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Ischemia-reperfusion Model of Acute Kidney Injury and Post Injury Fibrosis in Mice

Nataliya I. Skrypnyk[1], **Raymond C. Harris**[1], and **Mark P. de Caestecker**[1]

[1]Division of Nephrology, Vanderbilt University Medical Center

Abstract

Ischemia-reperfusion induced acute kidney injury (IR-AKI) is widely used as a model of AKI in mice, but results are often quite variable with high, often unreported mortality rates that may confound analyses. Bilateral renal pedicle clamping is commonly used to induce IR-AKI, but differences between effective clamp pressures and/or renal responses to ischemia between kidneys often lead to more variable results. In addition, shorter clamp times are known to induce more variable tubular injury, and while mice undergoing bilateral injury with longer clamp times develop more consistent tubular injury, they often die within the first 3 days after injury due to severe renal insufficiency. To improve post-injury survival and obtain more consistent and predictable results, we have developed two models of unilateral ischemia-reperfusion injury followed by contralateral nephrectomy. Both surgeries are performed using a dorsal approach, reducing surgical stress resulting from ventral laparotomy, commonly used for mouse IR-AKI surgeries. For induction of moderate injury BALB/c mice undergo unilateral clamping of the renal pedicle for 26 min and also undergo simultaneous contralateral nephrectomy. Using this approach, 50–60% of mice develop moderate AKI 24 hr after injury but 90–100% of mice survive. To induce more severe AKI, BALB/c mice undergo renal pedicle clamping for 30 min followed by contralateral nephrectomy 8 days after injury. This allows functional assessment of renal recovery after injury with 90–100% survival. Early post-injury tubular damage as well as post injury fibrosis are highly consistent using this model.

Keywords

Medicine; Issue 78; Immunology; Infection; Biomedical Engineering; Anatomy; Physiology; Kidney; Mice; Inbred Strains; Renal Insufficiency; Acute Kidney Injury; Ischemia-reperfusion; acute kidney injury; post injury fibrosis; mice; ischemia; reperfusion; fibrosis; animal model

Video Link

The video component of this article can be found at http://www.jove.com/video/50495/

Correspondence to: Nataliya I. Skrypnyk at Nataliya.Skrypnyk@vanderbilt.edu.

Disclosures

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Introduction

A variety of experimental models of acute kidney injury (AKI) have been developed to match the diversity and complexity of the human condition (see reference for a recent, comprehensive review[1]). Each of these models has its own strengths and weaknesses, and while each mimics the corresponding human conditions with varied efficiencies, none precisely model the pathophysiology of their human counterparts. Ischemia reperfusion (IR)-induced AKI has been developed as a model of acute ischemia-induced renal injury in rodents. While the severity of renal tubular injury seen in this model is rarely observed in patients with renal hypoperfusion injuries[2], despite its limitations and largely because of the relatively reproducible nature of this model, its extensive use has provided and, it is expected, will continue to provide important insights into many of the common basic mechanisms of AKI, repair, and therapy[3]. IR surgery requires familiarity with the mouse kidney anatomy, which we have illustrated in a simplified form in Figure 1. Renal ischemia-reperfusion (IR) injury surgery can be performed via ventral (laparotomy) or dorsal (retroperitoneal) approaches. We use a dorsal approach since it is less traumatic, allowing faster recovery times and improved survival (particularly when first learning the procedure). Renal IR injury can be performed unilaterally or bilaterally. However, differences between effective clamp pressures (which may result from interposition of perihilar fat between the clamp jaws) and/or differences in renal responses to ischemia between sides leads to more variable results. While this is not an insurmountable problem, it can increase variability between experiments, which is a major issue for this model. Unilateral IR can be performed with contra-lateral nephrectomy. This is our method of choice since it reduces variability in clamping between pedicles, and at the same time allows one to evaluate renal function, which is unaffected with unilateral IR alone. There has been discussion about what is the most practical vs. optimal method to assess renal function in mice. Blood urea nitrogen (BUN) provides a measure of renal function and is a useful "first look" marker in some models of AKI, including IR injury. However, BUN levels can be affected by volume status of the mice which may be affected, particularly following ventral approach IR injury, when delayed recovery reduces oral intake of fluids for a number of days post-surgery. Serum creatinine is less influenced by hydration status but is clearly affected by muscle mass. One of the difficulties with serum creatinine measurements has been problems with detection of non-creatinine chromogens in mouse serum using picric acid based techniques. As an alternative, a number of centers have developed an HPLC-based method to quantify mouse creatinine that is not affected by this artifact[4]. However, unlike BUN and picric acid creatinine assays, which only require 5–10 μl of serum, HPLC-assays require ~ 25 μl serum per assay, which if performed in duplicate will require ~100 μl of whole blood per assay. This can be limiting for mouse studies. Some centers have developed more sensitive HPLC and Mass Spectrometry-based methods that allow analysis of smaller sample volumes[5, 6]. However, these technologies are not widely available. An alternative, enzymatic cascade assay (which requires only 5–10 μl of serum) has been evaluated in mouse and rat serum samples and shown to closely parallel HPLC measurements of serum creatinine while picric acid assays always over-estimate creatinine values[7] . While this assay is not widely used in the AKI literature, the assay is commercially available, simple to use, and we find gives reliable results with this model of IR-induced AKI in mice.

