STAR Protocols

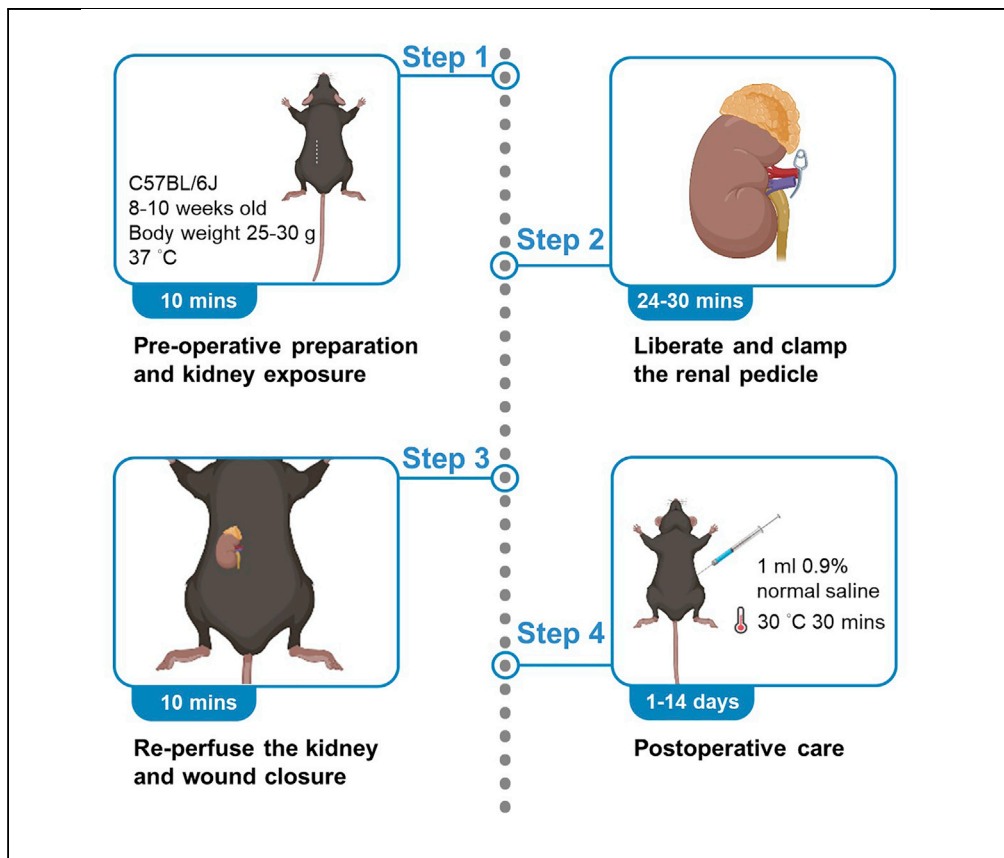**Protocol**

Protocol for renal ischemia-reperfusion injury by flank incisions in mice

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Highlights

Highly reproducible
procedure to study
renal ischemia-
reperfusion injury in
mice

Flank incisions
approach is
technically easier with
lower perioperative
injury

Detailed surgical tips
to avoid artificial
trauma or incomplete
pedicle clamping

Accessible renal
tissue for histologic
and molecular
analyses

Ischemia-reperfusion injury (IRI) contributes to acute kidney injury (AKI) and development of chronic kidney disease. We describe an IRI protocol for mice via flank incisions approach, using a pedicle clamp to cause ischemic injury. Compared with trans-abdominal approach, it is technically easier with lesser fluid loss and organ injury. Technical challenges during the dissection of renal pedicles are highlighted.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Protocol for renal ischemia-reperfusion injury by flank incisions in mice

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SUMMARY

Ischemia-reperfusion injury (IRI) contributes to acute kidney injury (AKI) and development of chronic kidney disease. We describe an IRI protocol for mice via flank incisions approach, using a pedicle clamp to cause ischemic injury. Compared with trans-abdominal approach, it is technically easier with lesser fluid loss and organ injury. Technical challenges during the dissection of renal pedicles are highlighted.

For complete details on the execution of this protocol, please refer to Lai et al. (2014).

BEFORE YOU BEGIN

Institutional permissions

The experiment should be approved by the Institutional Animal Care and Use Committee (IACUC) of laboratory animals to protect the welfare of animals. This study has been approved by the National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee (20210021). Adhere to the ethical guidelines and principles during the animal research.

