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An Effective Mouse Model of Unilateral Renal Ischemia-Reperfusion Injury

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TITLE:

An Effective Mouse Model of Unilateral Renal Ischemia-Reperfusion Injury

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KEYWORDS:

Renal failure, ischemia-reperfusion, renal tubules, renal artery, blood flow, fibrosis, inflammation

SUMMARY:

Renal ischemia-reperfusion injury is associated with high morbidity and mortality in hospitalized patients. Here, we present a simple and effective mouse model of unilateral renal ischemia-reperfusion injury and provide a sequential overview of representative pathological changes observed in the kidney.

ABSTRACT:

Ischemia-reperfusion injury (IRI) is the leading cause of acute renal failure and is a significant contributor to delayed graft function. Animal models are the only available resources that mimic the complexities of the IRI-associated damage encountered *in vivo*. This paper describes an effective mouse model of unilateral renal IRI that delivers highly reproducible data. Ischemia is induced by occluding the right renal pedicle for 30 min followed by reperfusion. In addition to the surgical procedure, a sequential overview of the expected physiological and histopathological changes following renal IRI will be provided by comparing data from seven different reperfusion times (4 h, 8 h, 16 h, 1 day, 2 days, 4 days, and 7 days). Critical data for planning experiments ahead, such as mean surgical time, average anesthetic consumption, and body weight changes over time, will be shared. This work will help researchers implement a reliable renal IRI model and select the appropriate reperfusion time that aligns with their intended investigative goals.

INTRODUCTION:

The kidneys are among the highest perfused organs in the body and are extremely susceptible to changes in blood perfusion[1]. Renal ischemia-reperfusion injury (IRI) remains the leading cause of acute renal failure[2,3] and is associated with high morbidity and high mortality in hospitalized patients[4]. With limited therapeutic options available,[4,5] renal IRI is currently the focus of several

research efforts in biomedicine[6,7] aiming for the development of novel therapeutic targets and the characterization of early and sensitive markers of renal injury[8–10]. Identifying a reliable, time-, and cost-effective animal model is considered essential to meet these needs. This paper presents a simple and effective mouse model of unilateral renal IRI. Ischemia is induced by clamping of the right renal pedicle for 30 min[11,12]. A crucial part of this model is choosing the most suitable reperfusion time that will reproduce the pathological events of interest, such as tubular necrosis, polymorphonuclear inflammatory cell infiltration, or fibrosis. Therefore, researchers are provided with this sequential overview of representative pathological changes expected in the IRI kidney.

PROTOCOL:

The following protocol describes a survival surgery. Therefore, the highest aseptic and surgical practice is applied. All animal experiments were performed in compliance with institutional guidelines and approved by the Institutional Animal Care and Use Committee. To eliminate gender- and strain-based differences in IRI effects, only male C57BL6 mice were used in the study. All animals were matched in age and weight to produce comparable results.

1. Preparation

NOTE: A timeline of the different experimental phases and interventions is shown in **Figure 1A**.

1.1. Clean and disinfect the surgical table before each procedure. Prepare and place all required materials (sterilized instruments and cotton swabs, gauze, prediluted anesthetics, heating pad, vascular clamp, and skin antiseptics and suture) on the surgical table (see the **Table of Materials**).

1.2. Anesthetize male C57BL6 mice (age range 11–13 weeks) by intraperitoneal injection of ketamine/xylazine (100 mg/kg and 20 mg/kg of body weight, respectively, previously diluted in sterile saline).

NOTE: Skilled animal handling is essential to minimize stress for the animal, as stress responses can negatively affect the action of anesthetics.

1.3. After ketamine/xylazine administration, shave the surgical area on the right flank using a razor blade and soap.

NOTE: Shaving the skin improves wound healing as well as the general outcomes of survival surgeries.

1.4. Disinfect the skin in the surgical area with 70% alcohol first and then with povidone iodine solution using a cotton swab.

1.5. After the skin preparation, place the mouse on a heating table in a ventral decubitus

position and stabilize the body temperature at 37 °C (monitored through rectal and pad sensor probes).

NOTE: Kidneys are more easily accessed and surgically exposed when placed in ventral decubitus rather than lateral.

1.6. While the body temperature is stabilized, apply eye ointment to the eyes of the mouse.

NOTE: Dissociative anesthetics, such as ketamine, cause the animal's eyes to remain open while anesthetized.

2. Surgery

2.1. Once the pain reflexes are absent (toe pinching with tweezers), perform an approximately 1 cm dorso-lateral surgical incision on the right flank using a scalpel blade. Start the incision behind the last rib and continue caudally approximately 1 cm parallel to the lumbar midline.

2.2. Transect the abdominal musculature using scissors to visualize the retroperitoneal space. Remove the small amounts of blood produced during the sectioning of the muscles using sterile cotton swabs.

NOTE: Because the dorso-lateral approach is used, the retroperitoneum, and not the peritoneal cavity, is accessed with this procedure.

2.3. Push the right kidney out from the abdominal cavity. Use Graefe forceps to expose the kidney carefully.

NOTE: Always keep forceps closed to avoid traumatic injury to the kidney when placed on the abdomen and use it only to carefully push and guide the kidney towards the surgical incision and out of it.

2.4. Slowly expose the right kidney and identify the renal pedicle. Carefully remove the adipose tissue around the pedicle.

2.5. To induce ischemia, place the vascular clamp over the renal artery and vein present in the renal pedicle, avoiding clamping the adjacent ureter. Use a Halsted-Mosquito hemostat for manipulating the vascular clamp.

NOTE: Ischemia is confirmed by the visualization of a change in color of the kidney from red-pink to dark purple (**Figure 1B**).

2.6. Cover the clamped kidney with sterile gauze soaked in saline to avoid desiccation and leave it for 30 min.

2.7. Monitor anesthesia depth and humidity of the gauze periodically during this time.

NOTE: The induction dose of anesthesia is sufficient to provide analgesia until the end of the ischemic event; hence, no additional anesthetic injections are required.

