Journal of Visualized Experiments

Renal denervation approach to prevent inflammation and fibrogenesis in chronic kidney disease

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60833R2
Full Title:	Renal denervation approach to prevent inflammation and fibrogenesis in chronic kidney disease
Section/Category:	JoVE Medicine
Keywords:	Denervation, norepinephrine, adrenergic receptor, ischemia/reperfusion, ureteral obstruction, inflammation, fibrosis, chronic kidney disease
Corresponding Author:	Babu Padanilam UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	bpadanilam@unmc.edu
Order of Authors:	Hee-Seong Jang
	Mi Ra Noh
	Jinu Kim
	Babu J Padanilam
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Omaha, Nebraska, USA

TITLE

A Renal Denervation Approach to Prevent Inflammation and Fibrogenesis in Chronic Kidney Disease

AUTHORSAND AFFILIATIONS

Hee-Seong Jang[1], Mi Ra Noh[1], Jinu Kim[1,2,3], Babu J. Padanilam[1,4]

[1]Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, NE, USA

[2]Department of Anatomy, Jeju National University School of Medicine, Jeju, South Korea

[3]Interdisciplinary Graduate Program in Advanced Convergence Technology & Science, Jeju National University, Jeju, South Korea

[4]Internal Medicine, Section of Nephrology, University of Nebraska Medical Center, Omaha, NE, USA

Corresponding Author:

Babu J. Padanilam (bpadanilam@unmc.edu)

Email Addresses of Co-authors:

Hee-Seong Jang (heeseong.jang@unmc.edu)

Mi Ra Noh (mira.noh@unmc.edu)

Jinu Kim (jinu.kim@jejunu.ac.kr)

KEYWORDS:

denervation, norepinephrine, adrenergic receptor, ischemia/reperfusion, ureteral obstruction, inflammation, fibrosis, chronic kidney disease

SUMMARY:

Described here is a protocol for renal denervation that is used to define the role of renal nerve-derived signaling in persistent renal tubular injury, inflammation, and fibrogenesis. It is focused on sympathetic nerve-mediated signaling.

ABSTRACT:

Chronic kidney disease (CKD) is affecting increased numbers of people across the world, and there remains no effective treatment strategy. Sympathetic nerve activation has been recognized as an important factor in the development and progression of cardiovascular disease, hypertension, and CKD. Catheter-guided renal denervation is useful to control blood pressure (BP) in patients with resistant hypertension and CKD. Sympathetic nerve-derived norepinephrine (NE) has been implicated in tissue homeostasis and the progression of various diseases, including CKD. The molecular mechanisms and signaling pathways triggered by sympathetic nerve activation, which drive renal inflammation and fibrogenesis in CKD progression, remain undefined. Presented here is the detailed methodology for renal denervation (RDNx) in experimental models of CKD. The results show that this method effectively ablates the renal nerve, as evidenced by the loss of tyrosine hydroxylase immunoreactivity and levels of kidney

NE. This results in the suppression of renal tubular injury, inflammation, and fibrogenesis in CKD models. Competence of surgeons performing surgical procedures to denervate the kidney is a requirement to achieve consistent results. RDNx can be utilized to study the roles of renal nerve, nerve-derived neurotransmitters, and factors, as well as unveil their downstream signaling pathways. Defining the molecular mechanisms and underlying functions will lead to the design of novel therapeutic interventions for CKD, regardless of its etiology (e.g., diabetes, hypertension, and cardiovascular complications).

INTRODUCTION:

CKD, characterized by tubular injury, persistent renal inflammation, and fibrosis, ultimately leads to end stage kidney disease (ESKD)[1-3]. Sympathetic nervous system governs both normal and pathological functions of diverse organ systems, including those in the kidney[4]. One type of catecholamine, norepinephrine (NE or noradrenaline), originates from sympathetic neurons and is an effector of the sympathetic nervous system[5]. In both patients and experimental models, increased sympathetic nerve activity and tissue levels of NE are hallmarks of CKD progression[6-8].