Experimental concerns

1. Be familiar with the anatomy of the abdominal cavity and retroperitoneal space of mice to avoid surgical damages to the blood vessels and organs. (Figures 1A and 1B).
2. Confirm the strain, gender, age, weight of mice, duration and bilaterality of kidney ischemia.

> ⚠ CRITICAL: All of these variables are important factors which affect the severity and outcome of kidney following ischemia-reperfusion injury (IRI), see discussion later (Lu et al., 2012; Müller et al., 2002; Yang et al., 2010).

> *Note:* Most of our experiences with optimal results were obtained in male mice of C57BL/6J genetic background with 8–10 weeks of age and body weight 25–30 g. For detailed design of animal model, please refer to Lai et al. and Chou et al. (Lai et al., 2014; Chou et al., 2020).

> *Note:* Surgical procedures can be designed as bilateral renal IRI or unilateral renal IRI with or without contralateral nephrectomy.
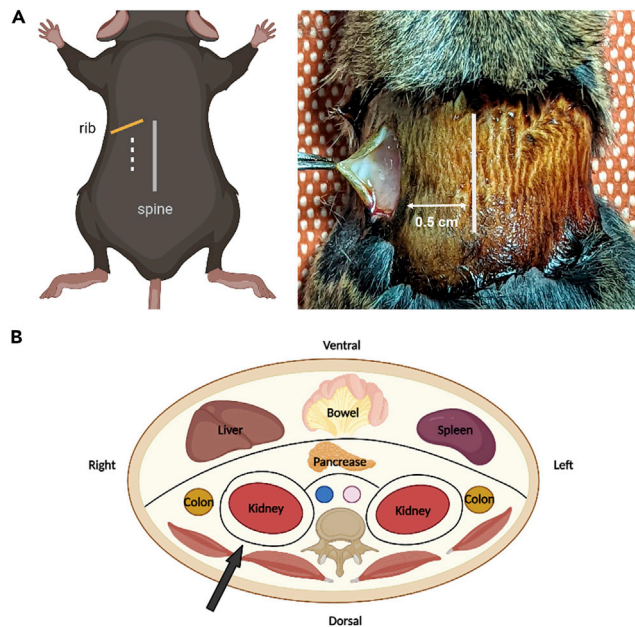
Figure 1. Anatomical landmarks for flank approach to the mouse kidney

(A) Landmark of flank incision. (B) Anatomy of mouse kidney. Black arrow indicates the route of dissection towards the kidney.

> *Note:* 4–6 mice/group were needed for each time point when the mice were euthanized. Kidneys from sham-operated mice were served as controls (n=4–6).

Preparations of surgery setup

🕐 Timing: 20 min

3. Clean the surgical table and instruments with 70% ethanol.
4. Preparation of narcotics and pain relief drugs, sterile surgical instruments, laboratory instruments, and equipment for specimen collection.
5. Prepare heating pads on the surgical platform pre-heated up to 37.0°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Ketamine	Pfizer	NA
Xylazine hydrochloride	Merck	23076-35-9
Povidone-Iodine solution	Sigma	CAS 25655-41-8
Normal saline solution	Sigma	7647-14-5
Tetracycline Hydrochloride ointment 1% (10 mg/g)	Genuine Chemical Pharmaceutical Co.	GMP G-12146
Buprenorphine	Bayer	NA
Formalin	Sigma	HT501128-4L
Alcohol	Honeywell	32221-1L
Paraformaldehyde solution (PFA)	Sigma	P6148-500G
Phosphate-buffered saline (PBS)	Gibco	14190-144
Optimal cutting temperature compound (O.C.T)	Leica	3801480

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
C57BL/6J, strain#:000664, male, 8–10 weeks old	The Jackson Laboratory	RRID:IMSR_JAX:000664
Other		
Platform balance	Accuris Mini	Z741398-1EA
DC temperature controller	FHC Inc.	40-90-8D
Heating pad 12.5 × 25 cm	FHC Inc.	40-90-2-02
Mini rectal thermistor probe	FHC Inc.	40-90-5D-02
3M Nexcare Micropore Tape	3M COMPANY	1535SP-1
Electrical razor	Favorita	GT104
Surgical scissors	Kent Scientific	INS750216-2
Iris forceps	Moria	11370-31FST
Sterile cotton swab 0.3 × 15 cm	Chemtronics	197-5028
Pedicle clamp	World Precision Instrument	501779-G
Autoclip applier 9 mm and wound clips	Fisher Scientific	BD427638
1 mL syringe with 25G 5/8 needle	Becton Dickinson	309626