2.8. Shortly before the end of the ischemia period, remove the gauze and uncover the kidney. Hold the Halsted-Mosquito hemostat, ready for clamp removal.

2.9. At minute 30, open the vascular clamp with the hemostat and remove it from the renal pedicle to allow reperfusion of the kidney.

NOTE: Reperfusion is confirmed by the visualization of a change in the color of the kidney from dark purple to red-pink (**Figure 1C**).

2.10. Perform the same procedures described above for sham animals without clamping of the renal pedicle.

2.11. After verification of the kidney color change, return the kidney to the abdominal cavity. Close the abdominal muscles with absorbable suture 5-0 using a cruciate pattern.

NOTE: A second injection of anesthetics may be required to maintain analgesia during the suturing of the muscles and the skin. Half of the initial dose has proved effective in providing analgesia until the conclusion of the surgery.

2.12. Close the skin with absorbable suture 5-0 using a horizontal mattress pattern. Clean the wound with a povidone iodine solution using a cotton swab.

3. Recovery and post-surgery

NOTE: As post-surgical time is the actual reperfusion time, proper post-surgical care is ethically mandatory and scientifically relevant. Reperfusion times can be selected as required by the researcher. Reperfusion times of 4 h, 8 h, 16 h, 1 day, 2 days, 4 days, and 7 days are compared to obtain a sequential overview of pathological changes induced by renal IRI.

3.1. Keep the mouse on the heating pad until it starts recovering from anesthesia.

NOTE: It is recommended to wait until the mouse begins moving its legs and attempts to move around. In cases when additional anesthetic injections are required during the surgery, the recovery time is longer. Atipamezole, an alfa-2 receptor antagonist, can be administered at a dose of 0.5 mg/kg of body weight intraperitoneally to reverse the xylazine effects and shorten the recovery phase. For pain management, buprenorphine (0.1 mg/kg of body weight, intraperitoneally) can be used. The use of non-steroidal anti-inflammatory drugs is discouraged as several drugs in this family induce nephrotoxicity and can, therefore, alter the results.

3.2. After recovery from anesthesia, place the mouse back in its cage with free access to water and food.

NOTE: Mashed food can be provided in a Petri dish and in material for hiding and playing (e.g., paper sheets, paper towel tubes).

3.3. Monitor the mouse daily to assess wound healing, food and water intake, body weight, and behavior.

NOTE: Wound healing status was assessed using the following scale: 1, dry; 2, wet; 3, partially opened; 4, opened. Fast wound healing was documented in this study, with more than 90% of dry wounds after day 2.

4. Euthanasia and sample collection

4.1. Euthanize the mice with sodium pentobarbital administered intraperitoneally at a dose that is twice the anesthetic dose for mice (100 mg/kg).

4.2. Collect fluid and tissue samples as required.

NOTE: Both kidneys, whole blood (for blood cell count), serum (for blood biochemistry), urine, the heart, and the lungs were collected. A few microliters of serum are needed for blood biochemistry analysis (blood urea nitrogen (BUN), creatinine, electrolytes). If required, 24 h before euthanasia, mice can be placed into metabolic cages to collect a higher urine volume that allows the determination of renal function parameters.

REPRESENTATIVE RESULTS:

Physiological parameters

Mice recovered from this unilateral renal IRI surgery uneventfully; appeared active and alert; and showed normal eating, drinking, and behavior by the following day. Some mice may have post-IRI body weight loss, although it is usually less than 10% of the initial body weight (**Figure 2**). Greater body weight losses (>10%) can be detrimental, and those animals should be removed from the study. Sham-operated mice did not show body weight changes post-surgery (measured 24 h after surgery). Most mice recovered their initial body weight between days 4 and 7 post-surgery (see IRI 7-day group, **Figure 2**). Renal function can be assessed using traditional markers such as blood urea nitrogen (BUN) and creatinine. Additionally, electrolyte levels in serum (sodium, potassium, and chloride) and an automated differential blood count were included in the analysis.

Histopathological changes

Assessment of histopathological findings was done using 4% paraformaldehyde-fixed, paraffin-embedded whole mid-sagittal sections of the kidney stained with hematoxylin/eosin (HE),

periodic acid Schiff, and Masson's trichrome stains. The most evident changes produced by this unilateral renal IRI model can be seen at the cortico-medullary junction, specifically in the proximal tubules, thick ascending limbs of Henle's loop, and distal convoluted tubules, as well as in the tubular interstitium (see the legend for **Figure 3**). Microscopic images showing the most characteristic lesions following IRI in the kidney can be seen in **Figure 3**. A list of the sequential histopathological findings is provided in **Table 1**.

A tubular injury scoring system was developed to categorize the damage over time (**Figure 4**). In this, five defined alterations were assessed by three different evaluators: 1) tubular epithelial attenuation; 2) brush border loss; 3) tubular necrosis; 4) luminal obstruction; and 5) presence of proteinaceous cast. An assignment of "1" indicates that the alteration is present, "0" that it is absent.

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental renal IRI model in mouse. (**A**) Phases of experiments and interventions (anesthesia induction, ischemia, and reperfusion) are shown. Please note the changes in the color of the right kidney to dark red during ischemia (**B**) to pink during reperfusion (**C**). (**D**) Macroscopic appearance of the IRI right kidney (red arrow) compared to the contralateral non-IRI kidney of the same animal 24 h after surgery. Red arrow in (**B**) shows the position of the hemostatic clamp. Abbreviation: IRI = Ischemia-reperfusion injury.

Figure 2: Body weight of mice before and after renal IRI. Individual data are shown. Abbreviations: IRI = Ischemia-reperfusion injury; h = hours; d = days.

