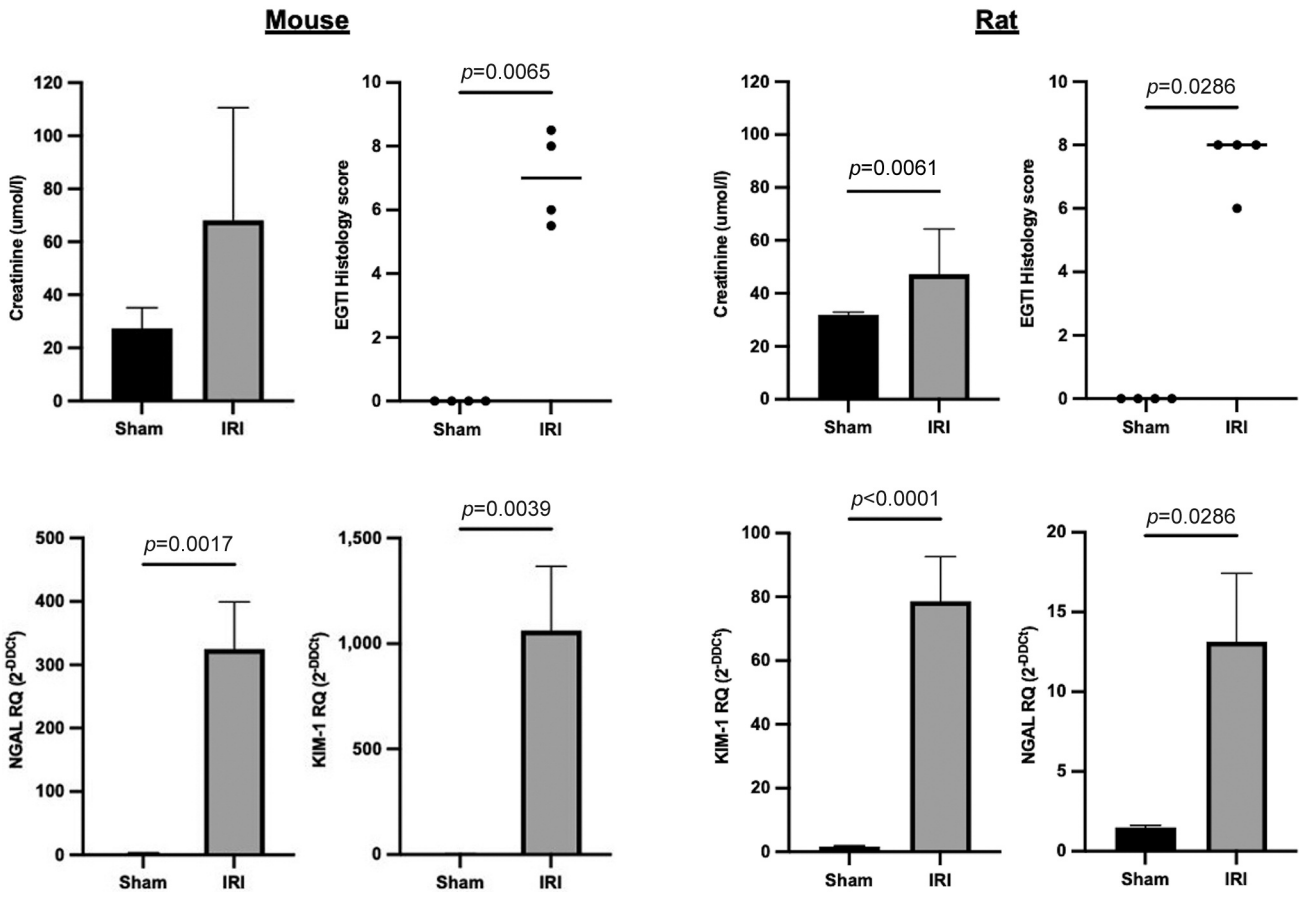

Figure 4. *Effect on Endothelial, Glomerular, Tubular, and Interstitial (EGTI) histology damage score, serum creatinine and renal injury markers NGAL and KIM-1 48 h after ischemia reperfusion injury (IRI) in the mouse (20 min; n=5 sham n=5 IRI (total 10 mice) and 48 h after IRI in the rat (45 min; n=5 sham n=5 IRI (total 10 rats). Serum creatinine, NGAL and KIM-1 was measured after 24-48 h and is plotted as mean±SD and analyzed by unpaired Student's t-test. Categorical data are expressed as median (and range) and analyzed using Mann-Whitney U-test. Significance level was pre-specified as p-value<0.05 and is annotated where this threshold is met. RT-qPCR analysis of NGAL and KIM-1 was performed following RNA extraction from whole kidney tissue and expression was normalized to GAPDH.*

1, NGAL and histology damage scores when compared to sham animals. In both models, we reliably reproduced mild-moderate kidney injury which WAS not present in sham animals. Although serum creatinine levels were increased in surgical IRI groups, NGAL, KIM-1 or histology damage scores may better reflect severity of disease.

