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TITLE:

A Modified Two Kidney One Clip Mouse Model of Renin Regulation in Renal Artery Stenosis

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KEYWORDS:

Two kidney one clip (2K1C), Renal artery stenosis, Renin Angiotensin Aldosterone System, renin expression, Kidney injury, mouse model

SUMMARY:

A modified 2 kidney 1 clip (2K1C) Goldblatt mouse model was developed using polyurethane tubing to initiate renal artery stenosis, inducing an increase in renin expression and kidney injury. Here, we describe a detailed procedure of preparing and placing the cuff onto the renal artery to generate a reproducible and consistent 2K1C mouse model.

ABSTRACT

Renal artery stenosis is a common condition in patients with coronary or peripheral vascular disease where the renin angiotensin aldosterone system (RAAS) is overactivated. In this context, there is a narrowing of the renal arteries that stimulate an increase in the expression and release of renin, the rate-limiting protease in RAAS. The resulting rise in renin expression is a known driver of renovascular hypertension, frequently associated with kidney injury and end organ damage. Thus, there is a great interest in developing novel treatments for this condition. The molecular and cellular mechanism of renin control in renal artery stenosis is not fully understood and warrants further investigation. To induce renal artery stenosis in mice, a modified 2 kidney 1 clip (2K1C) Goldblatt mouse model was developed. The right kidney was stenosed in wild type mice and sham operated mice were used as a control. After renal artery stenosis, we determined renin expression and kidney injury. Kidneys were harvested, and fresh cortices were used to determine protein and mRNA expression of renin. This animal model is reproducible and can be used to study pathophysiological responses, molecular and cellular pathways involved in renovascular hypertension and kidney injury.

INTRODUCTION

Renal artery stenosis (RAStenosis) is an intractable problem affecting about 6% of people over 65 and in up to 40% of people with coronary or peripheral vascular disease[1,2]. Current treatments for the disease are limited; therefore, there is a critical need to develop new therapies to treat renovascular hypertension or resistant hypertension induced by RAStenosis. Renin angiotensin aldosterone system (RAAS) is the key pathway involved in the pathogenesis of RAStenosis induced hypertension or renovascular hypertension[3,4]. Known therapies targeting RAAS, such as ACE inhibitors or angiotensin receptor blockers, do not work well in RAStenosis and deteriorate the renal function[5-7]. Renin catalyzes the rate-limiting step in RAAS; it converts angiotensinogen to angiotensin I. In atherosclerosis, plaque formation causes the narrowing of renal artery that drives renin secretion, resulting in renovascular hypertension and kidney damage[8]. A number of studies have reported increased levels of oxidative stress during renovascular hypertension in humans, which were corroborated with the two kidney one clip (2K1C) mice model as well as other hypertensive animal models[2,9-16]. The molecular mechanism of renin expression control during RAStenosis induced renovascular hypertension is not well understood and warrants further investigation.

Experimental animal models that reliably and reproducibly recapitulate RAStenosis are important in elucidating the cellular and molecular mechanisms of renin expression control for the development of novel therapies. The 2K1C mouse model is a well-established experimental model to study the pathogenesis of renovascular hypertension[17-20]. This model is generated by the constriction of the renal artery using a clip[17,20,21], therefore producing renal artery occlusion that results in an increase in renin expression and hypertension[17,19-21]. However, there are no technical reports available, which describe a step by step procedure to generate renal artery stenosis in animal models.

Conventional U-shaped silver clips, polyurethane tubes and other clips have been used to constrict the renal artery to induce renal artery stenosis. Some studies have shown that the design and material of this clip are critical to obtaining reliable and reproducible data with the 2K1C animal model. According to Lorenz et al., the use of conventional U-designed silver clips induces a low success rate of hypertension (40-60%)[21]. Due to the clip design, the renal artery is press laterally, triggering a few constrictions and greater probability to be dislodged from the renal artery. Silver malleability and ductility may allow changes in clip widths; therefore, causing different hypertension levels among mice. Silver dioxides on the clip can cause perivascular inflammation, intimal proliferation, and tissue granulation, altering the renal artery diameter[22]. Due to the variability in the levels of hypertension obtained with the conventional U-design silver clip, Warner et al. and Lorenz et al. have successfully used a rounder-design polyurethane tubing to initiate renal artery stenosis in mice, generating a more reliable and consistent induction two kidney one clip animal model[20,21].

In this report, we describe a surgical protocol to generate experimental RAStenosis in mice, using the polyurethane tubing to constrict the renal artery. The polyurethane round-design cuff is a more reproducible, reliable and low-cost clip to generate stenosis in mouse. The goal of this experimental model is to study and define the molecular and cellular mechanism of renin

expression control during renal artery stenosis. We confirmed the success of RAStenosis mice model by measuring renin expression and kidney injury marker neutrophil gelatinase-associated lipocalin (N-GAL).

PROTOCOL

Mice were housed and cared at the Vanderbilt University Medical Center (VUMC) Division of Animal Care following the National Institutes of Health (NIH) guidelines and the Guide for the Care and Use of Laboratory Animals, US Department of Health and Human Services. All animal procedures were approved by the VUMC Institutional Animal Care and Use Committee prior to starting the experiments.