Renal denervation (RDNx) is used as a therapeutic option for the treatment of drug-resistant hypertension[9-12], but the underlying molecular mechanisms are not yet fully understood. Moreover, studies[13,14] defining the role of renal nerve in CKD progression using chemical denervation or antagonists/agonists of sympathetic nerve-derived neurotransmitters (or its receptors) are limited, due to systemic effects that are not specific to the kidney. As reported[15-17], RDNx can overcome this limitation and has been successful in defining (in vivo) the roles of the renal nerve, renal nerve-derived factors, and downstream signaling pathways in eliciting renal inflammation and fibrosis.

Provided here is a detailed methodology and representative results for the use of RDNx to study renal tubular injury, inflammation, and fibrosis in a mouse model of CKD.

PROTOCOL:

Mice were cared for prior to and during the experiment in accordance with the policies of the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center (UNMC), and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All portions of the protocol received prior approval from the UNMC-IACUC.

1. Renal denervation

1.1. Use male (129S1/SvImJ) mice (8–10 weeks old) from the Jackson Laboratories.

1.2. Anesthetize mice using a cocktail containing ketamine (200 mg/kg body weight) and xylazine (16 mg/kg body weight), injected intraperitoneally[16,18].

1.3. Check for the lack of response to the toe pinch. Shave the left side of mouse's back with an electric shaver. Clean the region with an alcohol prep pad, and then ensure asepsis using an iodine-based prep pad.

1.4. Maintain the body temperature at 36–38 °C on a heating pad with a temperature controller.

NOTE: Use sterilized surgical instruments for all procedures.

1.5. Make an incision 1.5 cm long using a surgical blade on the mouse's back, 5 mm lateral to the spine.

1.6. Under a surgical microscope, expose the left kidney vessels connected to the kidney through left flank incision. Then, separate the artery and vein carefully from the surrounding connective tissue using micro-dissecting forceps.

1.7. For stripping the nerves, paint the kidney vessels with filter paper soaked in 95% ethanol for 2 min[15,16].

NOTE: Ensure that the vessels turn white in color under the microscope.

1.8. Wash the kidney vessels for 2 min with PBS. Close the muscular layer with absorbable sutures and close the wounded skin using an autoclip.

1.9. Perform a sham operation in the same manner as above, except strip the kidney nerves.

1.10. After surgery, inject 0.1 µg/kg body weight (BW) buprenorphine subcutaneously (SC) 1x/day for 3 days to reduce surgical pain.

2. Renal fibrosis models

2.1. Unilateral ureteral obstruction (UUO) model

2.1.1. Two days after renal denervation, anesthetize two male mice (129S1/SvImJ) aged 8–10 weeks as described in step 1.2.

2.1.2. Check for the lack of response to a toe pinch, then shave the left side of the mouse back.

2.1.3. Clean the region with an alcohol prep pad and ensure that the region is aseptic using an iodine-based prep pad.

2.1.4. Maintain the body temperature at 36–38 °C on a heating pad with a temperature controller.

2.1.5. Expose the left kidney using autoclaved cotton swab through a left flank incision using a surgical blade on the mouse's back, 1 cm lateral to the spine.

2.1.6. Ligate the left ureter completely near the kidney and pelvis using a 5-0 silk tie[15,18].

2.1.7. Close the muscular layer with absorbable sutures and close the wounded skin using an autoclip.

2.1.8. On the other mouse, perform a sham operation using the same surgical procedure, except for ligation of the ureter.

2.1.9. After surgery, inject 0.1 µg/kg buprenorphine SC 1x/day for 3 days to reduce surgical pain.

2.2. Ischemic acute kidney injury (AKI) to CKD transition model

2.2.1. Two days after the denervation, anesthetize male 129S1/SvImJ mice aged 8–10 weeks as described in step 1.2.

2.2.2. Check for the lack of response to a toe pinch and shave the left side of the mouse's back. Clean the region with an alcohol prep pad and ensure that the region is aseptic using an iodine-based prep pad.