STEP-BY-STEP METHOD DETAILS

Pre-operative managements

⏱ Timing: 10 min

The protocol is based on one mouse per time. The procedure can be performed two to four mice in a row, depending on personal skills, assistants and equipment. At least 6 mice per group is suggested to obtain statistically relevant results. Pre-operative preparations will be introduced in this section, including anesthetic agents, mouse positioning, and surgical site preparation.

1. Record body weight of each mouse.
2. Anesthetize the mouse by intraperitoneal injection of 10 uL anesthetic solution per gram of body weight for a mouse (Ketamine 0.1 mg/g and xylazine 0.01 mg/g).
    a. Prepare the anesthetic solution by adding 2 mL of Ketamine (50 mg/mL) and 0.2 mL of xylazine (50 mg/mL) in 7.8 mL of phosphate buffered saline.

   Note: The duration of immobilization and anesthesia was at least 40 and 28 min, respectively (Kawai et al., 2011).

3. Place the anaesthetized mouse in the prone position upon the feedback-controlled heating pads to maintain the mice body temperature at 36.8°C–37.3°C.

   Note: Secure the limbs with surgical tapes for fully extension.

4. Gently insert the mini rectal thermistor probe into the rectum to record body temperature.
5. Shave a 4 × 2.5 cm flank area in the surgical site or a 4 × 5 cm area for bilateral renal IRI by an electrical razor.
6. Disinfect the skin with cotton swabs soaked with povidone-iodine solution for 3 times. Remove the iodine solution with normal saline for 3 times.
7. Cover both of the mouse's eyes with lubricant tetracycline hydrochloride ointment 1%.

Expose and clamp the renal hilum

⏱ Timing: 30 min for a mouse (depends on the duration of ischemia)
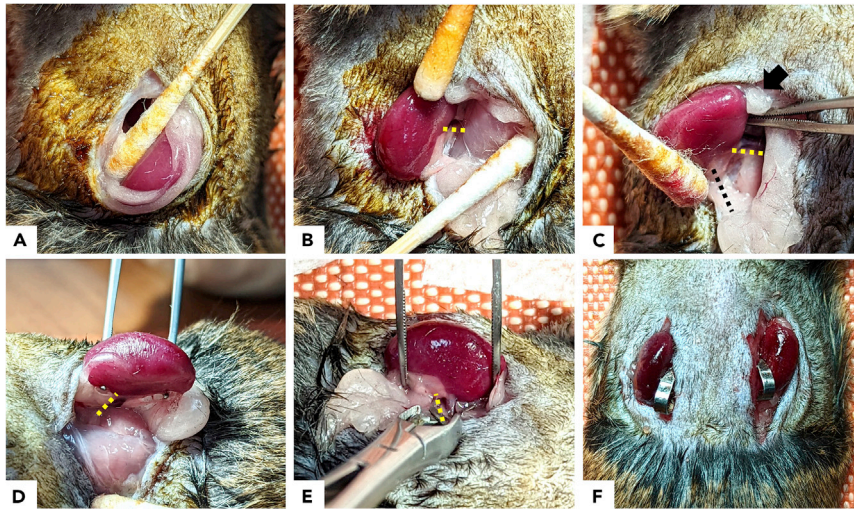
Figure 2. Main steps of the exposure of mouse renal hilum

(A) Expose the kidney from the retroperitoneal space by cotton swab in an outward direction.

(B–D) Liberate the renal pedicle (yellow dotted line) from the renal sinus fat with prevention of ureteral (black dotted line) and adrenal gland (black arrow) injury.

(E) Placement of a pedicle clamp over the renal pedicle.

(F) Repeat at the contralateral side as an optional procedure depending on the study design.

In this section, we introduce the surgical steps to expose the renal hilum via flank incisions, with minimum risk of intra-abdominal organ injury during dissection (Methods video S1).

> *Note:* The surgical procedures should be performed under aseptic conditions.

8. Make a vertical flank incision for 1.5 cm by surgical scissors layer by layer through the skin, fascia and then muscle layer (Figures 1A and 1B).