Figure 3: **Typical microscopic lesions observed in the cortex and the cortico-medullary junction of IR-operated mice.** Sham and different reperfusion times are shown (indicated above each picture). (**A**) Intact structures are shown in sham (magnification 40x; scale bar = 20 µm). Arrows in IRI 4 h indicate the presence of proteinaceous cast in the tubular lumen (magnification 40x; scale bar = 20 µm). Arrows in IRI 8 h show tubular dilatation (magnification 40x; scale bar = 50 µm). Black arrow in IRI 16 h shows tubular cast in medullary segments; white arrows show areas of cellular necrosis (magnification 40x; scale bar = 50 µm). Black arrows in IRI 1 d indicate tubular dilatation (magnification 10x; scale bar = 100 µm). Black arrow in IRI 2 d shows enlarged cell nuclei; white arrowheads show areas of lymphocytes and macrophages infiltration (magnification 40x; scale bar = 50 µm). White arrowheads in IRI 4 d indicate mitotic tubular cells (magnification 40x; scale bar = 50 µm). Black arrow in IRI 7 d shows an area of focal fibrosis; white arrowhead shows an area of regeneration (magnification 20x; scale bar = 100 µm). (**B**) PAS staining showing the renal cortex of mice during early reperfusion (4 h, 8 h, and 16 h). Note the progressive attenuation of the brush border (arrows). Magnifications 40x; scale bars = 50 µm (**C**) Masson trichrome staining of sham and IRI 7 d mice showing areas of interstitial fibrosis (white arrows). Magnification 40x; scale bars = 50 µm. Abbreviations: IRI = Ischemia-reperfusion injury; Glo = glomerulus; PCT = proximal convoluted tubule; DCT = distal convoluted tubule; CD = collecting duct; PAS = periodic acid Schiff; d = day.

Figure 4: Tubular injury score of sham- and IRI-operated mice. Scoring system scale 1 to 5 for tubular epithelial attenuation; brush border loss; tubular necrosis; luminal obstruction; and presence of proteinaceous cast. An assignment of "1" indicates that the alteration is present, "0" that it is absent. Individual values are shown. Bars represent mean ± SD (n = 4). Abbreviation: IRI = Ischemia-reperfusion injury.

Table 1: Most significant pathological changes over time. Diagnosed based on microscopic examination of 4–6 animals per group.

DISCUSSION:

Mouse renal IRI models are popular in biomedical research due to their relatively low operational costs and the availability of diverse transgenic models[12]. The unilateral renal IRI model presented here mimics characteristic pathological changes observed in human renal IRI such as tubular dilatation, necrosis, and fibrosis[13]. These results are based on varying reperfusion times.

Critical steps of this protocol include the maintenance of constant body temperature and correct placement of the vascular clamp in the renal pedicle. Body temperature influences the animal's metabolism[14], altering the experimental results both at the physiological and cellular levels[15]. In this model, the body temperature was stabilized before surgery using rectal and pad sensor probes. In addition, continuous monitoring of the body temperature during the whole surgical procedure is highly recommended, especially before placing the vascular clamp to induce ischemia.

The exposure of the kidney and the proper placement of the vascular clamp are also critical for the success of the experiment. Damage to the renal capsule by improper handling of the forceps during exposure of the kidney through the surgical incision will result in perirenal hemorrhage and inflammation. The vascular clamp should be placed on the renal pedicle occluding the renal artery and the renal vein without affecting the ureter and the suprarenal arteries. Critical for this step is the careful dissection of the adipose tissue surrounding the renal hilum[14,16].

This model is cost- and time-effective. Anesthetic consumption per mouse was 156.47 ± 37.88 µL (mean ± SD, n = 17) of a prediluted ketamine/xylazine cocktail (1:10 ketamine, 1:50 xylazine, in saline; stock solution concentration, 100 mg/mL both). Surgery can be performed in a relatively short period. The total surgery time per mouse was 53 ± 5.23 min (mean ± SD, n = 17). With trained personnel, several surgeries can be performed at the same time. In this group, one experienced researcher performed the surgery until the clamp was released from the renal pedicle, while a second one took over from wound closing until the recovery of the mouse. With this approach, we were able to perform a high number of surgeries on a single day. In this model, we used the dorsolateral approach, which results in less trauma and reduced fluid and heat loss from the abdominal cavity as compared with the midline approach[16].

Previously published protocols have described the renal pedicle clamping technique to induce acute kidney injury in mice[17–19]. However, in those studies, a contralateral nephrectomy was performed in addition to the unilateral IRI with ischemic times ranging from 15 to 26 min. In this

protocol, we induced unilateral ischemia for 30 min while preserving the contralateral kidney. This resulted in a survival rate of 100%. However, this model is not suitable to induce azotemic renal damage due partly to the compensatory effect exerted by the non-surgically intervened contralateral kidney. However, keeping one kidney unaffected in the same animal offers the advantage of using longer ischemia times with a higher survival rate. In addition to this, the contralateral kidney can be utilized to assess possible side effects of test drugs or treatments applied during the experimental procedure and to study kidney-kidney crosstalk effects[20,21]. For example, this model has been useful in showing reactive oxygen species-induced alterations at the cellular level both in the IRI and contralateral, non-surgically intervened kidney[11].

This model has a potential application in studies aiming to identify and characterize markers of unilateral renal damage, renal crosstalk effects, post-renal IRI-induced hemodynamic changes, and potential nephrotoxic effects of drug candidates to be used in renal IRI. This detailed description of the main pathological changes serves as a valuable tool to select the most suitable time to study specific cellular processes, from inflammation and necrosis (4 h to 2 days) to regeneration (4 days) and fibrosis (7 days and later).

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare that there are no conflicts of interest regarding this article.