2.2.3. Maintain the body temperature at 36–38 °C on a heating pad with a temperature controller.

2.2.4. Expose the left kidney using an autoclaved cotton swab through the left flank incision on the back, 1 cm lateral to the spine, using a surgical blade.

2.2.5. Subject the mice to 30 min of left renal ischemia by clamping the left renal pedicle with both the artery and vein with nontraumatic microaneurysm clamps, as described previously[16,19]. Close the incisions temporarily during this ischemic period using clamps.

2.2.6. Once the ischemic period is over, remove the clamps for reperfusion.

NOTE: Visually confirm reperfusion of the kidneys. The color of the kidneys will change from dark red to bright red upon reperfusion.

2.2.7. Close the muscular layer with absorbable sutures and close the wounded skin using an autoclip.

2.2.8. After the surgery, inject 0.1 µg/kg BW buprenorphine SC 1x/day for 3 days to reduce surgical pain.

2.2.9. Perform a sham operation with the same surgical procedure except clamping the renal pedicle.

3. Harvesting of kidneys and blood

3.1. At 1 h, 3 h, 6 h, or 12 h (or 1, 2, 3, or 10 days) post-UUO; or at 0.5, 1, 2, 4, 8, or 16 days post-IRI, anesthetize by placing the mouse's nose to a 50 mL conical tube with gauze containing isoflurane.

3.2. Collect either 1) 100 µL blood samples from the retroocular vein plexus using a heparinized capillary tube or 2) 500 µL of blood from the vena cava through a midline abdominal incision using a syringe (25 G needle) containing heparin.

3.3. For harvesting kidneys, expose the operated kidney by midline abdominal incision. Cut renal vessels and surrounding tissues with surgical blade and then remove the capsules.

NOTE: Ensure that UUO kidneys have urine filled the pelvis to confirm UUO induction.

3.4. Collect plasma from the blood by centrifugation at 8,000 *x g* for 3 min at room temperature (RT) using a heparinized capillary tube. Store at -20 °C until use.

3.5. For biochemical assays, snap-freeze the kidneys using liquid nitrogen immediately after removal. Store at -80 °C deep freezer until use.

4. Analysis of renal function

4.1. Anesthetize mice 15 days post-ischemia/reperfusion injury (IRI) as described above.

4.2. Expose the right kidney using autoclaved cotton swab through a right flank incision on the back, 1 cm lateral to the spine.

4.3. Remove the kidney (i.e., perform a nephrectomy) from the renal vessels and ureter using scissors, after ligation of the renal vessels and ureter[16,20].

4.4. Take blood samples from the retroocular vein plexus 6 h prior to placing the mouse in a metabolic cage.

4.5. To obtain urine samples, place the animals in mouse-specific metabolic cages for 18 h for analysis of glomerular filtration rate (GFR) by creatinine clearance[16].

4.6. Measure urinary and plasma concentrations of creatinine using a commercially available kit (**Table of Materials**). In brief, add 30 µL of plasma to a mixture of Reagents A and B (100 µL each). Then, read the optical density both 1) immediately and 2) at 5 min post-incubation at 510 nm and RT using a microplate reader. For urine, use 50 µL of Reagent A, 50 µL of Reagent B, and 100

µL of water per 5 µL of urine.

4.7. Calculate GFR as follows:

$$\mathrm{GFR} = \frac{urinary\ creatinine\ (mg/dl) \times urine\ volume\ (\mu l)}{plasma\ creatinine\ (mg/dl) \times urine\ collection\ time\ (min)\ \times body\ weight\ (g)}$$

4.8. Alternatively, measure GFR by transcutaneous measurement at 24 h post-Nx, as described previously[21]. In brief, under anesthesia with isoflurane, place a miniaturized fluorescence detector on the mouse's back, then inject fluorescein-isothiocyanate (FITC)-conjugated sinistrin (an inulin analog, 0.15 mg/g BW) through the retroocular vein plexus. In conscious and freely moving mice, collect data for the half-life of FITC-sinistrin for 1 h and perform the analysis.