1. Animal preparation and dissection

1.1. Turn on the germinator and water pump for the heating pad about 30 min before starting surgery.

1.2. Cut 0.5 mm length polyurethane tubing with a sharp scalpel. Remove 0.2 mm of the circumference by making a cut lengthwise to make a cuff.

NOTE: This is a critical part of the renal artery stenosis procedure that requires extreme precision, attention to details and patience. Try to cut several pieces of polyurethane tubing at a time. Perform all of this procedure under microscope.

1.3. Before proceeding any further, put on surgical sterile gloves and a surgical mask.

1.4. Use C57BL/6 wild-type (WT) mice of 6-8 weeks. Use an equal number of male and female mice to avoid any sex bias.

1.5. Record the weight of the mice before performing surgery. The ideal weight to perform renal artery stenosis with polyurethane tube is 18-22 g. Handle mice gently and do not agitate while injecting the anesthesia.

NOTE: While transferring the mice from their housing room to the surgery room, carry them with great gentleness and care to avoid agitation. Carrying mice cages in hands instead of a trolley is highly recommended.

1.6. Anesthetize the mice with a mixture of ketamine (100 mg/kg BW) and xylazine (10 mg/kg BW) via intraperitoneal (I.P.) injections.

1.7. Place the mouse back inside the cage until fully anesthetized. It takes about 4-5 minutes before the mouse is fully unconscious. Pinch the tail or toe with forceps to check if the mouse is fully unconscious and ready for the surgery.

1.8. Lay the mouse on its back on a paper towel. Remove the hair of the lateral abdomen using an electric hair clipper following the opposite direction of hair growth. Clean the shaved area with a sterile alcohol gauze pad and apply topical povidone-iodine solution.

NOTE: The hair removal procedure must be performed at some distance or preferably at a different bench than the surgery procedure bench to avoid any hair interference and hair contamination during the surgery procedure.

1.9. Bring the mouse to the surgical bench and place it on top of a water circulating heating pad, facing the mouse's abdomen towards the microscope. Maintain a constant pad temperature of 37 °C with circulating water. Cover the heating pad with a sterile sheet and place the mouse on it.

1.10. Open the sterilized bag containing all the surgical equipment. Using a dissecting microscope and sterile sharps scissors, make a small flank incision (close to 13th thoracic rib, the last rib in mouse) and about 0.5 cm away from vertebrae. Proceed along the lumbar vertebrae and make a 1 inch incision.

1.11. Pull back the skin and muscle to expose the kidney.

1.12. Clean and remove the surrounding fat using cotton swabs to isolate the renal artery. Isolate the renal nerve from the renal artery using curved forceps.

1.13. When performing the sham surgery, apply sutures to close the skin, use topical povidone-iodine and proceed to **Post-operative care.** If not, proceed with the following section to stenose the artery.

NOTE: Every experiment should have sham animals as controls of the surgical procedure. Sham animals consist of mice that have gone through the surgical procedure of exposing the renal artery without placing a cuff on it.

2. **Right renal artery stenosis**

2.1. Place two nylon sutures under the right renal artery, make loose knots, and then place the cuff around the main renal artery approximately equidistant between the kidney and aorta bifurcation

2.2. Close the cuff using the nylon sutures. Make three knots for each suture to avoid the probability of losing the sutures after the surgery.

2.3. Close the incision in the muscle by applying a simple continuous suture.

2.4. Make simple interrupted sutures to close the skin.

2.5. Apply povidone-iodine, and antibiotic to the wound, and administer painkiller (ketoprofen) intraperitoneally (I.P. dose: 5 mg/kg BW).

NOTE: Autoclave surgical tools before every use. If more than one surgery is being performed at once, wipe all used tools with a sterile alcohol gauge and place them in hot germinator for 15-30 s after every surgery. Change sterile gloves also for each mouse.

3. Post-operative care

3.1. Return mice to their cage and leave the cage overnight on a circulating water heating pad. Add gel diet recovery food inside the cage.

3.2. Administer painkiller (ketoprofen) intraperitoneally (dose: 5 mg/kg BW) during the next day.

3.3. Weigh the mice for the next two days; if some mice lose more than 20% of their weight consult with the veterinarian and decide if the animal needs to be euthanized following the appropriate AICUC authorized procedure.

3.4. Monitor the mice daily to assess for redness, swelling, pain or infection.

3.5. Apply wound clips and/or administer antibiotics if needed.

4. Tissue harvest

4.1. Record the weight of each mouse.

Place the mouse on a desiccation jar saturated with isoflurane to euthanize the animal with an anesthetic overdose.

CAUTION: Isoflurane must be used properly inside a fume hood vented to outdoors to avoid exposure.

4.2. Gently grasp the tail to remove mice from jar and place on to a sterile platform in supine position to dissect.

4.3. Secure and extend the limbs to limit movement.

4.4. Thoroughly spray the mice with 70% ethanol.

4.5. Make a midline incision to open the abdomen and chest area using sharp scissors.

4.6. Pull back the skin and peritoneal wall.

4.7. Carefully expose the heart and puncture the right ventricle and exsanguinate the mouse.

4.8. Remove both kidneys using forceps. Kidneys are located on the back of the mice.

NOTE: Do not mix both kidneys. Be aware of stenosed and contralateral kidneys.