Protocol

1. Autoclave all surgical instruments before surgery. Note that if one is performing multiple surgeries on different mice, rinse instruments after use and then sterilize using a hot bead sterilizer. It is not sufficient to soak in 70% ethanol.
2. Give 0.5 ml S/C sterile normal saline preoperatively and immediately postoperatively to compensate for loss of body fluid during surgery.
3. Weigh the mice.
4. Anesthetize mouse using IP Xylazine/Ketamine mixture. It usually takes 3–5 min for the mice to reach surgical plane anesthesia.
5. Shave the surgical site with enough border area to keep hair from contaminating the incision site. This needs to be performed in a preparatory area, not where the surgery is performed.
6. Apply ophthalmic lubricating ointment to the eyes to prevent drying during the procedure.
7. Place the mouse prone on the heated surface covered with absorbent bench pad. Tape legs to the surgical surface.
8. If using the water bath system, set heat at 38 °C 1 hr prior to surgery.
9. Aseptic preparation using Betadine solution swab stick: scrub from the center of the site towards the periphery 3×, each time followed by scrubbing with Nolvasan to remove the Betadine. All Betadine should be removed from the surgical field prior to incision by rinsing with Nolvasan. Before starting aseptic procedure gloves should be changed to be sterile.
10. Cover surgical field with a sterile surgical drape. This will prevent stray hair from entering the surgical field and provide an area on which to lay sterile instruments during surgery.
11. Palpate kidney location through the skin.
12. Cut the dorsal skin along the midline of mouse (approximately 1.5 cm) using scissors and forceps.
13. Separate skin and subcutaneous layers over the left and right dorsal sides through this incision by blunt dissection using scissors and forceps.
14. Make a small incision through the right flank muscle and fascia above the kidney and exteriorize the right kidney.
15. Carefully dissect the upper and lower poles of the kidney free from surrounding tissue. Note that fatty tissue from around the upper pole contains the adrenal gland carrying its own blood supply which can be pushed off the kidney but obviously should not be removed from the mouse (Figure 1). After liberating kidney from surrounding tissue, tie the 4-0 silk suture around the hilum of the right kidney using a double surgical knot. Leave one end of the suture ~2 in long to hold up the kidney

as you are cutting distal to the knot so you can visualize the site to be cut. Irrigate the stump with sterile saline.