> *Note:* The kidney is located at the middle 1/3 of the body, below the 13th rib and around 0.5 cm lateral to the spine.

9. Mobilize the kidney from the retroperitoneal fat with a 0.3 cm wide cotton swab in an outward direction (Figure 2A).

> ⚠ CRITICAL: Use normal-saline soaked cotton swabs to dissect the fascia and adipose tissue to prevent injury of kidney.

10. Dissect the peri-nephric fat with a cotton swab at the medial side of the kidney to expose the renal hilum (**yellow dotted line,** Figure 2B).

> ⚠ CRITICAL: Avoid grabbing the kidney with metallic forceps to prevent artificial trauma to the kidney during the dissection.

11. Drill into the renal hilar fat with cotton swabs or forceps above and below the renal pedicle in order to create adequate space for pedicle clamp (**yellow dotted line,** Figures 2C and 2D).

> ⚠ CRITICAL: Redundant renal sinus fat left behind to the renal pedicle may lead to incomplete renal ischemia as a cushion during pedicle clamping.
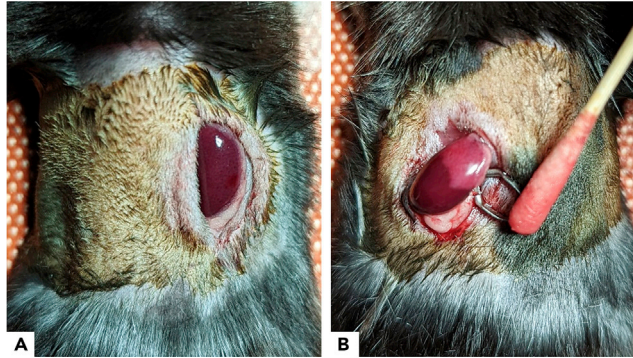
Figure 3. Expected changes of the kidney after ischemia-reperfusion injury
(A) Appearance of the kidney after successful clamping.
(B) Ineffective clamp leads to incomplete renal ischemia.

> ⚠ CRITICAL: Avoid injury to the ureter embedded within the peri-hilar fat during vascular clamping (black dotted line, Figure 2C).

> ⚠ CRITICAL: Avoid adrenal gland embedded within the adipose tissue above the upper pole of the kidney (black arrow, Figure 2C).

12. Apply the non-traumatic pedicle clamp to the renal pedicle gently (**yellow dotted line,** Figure 2E).
13. Set the timer immediately for the planned ischemia interval.
14. Ensure the successfulness of ischemia by observing the color change of the kidney into dusky appearance uniformly within few minutes (Figures 2F and 3A).
15. Replace the kidney to the retroperitoneal space.

> *Note:* Keep body temperature stable at around 37°C during the planned ischemia interval.

> ⚠ CRITICAL: Body temperature control during operation is very important. Cold ischemia will attenuate the ischemia injury of kidney and may make experiments non-reproducible (Figure 4).

16. Repeat the procedure at the contralateral side in the animal model designed for bilateral renal IRI (Figure 2F).

Accomplish and wound closure

> ⏱ Timing: 10 min for a mouse

17. Reopen the wound and release the pedicle clamp at the end of the ischemia interval.

> ⚠ CRITICAL: Successful kidney reperfusion should be confirmed by the observation of rapid color change to its original color.

18. Replace the kidney into the retroperitoneal space.
19. Give 1 mL of 37°C pre-warmed 0.9% saline into the retroperitoneal space before wound closure.
20. Close the wound with metallic staples, which will be removed after 7–10 days as the wound healed.

Post-operative managements

> ⏱ Timing: 1–14 days
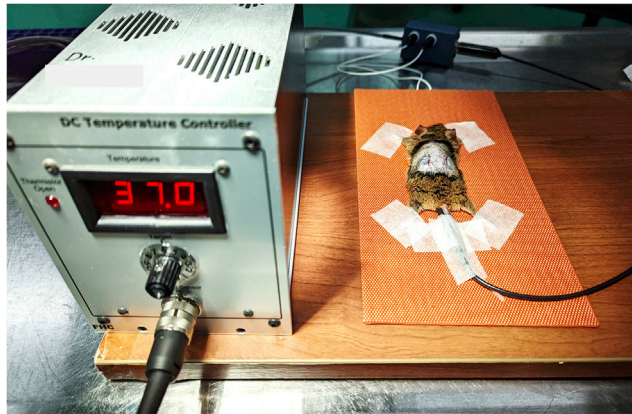
Figure 4. Stable body temperature intraoperatively with temperature controller

In this part, we introduce postoperative pain control, wound care and specimen collection.