5. Confirmation of renal denervation

5.1. Evaluation of kidney NE level

5.1.1. Homogenize kidneys frozen at -80 °C in a deep freezer with a glass tissue homogenizer. Use homogenizing solution containing 1 mM EDTA and 4 mM sodium metabisulfite in 400 µL of 0.1 N HCl.

5.1.2. Centrifuge the homogenates at 13,600 *x g* and transfer the supernatant to new tube.

5.1.3. Extract NE from samples by using a cis-diol-specific affinity gel, acylate, then convert enzymatically with a commercially available kit (**Table of Materials**)[15,16].

5.1.3.1. Transfer 300 µL of the lysates into the 48 well plate and add 250 µL of distilled water to each well, followed by adding 50 µL of assay buffer and 50 µL of extraction buffer.

5.1.3.2. Cover the plate with adhesive foil and incubate for 30 min at RT on a shaker (~600 rpm) then remove the foil, empty, and blot dry by tapping the inverted plate on a hand towel.

5.1.3.3. Wash the plate 2x for 5 min at RT using 1 mL of washing buffer by shaking on a platform shaker, followed by blotting dry as done in step 5.1.3.2.

5.1.3.4. Add 150 µL of acylation buffer and 25 µL of acylation reagent into the wells, then incubate for 15 min at RT on the shaker.

5.1.3.5. Empty the plate and blot dry as done in step 5.1.3.2, then wash 1x for 10 min as done in step 5.1.3.3, followed by blot drying as done in step 5.1.3.2. Add 150 µL of 0.025 M hydrochloric acid into the wells, cover the plate with adhesive foil, and incubate for 10 min at RT on the shaker.

5.1.4. Add 25 µL of enzyme solution to the pre-coated NE microtiter strips, followed by 20 µL of supernatant (as done in step 5.1.3.5) and standard solutions of NE (0 ng/mL, 5 ng/mL, 20 ng/mL,

75 ng/mL, 250 ng/mL, and 1,000 ng/mL) into each strip. Add 50 µL of the NE antiserum to the strips. Incubate for 2 h at RT on the shaker.

5.1.5. After discarding the buffer and washing the microtiter strips with 300 µL of washing buffer on the shaker, remove the buffer and blot dry as done in step 5.1.3.2. Incubate for 30 min at RT on the shaker with 100 µL of enzyme conjugate in each well.

5.1.6. After discarding the buffer and washing the strips, add 100 µL of substrate into each well. Incubate for 20–30 min at RT on a shaker, followed by adding 100 µL of stop solution.

5.1.7. Read the absorbance of the solution using a microplate reader at 450 nm and calculate the concentration using standard solution as control.

5.2. Immunohistochemistry for tyrosine hydroxylase

5.2.1. Fix kidneys in 4% paraformaldehyde and process the paraffin embedding with the central part of the fixed kidney with a cross section of 0.3–0.4 mm thickness.

5.2.2. Place the paraffin-embedded kidney on a microtome, cut to 3–4 µm thick, and attach the section to the microscope slide.

5.2.3. Deparaffinize the paraffin-embedded kidney sections with xylene. Rehydrate with 100%, 95%, and 90% ethanol (in this sequence) 2x at each step.

5.2.4. Permeabilize the kidney sections in 0.1% SDS for 5 min and wash 3x with PBS at RT.

5.2.5. Autoclave the permeabilized kidney sections in 10 mM sodium citrate at 120 °C for 10 min for antigen retrieval[16,22].