16. Close the muscle layer by using absorbable suture.
17. Make a small incision through the left flank muscle and fascia above the kidney and exteriorize the left kidney (Figure 2A).
18. Carefully hold kidney using blunt forceps while releasing renal pedicle from surrounding fat tissue using forceps (Figures 2B–2F). Too much pressure on the kidney using the blunt forceps may cause renal injury so one needs to remember this when designing "sham" controls for the studies. If you find that your sham controls are developing renal injury (as evidenced by a rise in serum creatinine), an alternative method you may consider to release the surrounding fat tissue from the renal pedicle is to gently roll the tissues with saline soaked sterile cotton-tipped swabs. In our experience the fat is fairly tightly attached to the renal pedicle and cannot easily be removed with cotton swabs, so this may not be the first approach you would want to use. However, like other aspects of this model, this is something that you will have to establish for yourself with practice and experience.
19. Clamp left renal pedicle using non traumatic vascular clamp using holding forceps (to facilitate clamp placement), use timer for different clamp times depending on the mouse strain and injury severity required (see Table 1).
20. Cover the left kidney with the skin (Figure 2H).
21. Cover skin incision with saline soaked gauze.
22. Exteriorize kidney, confirm uniform dusky appearance and release the clamp after the indicated times, and confirm that dusky appearance reverses uniformly throughout the kidney (Figure 2I/G). If the kidney does not rapidly and uniformly re-perfuse (pink up) the mouse should be excluded from further analysis. Gently push kidney back into the retroperitoneal space.
23. Close the muscle layer using absorbable suture.
24. Close the skin layer using monofilament nylon non-absorbable suture.
25. Administer buprenorphine analgesia.
26. Transfer mice away from the surgical field onto a heating pad until they wake.
27. Return the mice to animal room.
28. Closely monitor the mice and give additional doses of buprenorphine as indicated above every 8–12 hr for pain or discomfort. Clinical signs resulting in administration of analgesics include depression or other behavioral changes, abnormal appearance, or posture such as pilo-erection, hunched posture, lack of grooming and eating, or immobility.

Representative Results

Moderate IR-induced AKI

Unilateral IR with simultaneous contralateral nephrectomy substantially reduces variability in results, but with shorter clamp times necessary for the mice to survive this procedure, we still found that only 50–60% of mice developed the expected renal insufficiency 24 hr after injury (Figure 3A). In practical terms this creates difficulties evaluating data unless the studies involve treatment regimens that can be initiated at least 24 hr after injury, allowing time to assess renal function and discard mice that do not develop renal insufficiency.

Severe IR-induced AKI

In addition to performing unilateral IR with simultaneous contralateral nephrectomy, we perform unilateral IR alone. Mice can survive much longer periods of ischemia and tend to develop more severe post-injury fibrosis. With the same period of ischemia, mice subjected to unilateral ischemia develop more severe fibrosis than bilateral ischemia or unilateral ischemia with nephrectomy[8], so this model is also used as a model of post-AKI fibrosis. The disadvantage is that it is not possible to assess functional recovery after unilateral IR injury so there is no way of evaluating the severity of injury without harvesting kidney tissues and that may not be possible for longer term studies. For this reason we have developed a protocol for inducing severe unilateral IR injury, and assessing renal functional recovery by performing contralateral nephrectomy 8 days after the original injury. Using this approach 90–100% of mice survive the injury, but importantly the mice develop very consistent renal injury and can be evaluated for functional recovery following AKI from Day 9 onwards (Figure 3B). These findings are consistent with studies in rats indicating that longer renal pedicle clamp times induce more constituent renal tubular injury than shorter clamp times designed to induce mild to moderate AKI[9]. In addition, consistent with previous studies[8], these mice consistently develop post injury renal fibrosis (which we have assessed at Day 28). This is not associated with proteinuria at least on a BALB/c background: after severe IR-AKI at Day 28 post injury urinary albumin/creatinine ratio is 0.8± 0.04 (mean ± SEM in μg/mg). It is unknown whether the severe IR-AKI model gives rise to proteinuria on other backgrounds.