When considering which rodent model to use, there are clear differences in the responses between the mouse and rat model. These differences can be strain dependent, therefore optimization of the duration of IRI should be considered in different rodent strains.

Discussion

Although a variety of species have been used to model kidney disease (4, 7-10), we chose to develop both rat and mouse models of IRI. Rodents in general are reliable animals, easy to breed, simple to maintain within an institutional infrastructure and well tested over a variety of conditions and timepoints (11). In comparison to mice, rats are generally larger therefore, easier to operate on, easier for administration of novel drugs and test therapeutic interventions. Mice are much smaller, and marginally more challenging to operate on due to their size. However, they allow for evaluation of specific genes of interest due to a greater availability of transgenic and genetically modifiable strains.

Our model has been shown to produce functionally and structurally evident kidney injury and support the testing of novel treatment strategies such as Ischemic Preconditioning (IPC) in both acute and chronic injury in the rat model (5, 12). Moreover, we have previously shown that, by employing laser capture microdissection for isolation and analysis of specific cell types on tissue samples, this

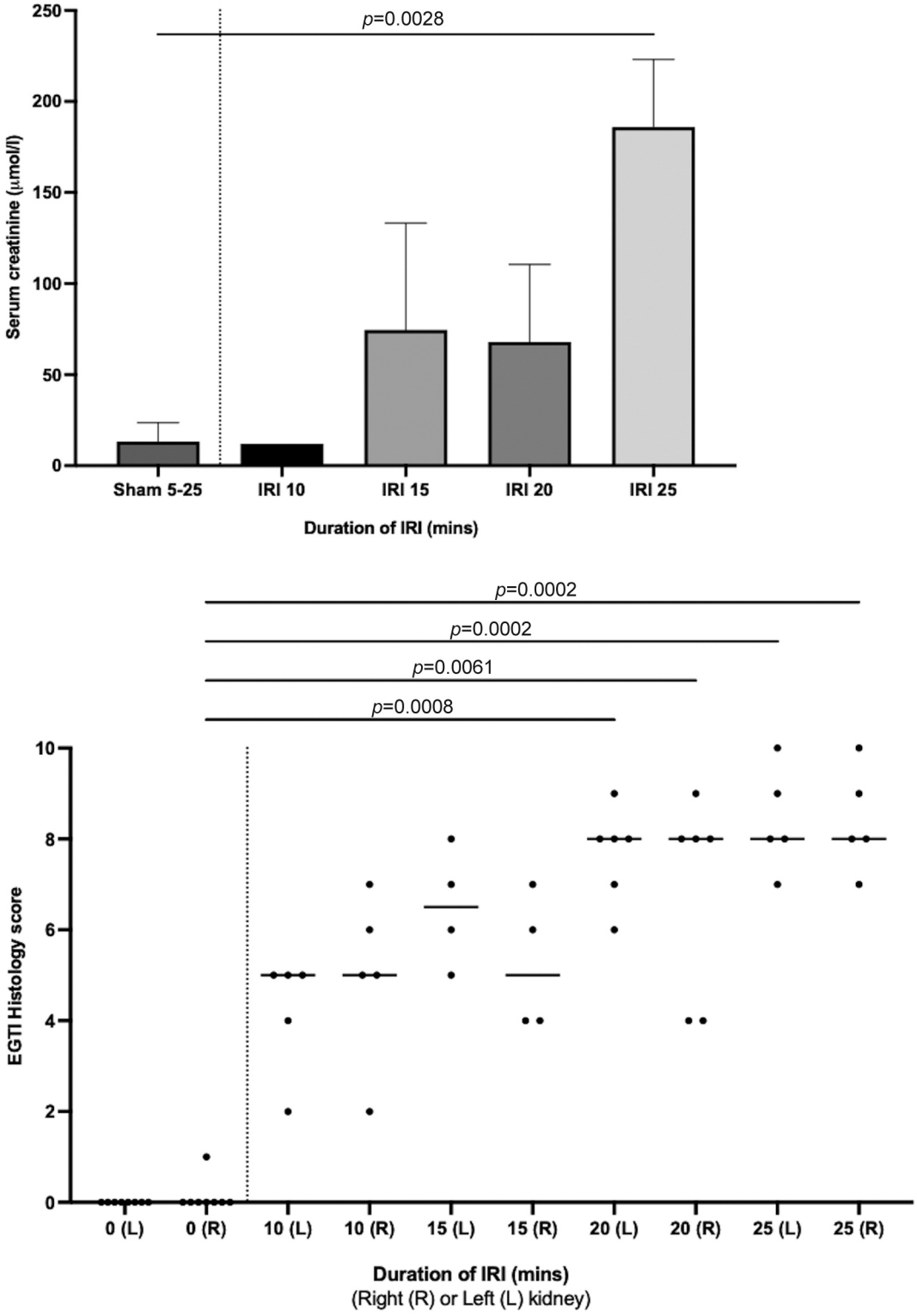

Figure 5. *Mouse creatinine [n=5 sham, n=5 ischemia reperfusion injury (IRI); 10 mice in total] and Endothelial, Glomerular, Tubular, and Interstitial (EGTI) histology scores (n=5 sham, n=1 IRI 10 min, n=3 IRI 15 min, n=3 IRI 15 min, n=5 IRI 25 min) for varying durations of IRI (10-25 min) compared to sham mice (control). Paired kidneys analyzed from the same animal and plotted as right (R) or left (L) kidney above. Categorical data are expressed as median (and range) and analyzed using the Mann-Whitney U-test. A. Creatine levels after IRI. EGTI histology scores in both the right and left kidney after 10-15 min of IRI.*