4.9. Remove the kidney capsule, clean them from any fat and record the weight of each kidney separately.

4.10. Cut a longitudinal section of both kidneys and fixed in 4% PFA overnight at 4 °C to be later processed for paraffin embedding, to perform in situ hybridization (ISH) and immunohistochemistry (IHC). Follow ISH and IHC protocols as reported[23,24].

4.11. Isolate the cortex of the remaining kidney and flash freeze in liquid nitrogen to perform western blot. Store samples at -80 °C until analysis.

4.12. Quantify renin, and N-GAL expressions with Western blot as described in literature[23,24].

5. **Statistics**

5.1. Use one-way or two-way ANOVA for experiments with three or more conditions followed by Bonferroni post-hoc tests for comparisons between individual groups. Consider a p-value equal or less than 0.05 significant. Use software (e.g., GraphPad Prism 8.2) to perform all statistical analysis.

REPRESENTATIVE RESULTS

Renal artery constriction increases renin expression in the stenosed kidney while repressing expression in the contralateral kidney. The two kidney one clip (2K1C) or Goldblatt model of stenosis induced renin expression increases and kidney injury. This is recognized as the best representative model of unilateral renal artery stenosis in humans.

Expression of renin and prorenin (precursor of renin) were measured using immunoblotting. The data show that renin and prorenin expression increased in the stenosed kidney comparing to contralateral and sham kidneys, suggesting that the cuff was constricting the renal artery causing changes in renal perfusion (**Figure 1**). To visualize the localization of renin expression, IHC was performed. IHC corroborated immunoblotting data showing increased expression of renin in clipped kidney (**Figure 2**). Moreover, juxtaglomerular (JG) cells recruitment along the afferent arteriole was seen in the stenosed kidney (**Figure 2**). To investigate the effect on renin mRNA expression levels, ISH was performed. The ISH data suggest increased renin mRNA and JG cells recruitment in the stenosed kidney comparing to contralateral and sham kidneys (**Figure 3**).

Another characteristic of renal artery stenosis is the upregulation of kidney injury markers due to changes in kidney perfusion, superoxide production and hypertension[2,25,26]. Neutrophil gelatinase-associated lipocalin (NGAL) is a well characterized acute injury marker and is

overexpressed during kidney injury[27,28]. Therefore, acute kidney injury marker NGAL was measured using immunoblotting. Immunoblotting data showed that N-GAL was highly upregulated in the stenosed kidney comparing to the contralateral and sham kidneys (**Figure 4**).

FIGURES LEGENDS

Figure 1. Renin expression. After 15- and 3-days of renal artery stenosis, mice were euthanized. Kidneys were harvested, and renin expression was determined by western blot. (**A**) is showing representative western blots images from 15-days stenosed (left panel) and sham mice (right panel). (**B**) is showing the densitometric analysis of prorenin (left panel) and renin (right panel) protein bands. Beta-actin was used as loading control. (**C**) is showing the representative western blots images from 3-day stenosed (left panel) and sham mice (right panel). (**D**) is showing densitometric analysis of prorenin (left panel) and renin (right panel) protein bands. Beta-actin was used as loading control. Data are presented as the mean ± SD. P calculated with two-way ANOVA followed by Tukey post-hoc test. *P<0.05, **P< 0.01, ***P< 0.001, N=3-6.

Figure 2. Immunohistochemistry analysis for visualization and localization of renin expression after renal artery stenosis. Kidneys were isolated after euthanizing mice from 3-day renal artery stenosis. I piece from longitudinal cut of the whole kidney was fixed with 4% neutral buffered formalin solution, after that dehydrated in a graduated ethanol series, and embedded in paraffin. Green staining represents renin protein expression; blue, nuclei. (**A**) Representative microscopy images of stenosed kidney (left side), contralateral kidney (right side) from stenosed mice. (**B**) Representative microscopy images of sham kidney (left side), and contralateral kidney (right side) from sham mice. Scale bar 30 microns. 90X magnification. (**C**) Representative microscopy images of stenosed kidney (left side), contralateral kidney (right side) from stenosed mice. (**D**) Representative microscopy images of sham kidney (left side), and contralateral kidney (right side) from sham mice. These images were mainly taken from cortex region. Scale bar 50 µm. 15x magnification. White dotted circles denote the location of glomeruli. G: Glomeruli, AfAr: Afferent arteriole, N=4.

Figure 3. In situ hybridization analysis of renin mRNA expression after renal artery stenosis. After 3-days of renal artery stenosis, mice were euthanized and kidneys were isolated and perfusion-fixed with 4% neutral buffered formalin solution, dehydrated in a graduated ethanol series, and embedded in paraffin. In situ hybridization was performed following the manufacturer's instructions. Dark red staining represents mRNA renin expression; blue, nuclei. (**A**)**.** Representative microscopy image of stenosed kidneys (left side), and contralateral kidney (right side) from stenosed mice. (**B**)**.** Representative microscopy image of sham kidney (left side), and contralateral kidney (right side) from sham mice. Scale bar 50 µm. White dotted circles denote glomeruli expressing renin. G: Glomeruli, AfAr: Afferent arteriole, N=4.