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Figure 1

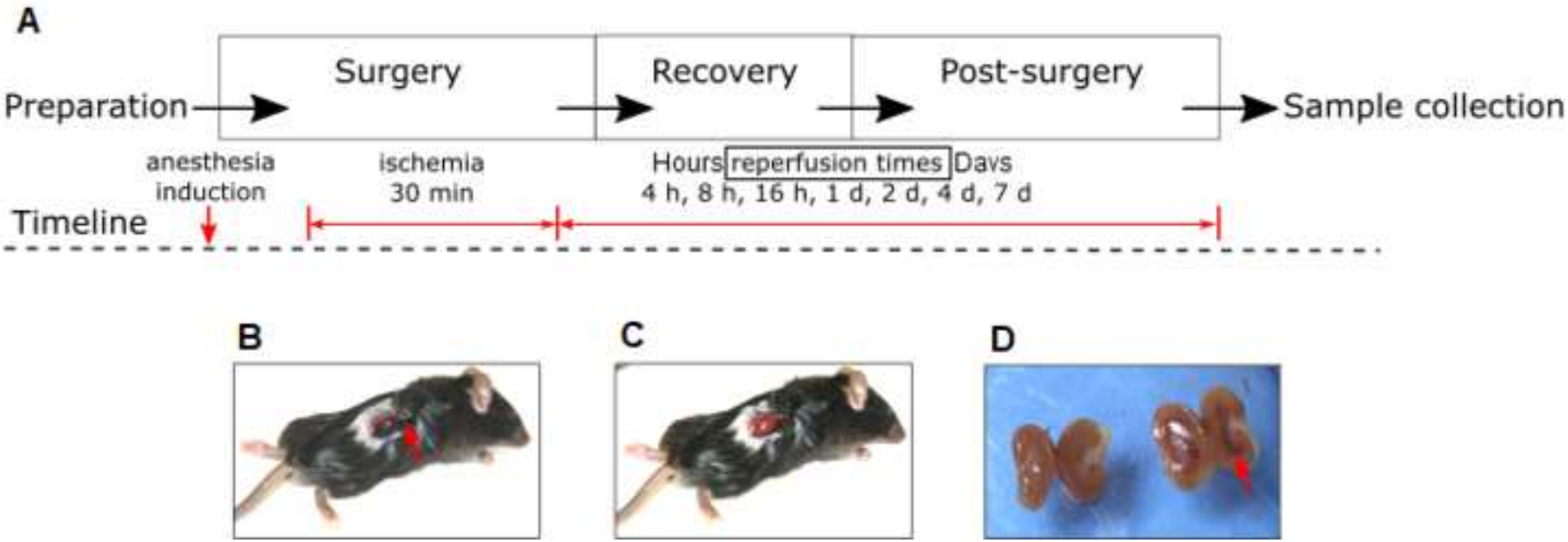

Figure 2

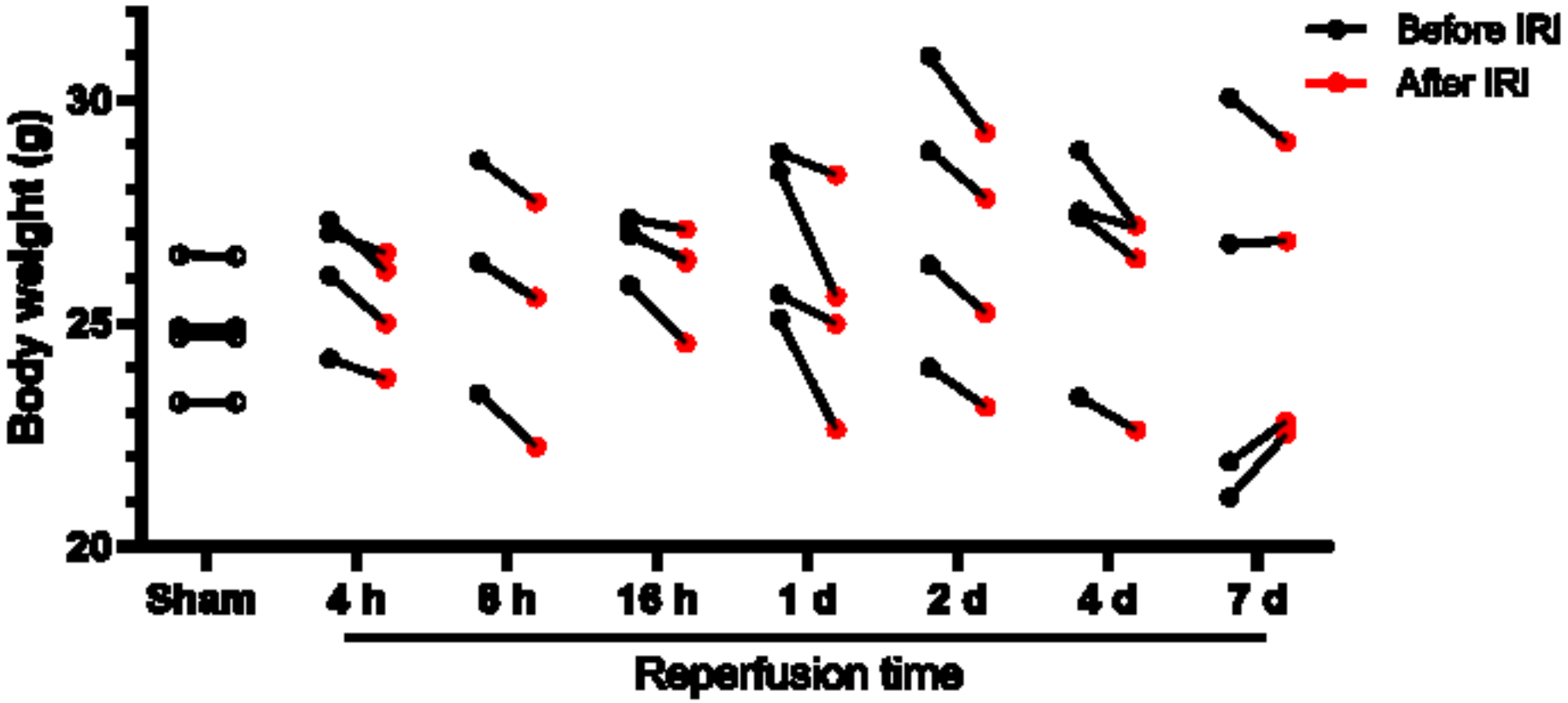