Discussion

We describe two models of IR-AKI to study the effects of moderate and severe renal injury. These models allow us to induce consistent and predictable injury with low mortality. Our protocol outlines many of the difficulties and pitfalls traditionally associated with this model. Moreover, we have shown that depending on the length of renal pedicle clamping, the model induces a largely reversible mild and moderate AKI, or more severe AKI with incomplete recovery and persistent renal fibrosis. Post-injury fibrosis in the severe IR-AKI model may result from the fact that mice are able to survive with more severe renal injury than would be possible if these longer ischemic times had been performed bilaterally. However, there is also evidence that the presence of an uninjured contralateral kidney for the first few days after IR enhances post-AKI fibrosis[8]. Therefore, while the mechanisms of cross talk between uninjured and injured kidneys remain to be established, this mechanism

may also account for enhanced post-injury fibrosis that we observe in this model. While it remains to be determined whether findings using these models can be translated into therapeutics for human disease, they provide reliable models to study mechanisms and therapeutics in IR-AKI and to prevent post-AKI renal fibrosis, a clinical problem that is emerging as a major contributor in progressive chronic kidney disease[10, 11].

There are a number of critical issues that can influence the severity of AKI following IR injury that need to be carefully attended to: mouse strain[12], gender[13], age[14] and weight of the mouse, the vascular clamp used, and heating systems[15]. Choosing the right vascular clamps can be a challenge. Efficiency of the clamp is assessed during the surgical procedure as the kidney should become uniformly black within 5–10 min of clamp placement. However, it is also important that the whole kidney is re-perfused (pinks up) rapidly after removing the clamp. We have found some of the heavier clamps work well with certain strains (*e.g.* BALB/c and C57Bl/6), but in others these clamps induce vascular injury and renal reperfusion is often incomplete (*e.g.*: CD1). A summary of the vascular clamps, clamp weights (pressure) and mouse strains that we have evaluated is listed in Table 1. For heating systems, a number of investigators use auto-regulated heating pads with temperature probes to monitor and maintain constant body temperature. While theoretically advantageous, we found this heating system can result in large swings in body temperature over the course of the surgery, giving rise to quite variable results. In addition, these systems are relatively expensive so may limit in the number of mice one can operate on at any one time. After a lot of trial and error we have moved to a constant temperature, circulating water heating system. The main advantage of this is that the heating platform gives stable temperatures (38 °C) throughout the surgery. With the large platform area it is also possible to operate on a number of mice on the surface at any one time (which is a practical requirement for most IR-AKI studies). Having said that, we did find there was still significant variability in injury following moderate (but not severe) IR-AKI. This is not a problem for studies in which treatments are initiated 24 hr or more post-injury, since mice developing insufficient or excessively severe injury can be removed from the study prior to randomization by analysis of serum creatinine. However, if mice are being treated pre-injury or if genetic models are being used, additional controls on experimental variability may need to be introduced. Since fluctuation in core body temperature is likely to be the major contributor to variability in IR injury in this model, one approach to limit variability would be to monitor core temperatures using rectal probes in order to determine appropriate animals to include or exclude in subsequent analyses. The final considerations are mouse age, weight, and gender. Most of our experience has been with BALB/c mice, and we have obtained the best results when mice reach stable body weight by 4–5 months of age, with body weights ~25–28 g. Finally, since males have greater susceptibility to IR-AKI than females[13], we perform all of our studies using male mice only. However, there are additional variables that are difficult to control, so each lab and each investigator will need to establish precise clamp times and conditions to induce the severity of injury they desire in the mouse strain of their choice.