Figure 4. Neutrophil gelatinase-associated lipocalin (**N-GAL**) **expression after renal artery stenosis.** After 3-day renal artery stenosis, mice were euthanized and kidneys were harvested, and N-GAL expression measure by Western blot. (**A**) Representative Western blots images from 3-day stenosed (left panel) and sham mice (right panel). Beta-actin was used as loading control.

(**B**) Densitometric analysis of N-GAL bands. Protein density values of N-GAL were normalized to β-actin. Data are presented as the mean ± SD. P calculated with two-way ANOVA followed by Tukey post-hoc test. **P<0.01, ***P< 0.001, N=3-5.

DISCUSSION

Renal artery stenosis is an important cause of secondary or resistant hypertension, and kidney injury[1,29]. The two kidney one clip (2K1C) Goldblatt model has been employed to study RAStenosis induced renovascular hypertension[1,17-19]. A number of previous studies using various animals models have shown that stenosis in the renal artery is a strong stimulator of renin overexpression and release, and kidney injury[18,30-35]. Moreover, this model is used to study immune cell infiltration, fibrosis, inflammation, and acute and chronic kidney injury markers[29].

Here, we described a detailed and step by step procedure to generate reproducible, reliable and consistent renal artery stenosis model in mice. Earlier, metal clips have been employed to initiate renal artery stenosis[36-38]. As an alternative, we used polyurethane round tubing (MRE 025; internal diameter (ID) = 0.30 mm; outside diameter (OD) = 0.63 mm; wall thickness, (WT) = 0.16 mm). We used tubing, since placement of a polyurethane cuff would result constriction in two dimensions (constriction) rather than one (flattening), as with a metal clip. Also, using polyurethane round tubing provides an advantage of uniform constriction in the renal artery. The critical step and challenges are to cut the right size of polyurethane tubing, which requires extreme attention to details that must be performed using a microscope. Another critical criterion is to keep mice between 18-22 g to fit the tubing onto the renal artery. Mice within this weight range normally have a renal artery outer diameter (OD) that is consistently within the range of the tubing cuff diameter. A limitation of the method is that heavy (above 25 g) or small (below 16 g) mice are difficult to perform surgery on because of the size of the tube and cuff made in it. However, when required, changes in the polyurethane tubing can be made to accommodate younger or older mice.

We have conducted 3-day and 15-day studies to initiate renal artery stenosis in mice with about 95% success rate. In our experience, induction of the renal artery stenosis using this method produced reliable, reproducible, and consistent results among the mice regardless of the sex. To confirm the constriction of renal artery, we measured renin expression and kidney injury. Our data suggest that renin expression significantly increased in the stenosed kidney.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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444

Figure

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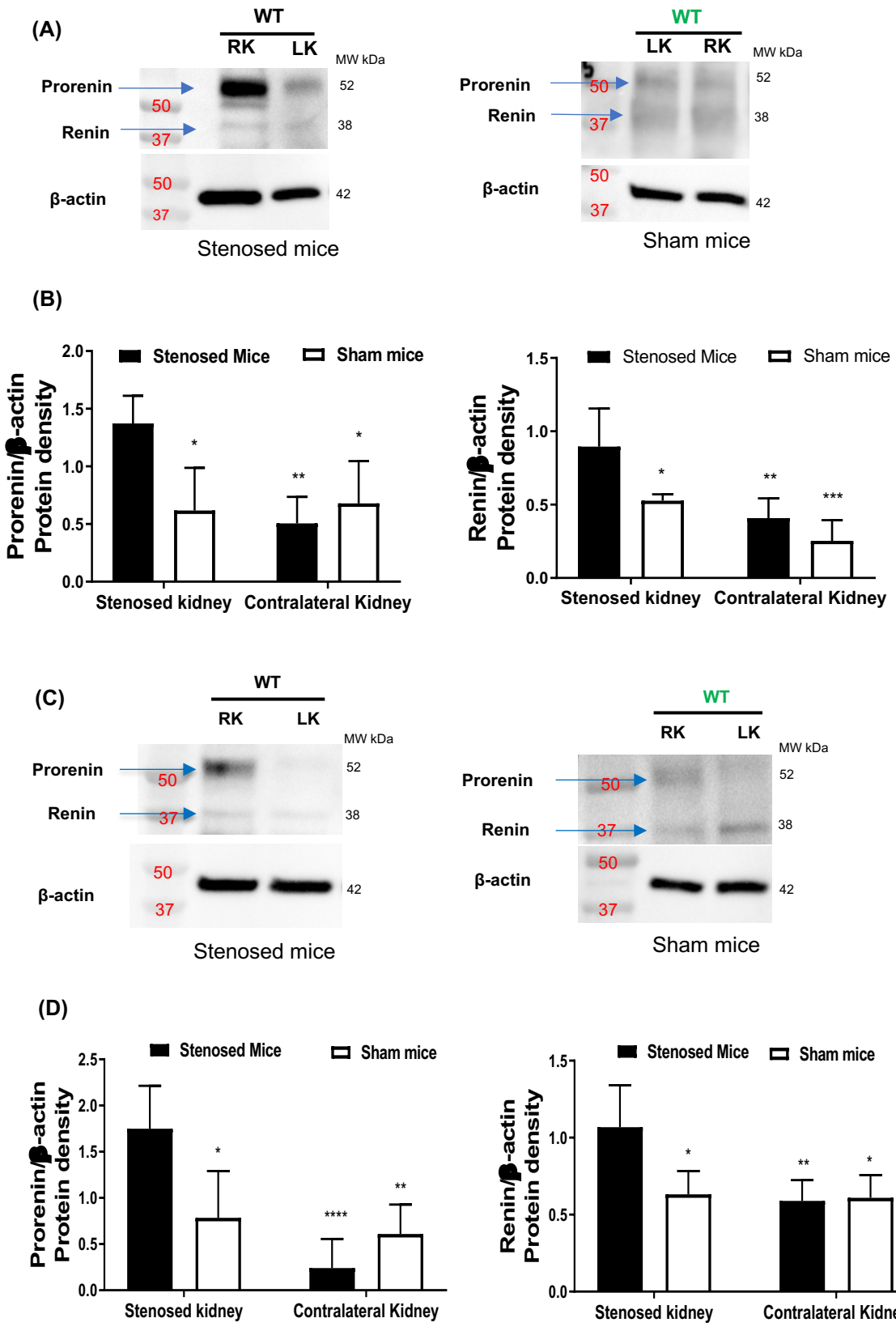