Figure 3

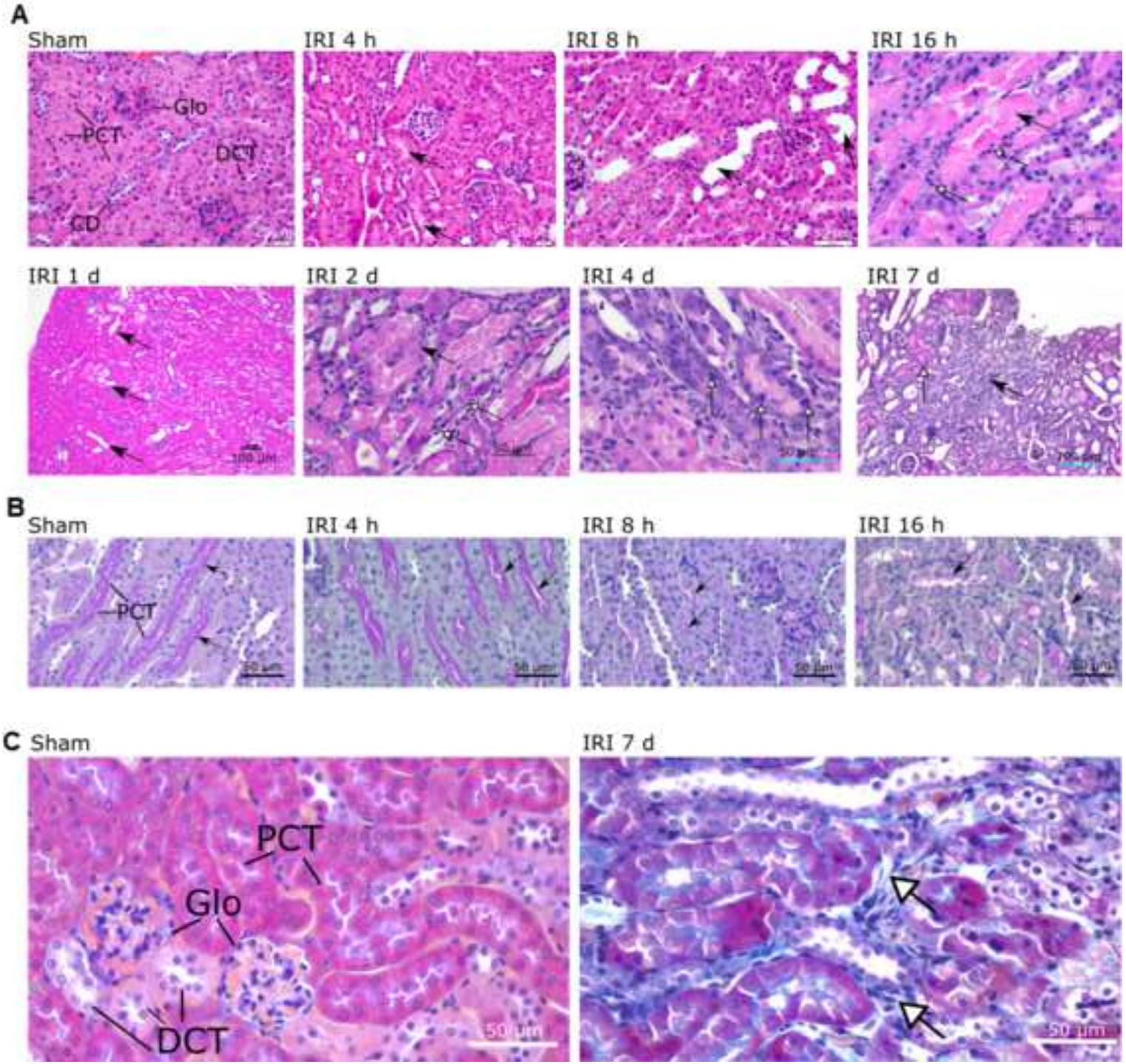

Figure 4

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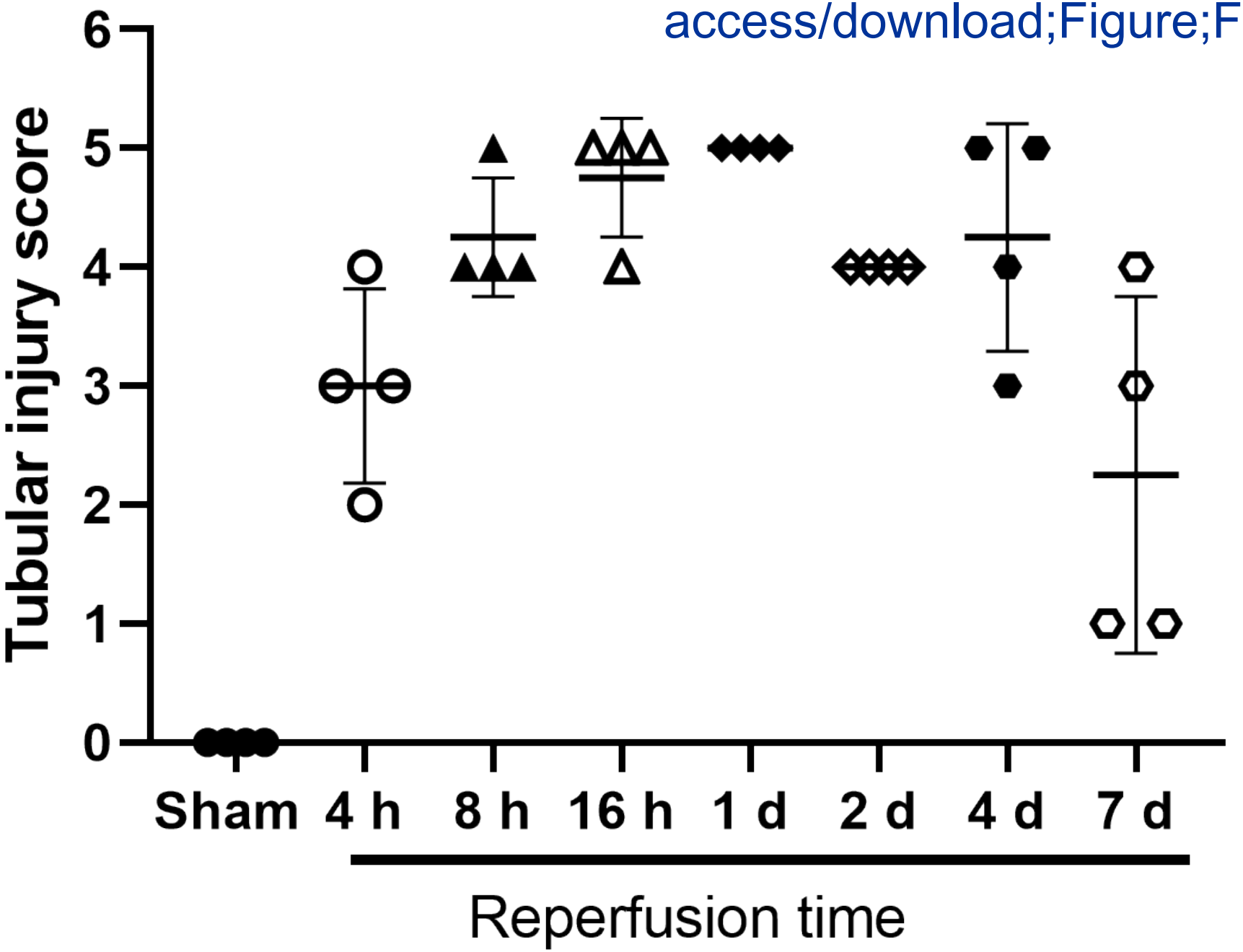