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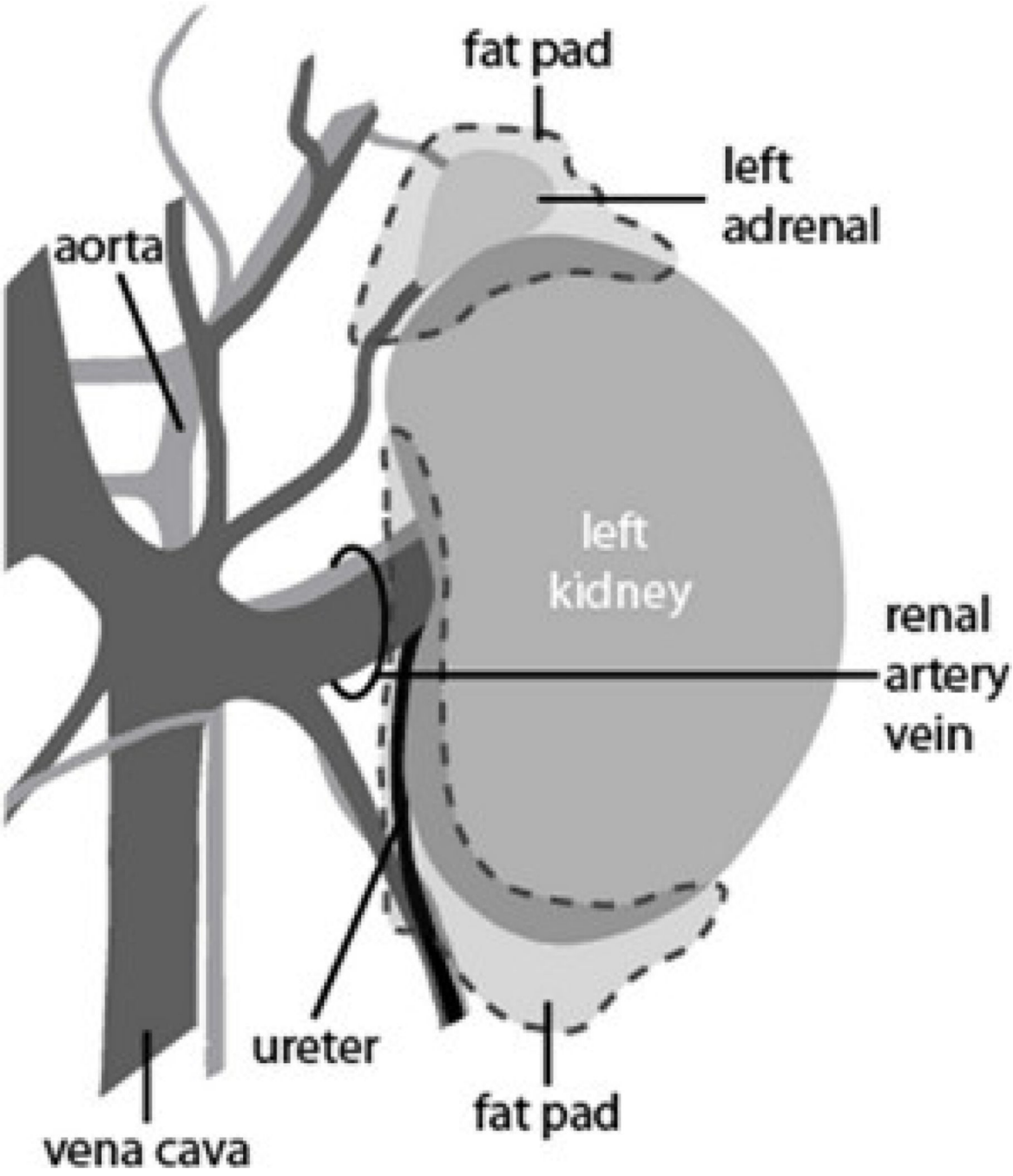

Figure 1. Anatomy of the mouse renal hilum

Illustrating position of the renal vascular sheath in relation to ureter, perinephric fat pads and the adrenal gland with blood supply. The black circle marks position of micro-clamp.