Figure 1.

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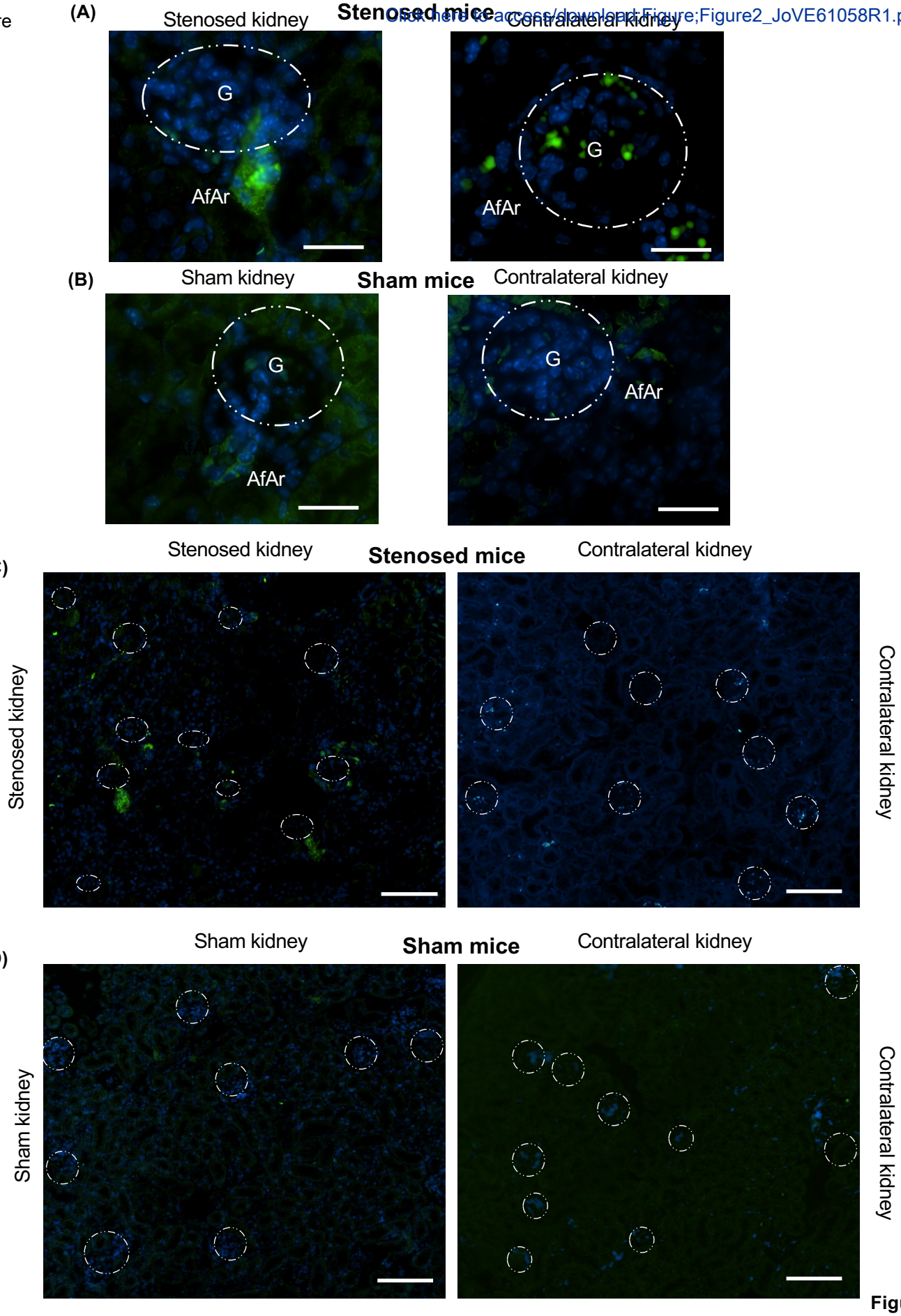

Figure 2.