Time after IRI	Most significant pathological changes
4 h	Tubular obstruction Protein cast in lumen
8 h	Tubular dilation Incipient necrosis Attenuation of epithelium
16 h	Cellular necrosis Tubular cast Neutrophils infiltration
1 day	Necrosis Tubular dilation Neutrophils infiltration
2 days	Tubular dilation Lymphocytes and macrophages infiltration Enlarged cell nuclei
4 days	Prominent mitotic activity in tubule cells
7 days	Focal fibrosis Areas of regeneration

Dear Dr. Bajaj,

We would like to thank you and the reviewers for the time and effort dedicated to providing feedback on our manuscript and for giving us the opportunity to address it.

We have considered all editorial and reviewers' suggestions and concerns and amended the manuscript accordingly; as a result, we feel that its quality has improved. Our point-by-point responses are listed below. Changes are included below (italicized) and tracked in the manuscript.

We thank you again for helping us to enhance the clarity and value of our article and are available in case further clarifications are needed.

Best regards,

Jose R. Godoy
Corresponding author

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you for this. The manuscript has been proofread and spelling and grammar errors corrected.

2. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

This point was addressed. The abstract contains 158 words.

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Point was addressed and numbering corrected.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Thank you for this. We changed all verbs into their imperative tenses. Text that could not be re-written in the imperative tense were added as "Notes".

5. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

This point was addressed.

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Fine Science Tools, Heidelberg, Germany, Vicryl, etc.

This point was addressed. All commercial names were removed from the manuscript.

7. Please revise the following lines to avoid overlap with previously published work: 37-39.

Thank you for this. This point was addressed and the lines re-formulated as follows:

L36-39 *"The kidneys are among the highest perfused organs in the body and are extremely susceptible to changes in blood perfusion [1]. Renal ischemia-reperfusion injury (IRI) remains as the leading cause of acute renal failure [2, 3] and is associated with high morbidity and high mortality in hospitalized patients [4]."*

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Addressed. More detailed instructions were included.

9. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We highlighted the parts of the protocol for filmable content (see manuscript sections highlighted in yellow, L88-133).

10. Please ensure the results are described in the context of the presented technique.

This point was addressed. All steps in the protocol follow a sequential logical order.

11. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

This point was addressed and critical steps included as follows:

L243-255 *"Critical steps of our protocol include the maintenance of constant body temperature and correct placement of the vascular clamp in the renal pedicle. Body temperature influences the animal's metabolism [14] altering the experimental results both at the physiological and cellular levels [15]. In our model, we stabilized the body temperature before surgery using rectal and pad sensor probes. In addition, continuous monitoring of the body temperature during the whole surgical procedure is highly recommended, especially before placing the vascular clamp to induce ischemia. The exposure of the kidney and the proper placement of the vascular clamp are also critical for the success of the experiment. Damage to the renal capsule by improper handling of the forceps during exposure of the kidney through the surgical incision will result in perirenal hemorrhage and inflammation. The vascular clamp should be placed on the renal pedicle occluding the renal artery and the renal vein without affecting the ureter and the suprarenal arteries. Critical for this step is the careful dissection of the adipose tissue surrounding the renal hilum [14, 16]."*

b) Any modifications and troubleshooting of the technique

Addressed as follows:

L260-266 *"With trained personnel, several surgeries can be performed at the same time. In our group, one experienced researcher performed the surgery until the clamp was released from the renal pedicle while a second one took over from wound closing until the recovery of the mouse. With this approach, we were able to perform a high number of surgeries on a single day. In our model we used the dorsolateral approach which results in less trauma and reduced fluid and heat loss from the abdominal cavity as compared with the midline approach [16]."*

c) Any limitations of the technique

Addressed and included as follows:

L271-273 *"However, this model is not suitable to induce azotemic renal damage due in part to the compensatory effect exerted by the non-surgically intervened contralateral kidney."*

d) The significance with respect to existing methods

Addressed and included.