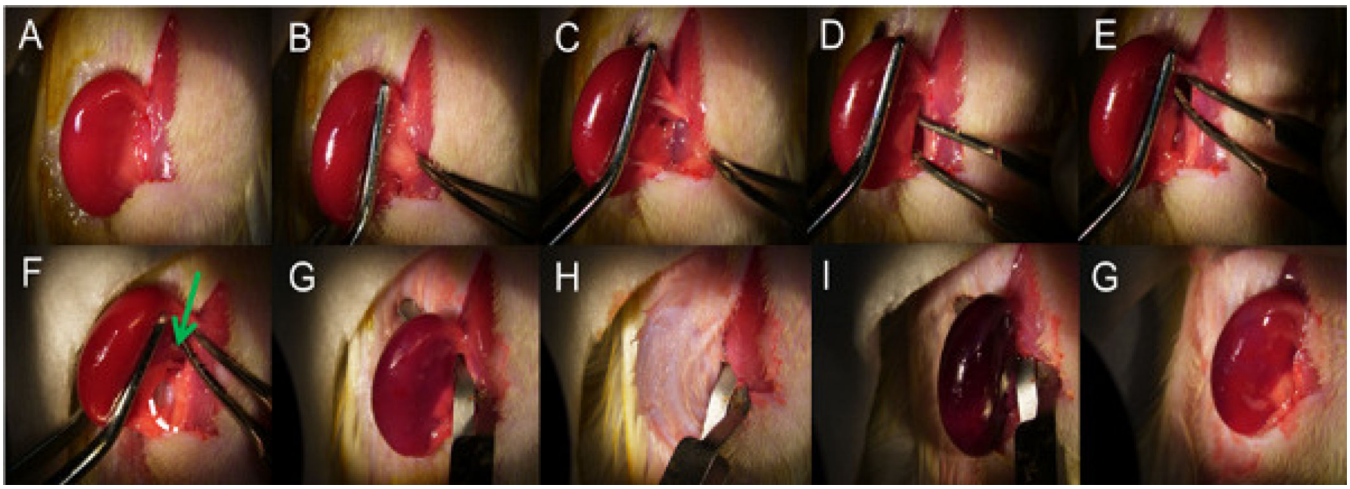

Figure 2. Exposure and clamping of mouse kidney vascular sheath

(A), exposure of the left kidney after dissection of skin and muscle layer; **(B – E)**, The left kidney is held gently using blunt forceps while perinephric fat is carefully removed using forceps. **(F)**, Exposed left renal pedicle (green arrow). The white dashed line marks the expected position of the ureter within the perihilar fat pad. This region needs to be avoided with the vascular clamp. **(G)**, Micro-clamp applied to the left kidney. **(H)**, Clamped left kidney is covered with the skin. **(I)**, **(G)**, Appearance of the kidney before **(A)**, during **(I)** and after **(G)** after removal of the vascular clamp.