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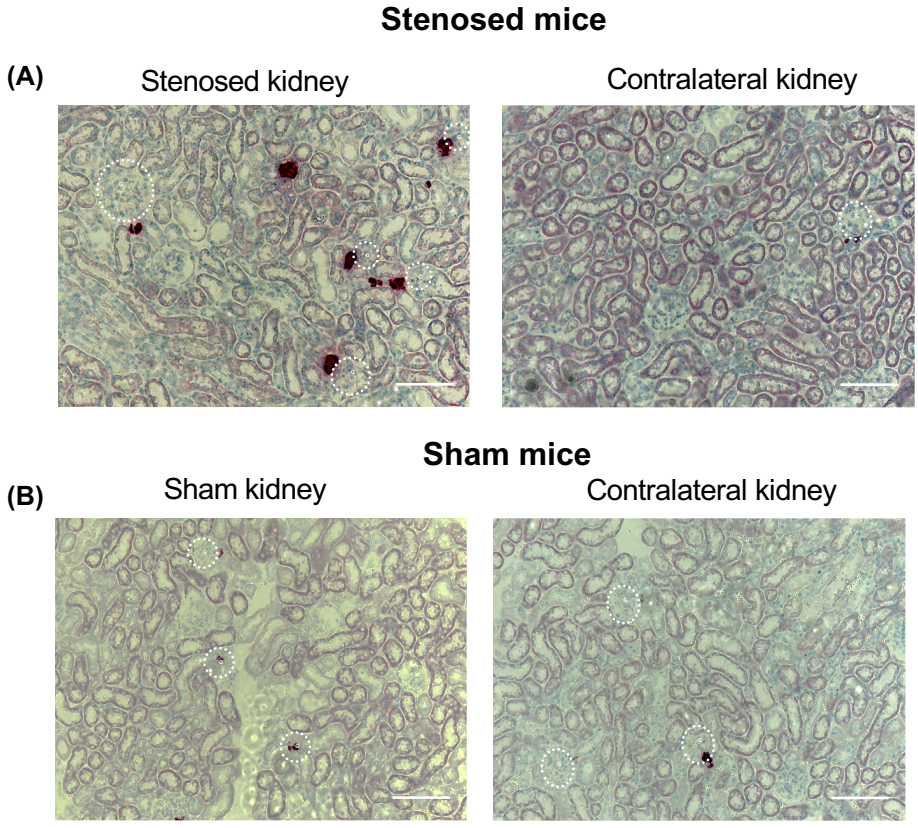

Figure 3.

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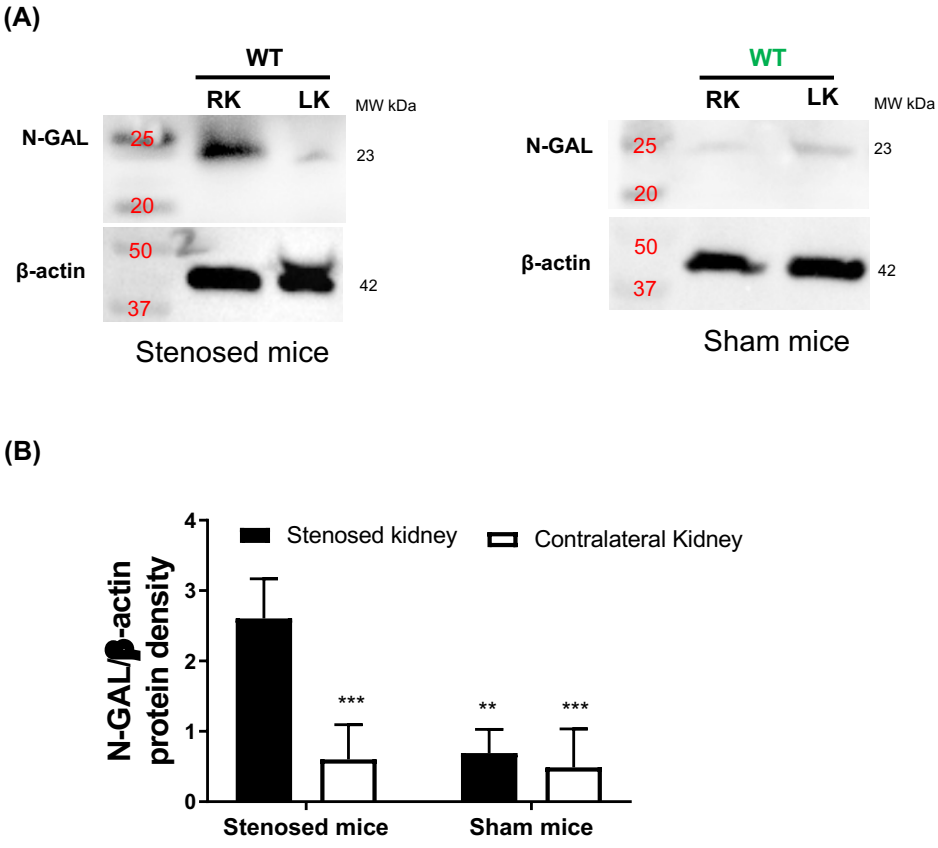

Figure 4.