L266-271 *"Previously published protocols have described the renal pedicle clamping technique to successfully induce acute kidney injury in mice [17–19]. However, in those studies a contralateral nephrectomy was performed in addition to the unilateral IRI with ischemic times ranging from 15 to 26 min. In our protocol, we induced unilateral ischemia for 30 min while preserving the contralateral kidney. This resulted in a survival rate of 100%."*

L273-276 *"But, keeping one kidney unaffected in the same animal offers the advantage of using longer ischemia times with a higher survival rate. In addition to this, the contralateral kidney can be utilized to assess possible side effects of test drugs or treatments applied during the experimental procedure, and to study kidney-kidney cross-talk effects [20, 21]."*

e) Any future applications of the technique

Addressed and included.

L280-285 *"This model has a potential application in studies aiming to identify and characterize markers of unilateral renal damage, renal cross-talk effects, post renal IRI induced hemodynamic changes, and potential nephrotoxic effects of drug candidates aimed to be used in renal IRI. Our detailed description of the main pathological changes serves as a valuable tool to select the most suitable time in order to study specific cellular processes, from inflammation and necrosis (4 h to 2 d) to regeneration (4 d) and fibrosis (7 d and later)."*

12. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.

This point was addressed. All trademark and registered symbols were removed from table of materials. All materials and equipment were sort in alphabetical order.

Reviewers' comments:

Reviewer #1

Manuscript Summary:

The authors present a protocol for a mouse model of unilateral renal ischemia-reperfusion injury, a time- and cost effective model that is commonly used for development of novel therapies and characterization of new markers for renal injury.

Major Concerns:

- The protocol is comparable to other protocols for unilateral renal ischemia-reperfusion injury in mice published earlier in JoVE (Skrypnyk et al., JoVE, 2013 and Hesketh et al. JoVE, 2014). The authors should prove that there is a substantial improvement of efficacy with their new protocol and what would be the advantage of using their proposed approached compared to these earlier protocols.

Thank you for this important point. We included the excellent works mentioned by the reviewer and discussed their main differences with our protocol. We believe the main advantage of our unilateral renal IRI without contralateral nephrectomy is that it allows the researcher to use clamping times of 30 min (or even longer) with a high survival rate. In addition to this, we use the non-surgically intervened

contralateral kidney to study kidney-kidney crosstalk effects. We included these important aspects in the discussion as follows:

L266-271 *"Previously published protocols have described the renal pedicle clamping technique to successfully induce acute kidney injury in mice [17–19]. However, in those studies a contralateral nephrectomy was performed in addition to the unilateral IRI with ischemic times ranging from 15 to 26 min. In our protocol, we induced unilateral ischemia for 30 min while preserving the contralateral kidney. This resulted in a survival rate of 100%."*

L273-278 *"…keeping one kidney unaffected in the same animal offers the advantage of using longer ischemia times with a higher survival rate. In addition to this, the contralateral kidney can be utilized to assess possible side effects of test drugs or treatments applied during the experimental procedure, and to study kidney-kidney cross-talk effects [20, 21]. For example, this model has been useful in showing reactive oxygen species-induced alterations at the cellular level both in the IRI and contralateral, non-surgically intervened kidney [11]."*

- The authors use an ischemia time of 30 minutes. However, also body temperature is crucial during an ischemic injury as already minimal body temperature variations alter functional and morphological outcome (Pelkey TJ et al., J Vasc Surg, 1992; Le Clef N et al., PLoS One, 2016; Delbridge MS et al., Transplant Proc, 2007). Therefore, the authors should stress the importance of temperature follow-up during surgery.

Thank you for this suggestion. This point was addressed and included in the text as follows:

L244-249 *"Body temperature influences the animal's metabolism [14] altering the experimental results both at the physiological and cellular levels [15]. In our model, we stabilized the body temperature before surgery using rectal and pad sensor probes. In addition, continuous monitoring of the body temperature during the whole surgical procedure is highly recommended, especially before placing the vascular clamp to induce ischemia."*

- Surgery step 5: The authors should mention an additional step to describe how the renal pedicle should be dissected free from surrounding tissue to improve clamp placement and avoid ureter or adrenal gland obstruction.

Thank you for allowing us to provide a clarification on this. We included the following text on steps 5 and 6:

L104-108

"2.5. Slowly expose the right kidney and identify the renal pedicle. Carefully remove the adipose tissue around the pedicle.

2.6. To induce ischemia, place the vascular clamp over the renal artery and vein present in the renal pedicle, avoiding clamping the adjacent ureter. A Halsted-Mosquito hemostat is useful for manipulating the vascular clamp"

We also included this as a critical point in the discussion as follows:

L249-255 *"The exposure of the kidney and the proper placement of the vascular clamp are also critical for the success of the experiment. Damage to the renal capsule by improper handling of the forceps during exposure of the kidney through the surgical incision will result in perirenal hemorrhage and inflammation. The vascular clamp should be placed on the renal pedicle occluding the renal artery and the renal vein without affecting the ureter and the suprarenal arteries. Critical for this step is the careful dissection of the adipose tissue surrounding the renal hilum [14, 16]."*

Minor Concerns:

- The authors mention that it is important to eliminate gender differences and match animals for age and weight to produce reproducible results. However, it has been shown that also the mouse strain influences IRI effects (Burne MJ et al., Transplantation, 2000). This information should be added to the manuscript.

Thank you. We addressed this point and added strain-based differences as follows:

L56-58 *"General remarks. In order to eliminate gender- and strain-based differences in IRI effects, only male C57BL6 mice were used in the study. All animals were matched in age and weight in order to produce comparable results."*

- Please improve overall quality of the figures (mainly figure 1, 2 and 3).

We sincerely apologize for this. We provided all original figures as high-resolution individual images. We opened each image and diagram and the resolution is very high. However, during the conversion of the original files to a single PDF document, the quality of the figures was dramatically reduced. Besides this, the reader can open the original high-resolution images by clicking on the link placed on each figure in the PDF.

- The table of material should be completed as some crucial equipment is currently missing, e.g. razors, iodine, 70% ethanol, and others.