Table II. *Table of expected adverse outcomes.*

Expected adverse effects
Post-operative pain: This will be possible in all cases. There will be regular observation to look for signs of distress, such as piloerection, lack of mobility, inactivity, or appearance of weakness. Animals will be treated with administration of analgesia under the guidance of the Named Veterinary Surgeon (NVS). There will be regular observation of the animal's wellbeing, checking for clinical signs described in the score sheet. If clinical signs continue, indicating that the analgesia has not been successful in controlling pain, animals will be killed by a Schedule 1 method.
Wound breakdown: Minimal frequency expected but recognized by signs of an open wound. There will be regular observation of the animal's wellbeing and inspection of the wound. If there is superficial dehiscence, within reason and in agreement with named veterinary surgeon, the wound can be re-sutured on one occasion. If there is complete wound dehiscence, then the animal will be killed by a Schedule 1 method.
Uremia: Minimal frequency which is usually expected in the first 24-48 h. In our previous experiments, we have not encountered uremia to be a major problem within the observation period. There are no specific reliable signs or symptoms of uremia, however regular observation of the animal's general wellbeing should be recorded. Animals will be provided with appropriate fluids *via* the feeding bottle as required and regularly observed. Animals will be killed by a Schedule 1 method if there are signs that the wellbeing of the animal is in decline (as per discussion with the named veterinary surgeon).
Partial kidney ischemia: This is often secondary to incomplete occlusion of renal vessels from initial clamping or anatomical variation of an accessory renal vessel. Firstly, identify incomplete ischemia by the lack of uniformity in the ischemia discoloration of the kidney. Once partial kidney ischemia is identified, remove the clamp, and identify vasculature supply to the kidney and clamp the entire renal vascular supply to the kidney on a second attempt and observe uniform discoloration of ischemic kidney.
Bleeding: Excessive bleeding can occur from several surgical sites. However, in our experience bleeding within the rodent animal is rare unless there has been direct iatrogenic injury to the organs, muscle, or vasculature structures. Bleeding from these structures can lead to major hemorrhage and necessitate termination of an animal. However, small amounts of bleeding from the abdominal wall can be managed by applying gauze to the area until bleeding is managed.
Apnea: Apnea can occur at any time during anesthesia. However, careful titration of anesthetic to ensure appropriate levels are maintained is essential to prevent over-anesthesia. If apnea is observed, a reduction in inhalation agent should be initiated to prevent hypoxia whilst maintaining appropriate levels of anesthesia.

protective IPC signal originates from the proximal tubular cells (5). These animals typically recover very well from the surgery with minimal adverse outcomes (Table II). The ability of these models to demonstrate robust kidney injury (both AKI and chronic fibrosis) and then attenuate it with effective treatment strategies, makes them very attractive to study kidney disease and test treatments.

There are some limitations worthy of note implicated with the use of this surgical animal model, including the cost of maintenance, surgical training time and translation to human pathology. However, by adhering to the principles of the 3Rs of reduction, replacement, and refinement (13), these limitations are offset by the ability of these models to advance our understanding of disease and accelerate drug discovery and development of novel therapeutics. Furthermore, these refined models allow for replacement of non-functional models of AKI.

There are significant advantages of using these rodent bilateral renal IRI models. First, rats and mice are easy to maintain and house. Larger mammal species require a more expensive and labor intensive set up, limiting their use. Although, this is a surgical model that is amenable to both unilateral and bilateral injury, the bilateral model more closely mimics the clinical disease and is preferred. In addition, it allows for both functional and structural evaluation of kidney injury which is only possible in the bilateral model. Furthermore, this model is well validated by a range of research groups, with reliable and reproduceable results across many species. Our model has been designed over several years to enable single operator use and it has been used extensively within our research group with minimal complications. These animal models enable us to investigate complex disease processes in a controlled environment.

Conclusion

We present the iterative development of a protocol for bilateral IRI in mice and rats. The protocol can be adapted to support bilateral protective ischemic preconditioning, indirect preconditioning utilizing ischemia of muscle, or intraperitoneal/intravenous/subcutaneous drug administration.

Conflicts of Interest

The Authors declare no competing interests in relation to this study.

Authors' Contributions

R.C., D.F., and U.K. conceived and designed the research. T.S., A.Z., C.V.M.B., and U.K. conducted data collection and collected samples. U.K. and G.P.C. performed sample and data analysis. T.S., A.Z., D.F., and U.K. wrote the manuscript, D.F, T.B, R.C, and S.M. reviewed the manuscript. All Authors read and approved the final manuscript.

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