Table of Materials

Name of Material	Company	Catalog number
Diet Gel	ClearH2O	Diet-Gel 76A
EMC Heated Hard pad	Hallowell	000A2788B
Ethilon Nylon Suture	Ethicon	662G
Ethilon Nylon Suture	Ethicon	2815 G
Germinator 500	Braintree Scientific Inc.	GER 5287
Ketoprofen	Zoetis	Ketofen
Polyurethane	Braintree Scientific Inc.	MRE-025
Povidone-iodine antiseptic swabsticks	Medline	MDS093901
Reflex 7 Clip Applier	Roboz Surgical Instrument Co	204-1000
Sterile towel drapes	Dynarex	4410
Water pump	Stryker	T/pump Professionals

Comments/Description

Surgery recovery diet
Heating pads were used to keep mice warm
4-0 (1.5 metric), This suture was used to close the peritoneum, and skin
8-0 (0.4 metric), This suture was used to close cuff to tie and constrict the artery
Sterilize surgical tools between surgeries
Painkiller
This tube was used to initiate stenosis
It was applied after hair removal and surgery on the skin
This clip applier was used to apply clip in case one or more sutures went off
It was used as a bedsheet for mice during surgery
Used to warm and circulate water in the heating hard pad to keep mice warm during and post-surg

jery

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Please revise lines 286-289, 343-344, and 386-391 to avoid textual overlap with previously published work.
3. Please reference Figure 1A, B in the protocol section.
4. References: Please do not abbreviate journal titles; use full journal name.

We appreciate the editorial comments and to correct the references issues, we followed the JoVE reference style available in the EndNote.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This is a description of a method to create 2K-1C hypertension in the mouse

Major Concerns:
I don't see a 10 mmHg increase in blood pressure after 1 week in the stenosed mice (Figure 1C) as the authors claim in their response to the reviewers. It actually looks like the sham mice have a greater increase in blood pressure over basal levels after 1 week (~100 to 120 mmHg?). The variability in the baseline and 1 week blood pressure measurements obtained by the tail-cuff method is also very high as depicted in this figure. This emphasizes the point that blood pressures need to be measured by a direct method. The clipped mice should be clearly hypertensive at this point and they are not, at least represented in Figure 1C.

The authors claim that implantation of telemeters would increase mortality in this model; however, the telemeters can be placed PRIOR to the 2K, 1C surgery. The mice could easily be allowed to recover for 1-2 weeks after this surgery as it only requires a small incision in the neck and the telemeters are positioned subcutaneously. That way blood pressure could be continuously monitored throughout the whole study. Direct measurement of blood pressure is required for true validation of this model. The authors surely could find someone at their major medical institution who measures blood pressure in mice by telemetry to aid with these measurements.

We appreciate the reviewer suggestions. We are dropping the idea to report the blood pressure data in this pedagogical article. Reason 1: The main objective of the article is to describe the technical and methodological steps of producing renal artery stenosis in mice using 2K1C approach. Later, the critical steps required to produce renal artery stenosis will be filmed to show visual procedures and will be published with the article. Reason 2: Presently, we are not able to arrange telemetry units to measure blood pressure.

Reviewer #3:
The present manuscript brings a method to induce Goldblatt hypertension (two kidneys one clip) in

mice. The topic is relevant and of scientific interest. However, what is the purpose of the journal? is it pedagogical or scientific? If it is pedagogical the manuscript makes sense, however, if it is scientific the manuscript does not make sense. Similar work has already been published by John N. Lorenz and colleagues (Renovascular hypertension using a modified two-kidney, one-clip approach in mice is not dependent on the α1 or α2 Na-K-ATPase orabain-binding site, Am J Physiol Renal Physiol. 201; 301(3): F615-F621.). In addition, the analyzes made in the present study extended up to two weeks, and some analyzes only until the third day after clipping. Below are other important points that should be considered by the authors.

We acknowledge the limitations. Since the main objective of the paper is to describe the methodological and technical aspects required to produce renal artery stenosis, we did not focus much on the length of the study, our main focus is to describe the 2K1C modified technique and present data for renin and prorenin expression and kidney injury. Furthermore, we present analysis of the different expression of renin and prorenin differences between the stenosed and contralateral kidney and comparison to kidneys from sham animals.

1. In the introduction, the authors describe: "... silver is malleable and can be reshaped; and the silver dioxides of the clip can cause perivascular inflammation, intimal proliferation, and tissue granulation, altering the renal artery diameter. "The respective statement is reasonable, however, the references pointed out by the authors, that is, 4 and 5, do not describe studies who reported such characteristics in animals submitted to renovascular hypertension through the use of silver clips. Please insert correct references. Furthermore, how are the authors sure that the polytetrafluoroethylene tube did not cause similar effects? Do they have histological analysis of the respective arteries?

We apologize for our references oversight; these have been amended. We are sorry, we do not have the histological analysis of the arteries, however we changed the text in the introduction about silver clips. The main reason to prefer polyurethane tubing over silver clips is that Lorenz et al 2011, recently reported that using U-shaped conventional silver clip results in inconsistent results and lower reproducibility.

2. The authors state that they had a 95% success rate in inducing renovascular hypertension using the method, however, I consider that the number of animals used in each group was extremely low. Considering the purpose of the study, increasing the sample size is crucial and can strengthen the use of the method by other research groups.

We appreciate the reviewer suggestion, and now we have increased the sample size.