21. Administer buprenorphine 0.05–0.10 mg/kg immediately and then every 8–12 h subcutaneously for 3 days or more for post-surgical pain control.

   Note: Monitor the presentation of pain by using the following criteria: poor appetite with absence of feces, dehydration with skin tenting, loss of mobility with a limb protecting the incisional site, failure to groom with dirty appearance, aggressive behaviors such as squealing or biting…etc.

22. Observe the mice in the recovery rack with a temperature of 25°C–30°C for 30 min.
23. Monitor the surgical wound every or every other day within one week.

   Note: Euthanize the mouse if wound infection or dehiscence is noticed.

24. Sacrifice the mice according to the experimental design. Collect the kidney specimen for histology and protein (Figure 5).
   a. For histopathological analysis: store in 10% formalin at 15°C–30°C for 24 h. Change to 70% alcohol at 4°C on the next day.
   b. For frozen section: soak the sample in 4% PFA/PBS for 1–1.5 h, then change to 30% sucrose in PBS at 4°C for 24 h. On the next day, put the sample into OCT block, and then store with liquid nitrogen at −80°C immediately.
   c. Snap frozen for further protein and RNA analysis: drop the specimen in liquid nitrogen, and then store at −80°C.

EXPECTED OUTCOMES

Selection of the methods to clamp renal pedicle depends on the experimental design, including: unilateral IRI, unilateral clamping with immediate contralateral nephrectomy (uIRIx) and bilateral IRI (Shiva et al., 2020; Yang et al., 2010). After renal IRI, the damaged tubules can be either recovered or dedifferentiated and progressed to interstitial inflammation and fibrosis, according to the severity of ischemia injury. There are also several crucial factors affecting the susceptibility to renal ischemia, including genetic factor, age, gender, body weight, anesthetic agents, vascular clamps and heating systems. For instance, C57BL/6 mice are more sensitive to IRI than BALB/c, NIH Swiss or 129/Sv mice; older mice are more sensitive to IRI than the younger mice; male mice are more susceptible to ischemia-induced renal damage (Burne et al., 2000; Lu et al., 2012; Shiva et al., 2020; Yokota et al., 2003; Xu et al., 2014). Volatile anesthetics such as isoflurane or halothane had been reported
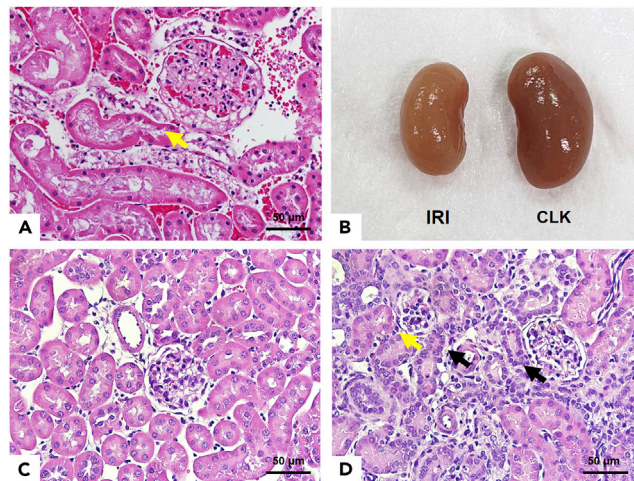
Figure 5. Expected changes of the kidney after 30-min unilateral ischemia-reperfusion injury
(A) Histopathological changes of kidney by Hematoxylin and Eosin (H&E) stain. Significant sloughing epithelial cells (yellow arrow), tubular necrosis and cast formation of the post-IRI kidney with H&E stain on day 1. Tubular injury score grade 2. Scale bar: 50 μm, magnification: 40×.
(B) Reduced mass of the post-IRI kidney compared with contralateral kidney (CLK) on day 14.
(C and D) Histopathological changes of the CLK (C) and post-IRI kidney (D) on day 14. Injured and atrophic renal tubules (black arrow) with interstitial inflammatory cells infiltration (yellow arrow) are evident in post-IRI kidneys. Tubular injury score grade 4. Scale bar: 50 μm, magnification: 40×.

to protect from renal IRI compared with injectable anesthetics (ketamine or pentobarbital) (Lee et al., 2004).