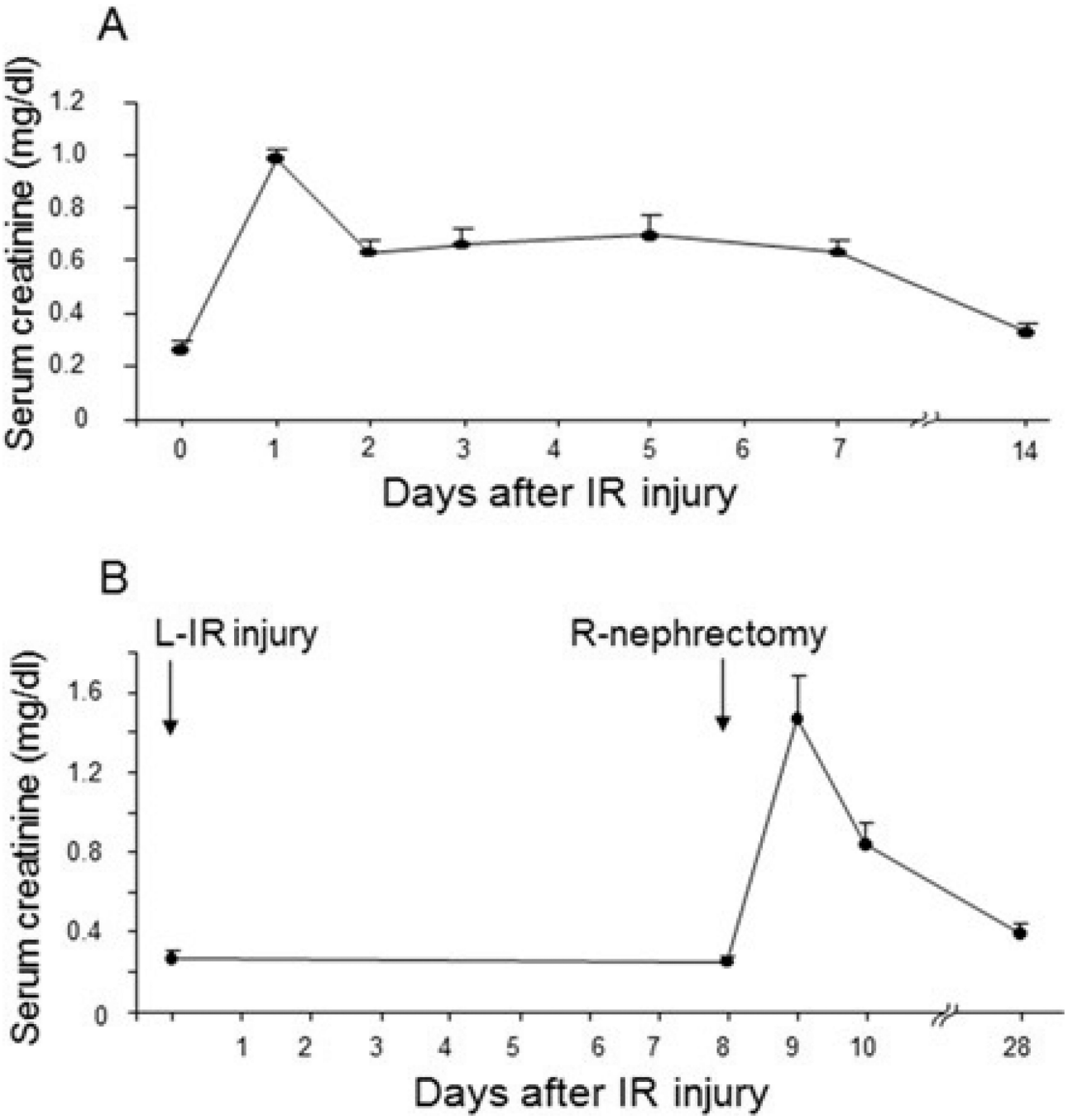

Figure 3. Functional recovery after AKI

Changes in serum creatinine (by enzymatic assays) after moderate IR injury (26 min clamp time) and contralateral nephrectomy **(A)**, changes in serum creatinine after induction of severe injury (30 min clamp time) and contralateral nephrectomy at Day 8 **(B)**. Studies performed in BALB/c mice, n=6 per group. Results expressed as mean+/−SEM serum creatinine in mg/dl, measured using enzymatic assay.

Table 1

Recommended vascular clamps, ischemia times, and mouse strains that we have evaluated.

Mouse strain	Approximate Mouse age	Approximate Mouse weight	Clamp type	Company and catalogue number	Ischemia time for severe injury	Ischemia time for moderate injury	Heating pad temperature
BALB/c	4–5 months	25–28 g	Vascular clamp (795 g pressure)	ROBOZ, RS-5459	30 min (or longer)	26 min	38 °C
C57Bl/6	4–5 months	24–27 g	Vascular clamp (795 g pressure)	ROBOZ, RS-5459	29 min (or longer)	26 min	38 °C
CD1	4–5 months	36–39 g	Vascular clamp (75–85 g pressure)	FST, 18055-02	29 min (or longer)	26 min	38 °C