3. As it is a methodological approach, the insertion of schematic figures of the described processes is vital for the reader's clear understanding. Please provide diagrams, as well as a representative image of the polytetrafluoroethylene tube inserted in the renal artery.
Based on the reviewer comment, the critical steps required to produce renal artery stenosis will be filmed to show visual procedures and will be published with the article. We will emphasize in the video the critical steps and give specific suggestions and advice on how to perform the procedure.

4. What was the internal diameter of the polytetrafluoroethylene tube? This characteristic must be considered. The internal diameter of the clip is a crucial point in determining the plasma renin activity and blood pressure (J Appl Physiol (1985). 2012 Feb;112(3):362-6. doi: 10.1152/japplphysiol.01015.2011).

We appreciate the reviewer comment, these are the measurements of the tubes which we used: Catalog # MRE 025, Braintree Scientific; **internal diameter** (ID) = 0.30 mm; outside diameter (OD) = 0.63 mm; wall thickness, (WT) = 0.16 mm]. We have included this information in the paper as well.

5. Why did the authors not observe an increase in blood pressure one week after the induction of renovascular hypertension? Was there an increase in intrarenal renin after 7 days of disease induction? Was plasma renin activity elevated after the first and second weeks of induction?

We understand the reviewer's concern with respect to plasma renin activity and blood pressure. Stenosed mice exhibited an increase in blood pressure after one week of surgery, however it was not significant. We are sorry but we do not have the explanation why mice did not show significant increase after one week of the renal artery stenosis. We did not measure renin expression after 7 days of the surgery. We also did not measure plasma renin activity after the surgery. In the new revised version, we shifted the focus of the 2K1C procedure to be a reliable method to study renin expression and kidney injury induced by renal artery stenosis.

6. Why were the analyzes shown in figures 3, 4 and 5 not performed 15 days after induction of hypertension? Considering the subsequent analyzes, this information seems incomplete and such data must be inserted.

We understand the reviewer comment. However, since this is a methodological article and we wanted to show the step-by-step procedure to induce renal artery stenosis, we focused on to show the procedure by conducting analysis of renin and prorenin expression as well as kidney injury at 3- and 15-days after renal artery stenosis.

7. The legend of figure 5 is wrong. Figure 5 instead of Figure 2.

We appreciate the comment and we are sorry for the oversight and corrected the error.

8. Only two weeks of observation is not enough to assess cardiovascular and renal changes in renovascular hypertension, a longer observation period, at least up to 4 weeks is recommended.

We acknowledge the reviewer comment. As we mentioned before, this is a methodological article and we wanted to show the step-by-step procedure to induce renal artery stenosis, so we focused on changes in renin expression and kidney injury found after 3- and 15-days after renal artery stenosis.

Reviewer #4:

Manuscript Summary:

Although the 2 kidney 1 clip model (2k1c) is an accepted model for renovascular hypertension and is widely used in several laboratories worldwide, a comprehensive technical description of the procedure has not yet been published. Therefore, the authors present a step by step protocol as well as results demonstrating the feasibility of a 2k1c model.

Major Concerns:

none

Minor Concerns:

In the introduction (starting at line 90) the advantages of the tail cuff method over telemetry are listed. This presentation is too one-sided as it does not show the disadvantages of the tail cuff method (fixation

of the mice, warming of the whole mouse or the tail, measurement during the day and thus disturbance of the resting phase, etc.).) nor the advantages of telemetry (continuous measurement during rest and activity phase, direct measurement of systolic and diastolic pressure, recording the activity of the mice, etc.). This should be corrected.

We agree with this comment, we apologize for this oversight, we made changes to the introduction to correct this issue.

Since the 2k1c model is widely used in renin research and therefore plasma renin activity might be a relevant parameter for researchers using this model, it should be mentioned that plasma renin activity is markedly stimulated by stress and by anesthesia and that blood sampling should be done preferably in conscious mice, for instance by puncture of the facial vein.

We completely agree with the reviewer that plasma renin activity is an important parameter for 2K1C mode, so we will explain this important parameter in the 'discussion' section. In this study we focus our attention to the expression of renin and prorenin in the kidney.

The authors state that "renal artery constriction increases renin expression in the stenosed kidney while represses expression in the contralateral kidney" (line 292). While this is of course true, the data of the present study do not show this. It is clearly shown that renin protein is stimulated in the constricted kidney (figure 2). However, renin protein is not reduced in the constricted kidney compared with sham kidneys (fig.2). Therefore, the data do not allow concluding that renin is repressed in the contralateral kidney. Please comment on this issue.

We appreciate the comments by the reviewer, we agree that renin can be seen in the contralateral kidney in stenosed mice which is similar to the sham kidneys Figure 1A and B former Figure 2A. This is true only for the 15-day study, the same trend in present after 3-days of renal artery stenosis (Figure 1D). However, renin expression in the contralateral kidneys in stenosed mice is almost undetectable when measured with immunofluorescence and *in situ hybridization* in Figure 2A, C (right panel) and 3A (right panel). We have made changes to the article to reflect the results we present in the paper.

Fig. 3: please show an overview of a larger area of kidney cortex including more than 1 glomerulus.

We thank the reviewer for the comment. Now we added images showing a broader cortex image which including several glomeruli (Figure 2 C, and D).