The duration of ischemia is the major cause affecting the severity of renal injury. Serum creatinine and blood urea nitrogen elevated significantly as the ischemic duration increased by 2-min increments (Hesketh et al., 2014). AKI can be observed within the first 24 h after renal reperfusion, including inflammatory cells infiltration and tubular epithelial cells necrosis. After bilateral renal ischemia for 25–30 min, tubular injury and fibrotic change are found to be progressive (Park et al., 2003). Biomarkers such as blood urea nitrogen, creatinine clearance, kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), interleukin-6 (IL-6), tissue inhibitor of metalloproteinase-2 (TIMP-2) etc. are used to assess the extent of AKI (Beker et al., 2018).

Histopathological changes of the kidney are examined by paraffin embedded tissue sections stained with Hematoxylin and Eosin (H&E) and/or Periodic-acid Schiff (PAS) stain. Tubular injury score is graded depended on the percentage of injured area (0: no tubular injury; 1: ≤10% injured tubules; 2: 11%–25% injured tubules; 3: 26%–50% injured tubules; 4: 51%–74% injured tubules; and 5: ≥75% injured tubules (Figure 5). Other common evaluations include platelet-derived growth factor receptor-β and α-smooth muscle actin immunofluorescence stain for renal fibrosis (Figures 6A and 6B), F4/80 immunofluorescence stain for macrophage infiltration (Figure 6C), picrosirius red staining for collagen deposition (Figure 6D) (Dong et al., 2019; Goujon et al., 1999).

LIMITATIONS

Outcomes of kidneys may not be consistent in unskilled surgeons or mice with varying genders, ages and body weight. To draw a robust result from the experiment, it is important to be familiar with the procedure and decrease the variability among animals. Different tolerance to ischemia between species may result in different molecular or anatomical changes in respond to ischemia and reperfusion injury (Lieberthal and Nigam, 2000). Although the pig kidney is a better model to simulate
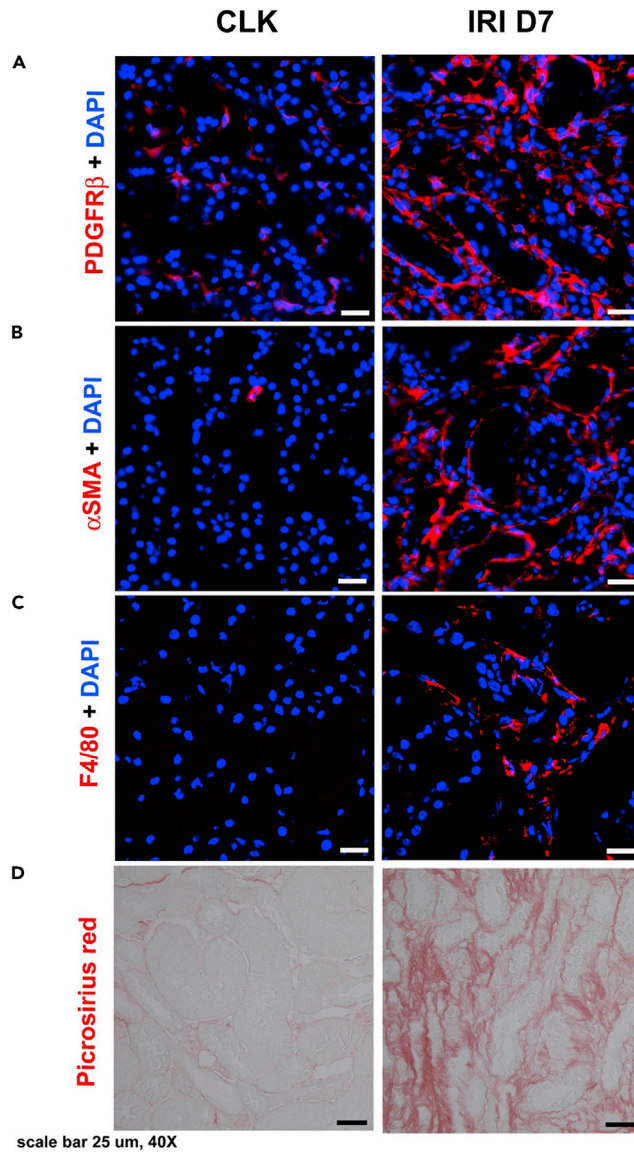
Figure 6. Immunofluorescence and picrosirius red staining of the kidney at 7 days after 30-min unilateral ischemia-reperfusion injury

(A) Platelet-derived growth factor receptor-β (PDGFR-β) were upregulated in renal mesenchymal cells during renal fibrosis.