5.2.6. Cool down the autoclaved kidney sections for 30 min at RT and wash 3x with PBS at RT.

5.2.7. Incubate with 3% BSA in PBS for 30 min at RT to prevent non-specific binding.

5.2.8. After removal of BSA by suction, incubate the kidney sections with antibody (1:1000) against tyrosine hydroxylase overnight at 4 °C[15].

5.2.9. Next day, wash the kidney sections 3x with PBS at RT and incubate with HRP-conjugated anti-rabbit antibody for 1 h at RT.

5.2.10. After washing 3x with PBS at RT, apply 3,3 –diaminobenzidine (DAB) to the kidney sections and stain for up to 2 min.

5.2.11. After washing 3x with PBS at RT, dehydrate the kidney sections with 90%, 95%, and 100% ethanol and xylene for 5 min each. Mount the coverslips with a 1:1 mixture of mounting medium and xylene.

5.3. Western blot analysis for tyrosine hydroxylase

5.3.1. Isolate whole kidney proteins using lysis buffer with phosphatase inhibitor and protease[16,18].

5.3.2. Run 30 µg of kidney protein on PAGE gel in Tris-glycine-SDS buffer at 80 V for 1 h.

5.3.3. Transfer the protein to PVDF membrane from gel in Tris-glycine buffer at 80 V for 100 min at 4°C.

5.3.4. Block the nonspecific proteins on the membrane using 5% skim milk for 30 min at RT.

5.3.5. Incubate membranes with diluted antibody (1:2,000) against tyrosine hydroxylase in 10 mL of a commercially available blocking solution[15,16].

5.3.6. Use anti-β-actin antibody as loading controls on stripped membranes.

5.3.7. Expose membranes to the chemi-luminescence substrate solution. Quantify the bands using analysis software.

6. Analysis of tubular injury

6.1. Tubular damage: section paraffin-embedded kidney into 3 µm thick sections using microtome and stain using a periodic acid/Schiff (PAS) stain. First, incubate the sections with 5% PAS stain for 5 min. Then, rinse with distilled water and incubate with Schiff reagent for 15 min.

6.1.1. Wash PAS-stained kidneys with tap water for 5 min, counterstain with hematoxylin for 1 min, and dehydrate after washing with tap water for 5 min. Cover with mounting medium.

6.1.2. Select five fields from each PAS-stained kidney randomly in a blinded manner and photograph using light microscopy.

6.1.3. Score the damaged tubule with flattened lumen according to the loss of brush border, proteinaceous cast, loss of tubular nucleus, and dilation as follows: grade 0 = normal; grade 1 = >10%; grade 2 = 10%–25%; grade 3 = 26%–50%; grade 4 = 51%–75%; grade 5 = <75%[15,16,18].

6.2. Apoptotic cell death: analyze apoptotic cell death in paraffin-sectioned kidney samples by performing a TUNEL assay using a commercially available kit (**Table of Materials**), following the manufacturer's protocol.

7. Inflammation

7.1. Analysis of neutrophil

7.1.1. Prepare kidney sections as described in step 5.2 and incubate with antibodies against polymorphonuclear neutrophil overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibody[15,16].

7.1.2. Perform DAB staining and dehydration as described in steps 5.2.10 and 5.2.11.

7.1.3. Take photographic images from cortical or outer medullary regions in a blinded manner.

7.2. Analysis of macrophage

7.2.1. Prepare kidney sections as described in section 5.2.

7.2.2. Incubate the kidneys with antibodies against F4/80 overnight at 4 °C. Incubate with an HRP-conjugated secondary antibody[15,16].

7.2.3. Take images from cortical or outer medullary regions in a blinded manner.

7.3. Analysis of inflammatory cytokine level

7.3.1. Following section 5.3, prepare kidney samples.

7.3.2. Incubate the kidney samples with antibodies against ICAM-1, TNF-α, IL-1β, IL-6, and TLR4 overnight at 4 °C. Then, incubate with the corresponding secondary antibodies[15].