We included all crucial equipment and medicaments (vascular clamp, heating pad, ketamine, buprenorphine, etc) in the table but excluded basic surgical supplies that are commonly found in a standard laboratory setting (70% ethanol, iodine, cotton swabs, gauze, etc.).

- Figure 1d: Please mention in the figure legend the reperfusion time that was applied for this kidney.

Thank you for pointing this out. We included this information as follows:

L 213 *"... 24 h after surgery."*

Reviewer #2:

Manuscript Summary:

The authors have presented a mouse model of unilateral renal ischemia-reperfusion injury (IRI) in this study. The right kidney was retroperitoneally exposed in mice for inducing 30-min of unilateral renal ischemia via clamping pedicle, which followed by reperfusion periods of 4 h, 8 h, 16 h, 1 d, 2 d, 4 d, and 7 d in different groups in order to demonstrate sequential renal histopathological changes.

Major Concerns:

1- The unilateral renal ischemia-reperfusion is not a proper model for studying the effects of renal IRI on the plasma and urine variables, because the contralateral kidney exhibits compensatory function and prevents the plasma and urinary levels of creatinine, BUN and electrolytes to be changed.

We agree with the reviewer. We included the following sentence as one limitation of our model:

L 271-273 *"However, this model is not suitable to induce azotemic renal damage due in part to the compensatory effect exerted by the non-surgically intervened contralateral kidney."*

2- The quality of representative light microphotographs is poor and most of the mentioned lesions are not recognizable in them. In addition, the presented microphotographs for different reperfusion periods are not from the same region of the kidneys and, hence, they cannot be well-compared to each other.

We sincerely apologize for this. We provided all original figures as high-resolution individual images. We opened each image and diagram and the quality is very high. However, during the conversion of the

submission files to a single PDF document, the quality of the figures was dramatically reduced. By clicking on the link placed on each figure, the reader can open the original high-resolution images.

We agree with the reviewer with the fact that micrographs from the same regions of the kidney allow a better comparison between different animal groups. For this publication, however, we aimed to show the most representative results at each specific reperfusion time.

3- Figure 4 presents tubular injury scores at different reperfusion time. Scoring system scale 1 to 5 (see main text for a detail description). However, the methods for the grading and scoring of different renal tissue damages have not been explained.

Grading and scoring is now explained in the Results section as follows:

L 199-202 *"We developed a tubular injury scoring system in order to categorize the damage over time (fig. 4). In this, five defined alterations were assessed by three different evaluators: 1) tubular epithelial attenuation; 2) brush border loss; 3) tubular necrosis; 4) luminal obstruction; and 5) presence of proteinaceous cast. We assign "1" if the alteration is present or "0" if absent."*

4- One of the aim of this study is demonstrating sequential renal histopathological changes over different reperfusion times. Therefore, it could be much better that the levels of each type of renal tissue lesions to be quantified at cortex, outer medulla and inner medulla and also total histopathological score to be calculated for all reperfusion periods.

Thank you for this suggestion. We are working on a scoring system for the renal medulla. In this work, we wanted to focus primarily on the surgical technique necessary to recreate our mouse model of unilateral renal ischemia-reperfusion injury, and on the most representative pathological changes observed following renal IRI. Significant microscopic findings were confined primarily to the deep cortex and the outer medulla. In our current scoring system therefore, we utilized the term "corticomedullary junction" to refer to the region of the kidney most affected by tubular injury.

Reviewer #3:

Manuscript Summary:

The authors presented a mouse model of unilateral renal ischemia-reperfusion injury and provided a sequential overview of representative pathological changes observed in the kidney.

Major Concerns:

1.The IR-induced tubular injury is not uniform over the whole kidney. In general, juxta-medullary region (or named outer medulla) including primarily S3 proximal tubule and TAL has the most severe injury. Thus, the renal pathology should be described and scored in different regions e.g. cortex, outer medulla, and inner medulla, respectively.

We agree with the reviewer. Nephron segments show a highly diverse response to IRI and, hence, pathological changes are not uniform over the whole kidney. In this study, we aimed to describe our method, the surgical details for an easy replication of the model, as well as the most representative results after different reperfusion times. We found that most of the pathological changes after IRI are confined to the cortex and cortico-medullary junction (juxta-medullary region) (figure 3). A zonal separation of the kidney is a valid approach and can provide valuable information. However, we found most of the damage in tubular segments that span the deep cortex and the outer medulla such as the straight parts of proximal tubules and the TALs. We, hence, focused on the tubular segments that showed most of the damage.

2. A video showing the surgery procedure is necessary, especially for the publication in JOVE.

We agree with the reviewer and we will work with the JoVE filming team to scheduling time for video recording the procedure.

Minor Concerns:

A 30-minute ischemia at 37 degree could induce very severe AKI without a full recovery, followed by a progression to CKD. Please see references [PMID: 31116604, PMID: 32567350, PMID: 29949392] and discuss this in details.

In addition, it would be much better if the authors can repeat the model with stepwise renal ischemic time (e.g. 15 minutes and 22 minutes) and present the outcomes.

Thank you for this point. Renal IRI is associated with a high mortality rate if performed in both kidneys at the same time (PMID: 31116604, PMID: 32567350) or if performed in one kidney with simultaneous contralateral nephrectomy (PMID: 31116604). In those studies, clamping of the renal pedicle was less than 30 min in order to improve survival rate. In our study, we used a unilateral renal IRI with complete preservation of the contralateral kidney and 100% survival rate. This model has been useful to study unilateral renal IRI damage, renal-induced hemodynamic changes, and remote responses in the contralateral non-intervened kidney (kidney-kidney cross-talk effects).

We apologize, but under the current circumstances, we are not able to repeat the model with different ischemic times. In our study, we used 30 min ischemia and provide a detailed description of the most representative changes 4 h, 8 h, 16 h, 24 h, 2 d, 4 d, and 7 d following unilateral renal IRI. We sincerely hope to make a real contribution to researchers aiming to replicate this model.