(B) α-smooth muscle actin (α-SMA) as a marker of myofibroblasts expressed on fibrosis tissue.

(C) F4/80 antibody for macrophage staining.

(D) Picrosirius red staining could visualize collagen I and III fibers in renal fibrosis. Scale bar: 25 μm, magnification: 40×. CLK: Contralateral kidney.

human kidneys, there are still advantageous aspects of low expense, convenience and better availability of genetic information in rodent models.

TROUBLESHOOTING

Problem 1
Sticky fat adhesive to the kidney- "expose and clamp the renal hilum" section, step 10.
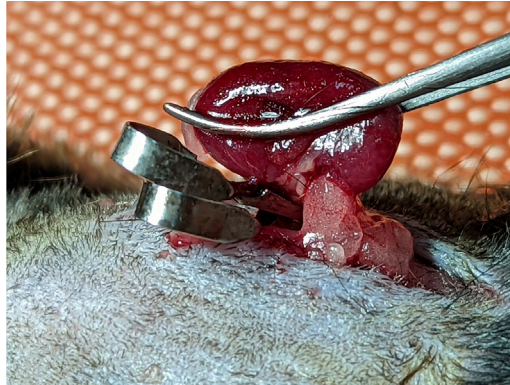
Figure 7. Two pedicle clamps to confirm complete occlusion of the renal pedicle

Potential solution

It is a common situation encountered in tubby mice, whose retroperitoneal fat is thicker making renal pedicle hardly to be identified. Incise a longer wound for better intraoperative view. Use a pair of forceps to grasp the skin and retroperitoneal fat simultaneously, then peel of the kidney from the fat with a cotton swab gently. Dissect above and below the renal pedicle back and forth via the anterior and posterior aspect of the kidney (Methods video S1).

Problem 2

Incomplete ischemia of kidney (Figure 3B)- "expose and clamp the renal hilum" section, step 12–14.

Potential solution

It is recommended to use the same brand of vascular clamp during the procedure and renew it every ten procedures to avoid the clamp from wearing down. Incomplete occlusion of the renal pedicle will lead to lesser ischemic injury and should be excluded from the analysis. If the renal pedicle is completely isolated without adipose tissue impediment, change the pedicle clamp to another effective one or apply two pedicle clamps to confirm the complete occlusion (Figure 7).

Problem 3

Bleeding over the renal parenchyma or renal pedicle- "expose and clamp the renal hilum" section, steps 10–11.

Potential solution

Compress the oozing point with gauze or cotton swab. Most bleeders can be controlled by adequate compression. However, hemorrhagic shock-induced AKI could occur if bleeding amount more than 0.4 mL and should be euthanized and excluded from the analysis. High mortality rate up to 50% was noted if more than 0.5 mL blood loss from mice.

Problem 4

Failed of kidney reperfusion after clamp removal- "accomplish and wound closure" section, step 17.

Potential solution

The condition may indicate possible blood clot formation or vascular rupture, which should be euthanized and excluded from analysis.

Problem 5

Traumatic damage seen in sham controls- "post-operative managements" section, step 24.

Potential solution

Blunt forceps should be used when retrieving the kidney to avoid artificial trauma. May replace the forceps with saline soaked cotton tipped applicators to lessen the pressure injury.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be provided by the lead contact, Chun-Fu Lai, (601540@ntuh.gov.tw).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new code or data.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101678.

AUTHOR CONTRIBUTIONS

C.-F.L. conceptualized the study; Y.-T.C. and Y.-C.T. performed animal experiments; Y.-T.C. and Y.-H.C. prepared the figures; Y.-T.C. and Y.-C.T. wrote the original draft; Y.-H.C. and C.-F.L. revised the manuscript. All authors discussed and approved the final manuscript.

DECLARATION OF INTERESTS

The authors have disclosed that they do not have any potential conflicts of interest.

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