8. Evaluation of fibrosis

8.1. Sirius red stain

8.1.1. Following section 5.2, rehydrate the kidney sections.

8.1.2. Stain the kidney sections with Sirius red solution (0.5 g of Direct Red 80 + 1.3% picric acid in 500 mL of distilled water) for 30 min[15,16,18].

8.1.3. After washing 2x with acidified water (0.5% acetic acid, glacial), physically remove the water from the slides.

8.1.4. After mounting (section 5.2), take images of cortical or outer medullary regions in a blinded manner.

8.2. Evaluation of fibroblast activation and fibrogenic factors

8.2.1. Following section 5.3., prepare kidney samples.

8.2.2. Incubate the kidney samples with antibodies against alpha-SMA, fibronectin, TGF-β, and phospho-Smad3 overnight at 4 °C. Incubate with the corresponding secondary antibodies[15].

8.2.3. Use anti-β-actin antibody as a loading control on stripped membranes.

8.2.4. Quantify the bands using analysis software.

REPRESENTATIVE RESULTS:

Removal of renal nerve by renal denervation surgery

Renal denervation (RDNx) was carried out 2 days prior to IRI or UUO to define whether renal nerve contributes to the initiation and development of kidney fibrosis and inflammation. Mice were subjected to either 1) 30 min IRI followed by reperfusion for 1, 2, 4, 8, or 16 days or 2) UUO for 1 h, 3 h, 6 h, or 12 h; 1, 2, 3 or 10 days.

To confirm whether RDNx was successfully applied, tyrosine hydroxylase (TH)-positive sympathetic nerve fibers in adventitia of intrarenal arteries were observed at 10 days post-UUO by immunohistochemistry (**Figure 1A**). Furthermore, RDNx markedly reduced TH expression (**Figure 1B**) in the kidney. To determine the level of norepinephrine (NE), which is released from the sympathetic nerve fiber terminus, ELISA method was used. During UUO injury, intrarenal level of NE continued at the same level in both sham and UUO kidneys, but RDNx markedly suppressed its level (**Figure 1C**). Similarly, RDNx inhibited TH expression almost completely in both sham and IRI kidneys (**Figure 2A**). In IRI kidneys, NE level was increased at earlier timepoints and continued over time (at least up to 16 days post-IRI) (**Figure 2B**). These results show the successful removal of sympathetic nerve fibers and subsequent blockage of NE release by RDNx.

Effect of renal denervation in renal tubular injury, inflammation and fibrosis.

Renal tubular injury, inflammation, and fibrosis were analyzed to confirm the effects of RDNx in established renal fibrosis models. Since renal tubular injury is an initial factor of renal inflammation and fibrosis, tubular necrosis and apoptosis in UUO and IRI kidneys were observed. Tubular damage was evaluated using PAS-stained kidney sections, while tubular apoptosis was evaluated using the TUNEL assay.

UUO-kidneys showed severe necrotic cell death in all renal tubules (**Figure 3A**), but this was limited to the proximal tubules of outer medulla (**Figure 4A**) in IRI kidneys. Tubular apoptosis was similar with those of necrotic cell death (**Figure 4B**). Kidney function was preserved in IRI-kidneys with RDNx, compared to non-RDNx (**Figure 4C**). Kidney inflammation was analyzed by immunohistochemistry of PMN for neutrophils and F4/80 for macrophages, as well as western blotting for proinflammatory cytokines (i.e., IL-1β, IL-6, and TNF-α). Expression of neutrophil, macrophage, and proinflammatory cytokines were highly upregulated in both IRI and UUO kidneys (**Figure 3B-D and 4D-G**).

To determine fibrosis progression, Sirius Red Stain and western blotting for profibrotic cytokines (p-Smad3 and TGF- β), myofibroblast activation (α-SMA), and extracellular matrix (fibronectin) were assessed. These markers of fibrosis were markedly increased in both UUO (**Figure 5**) and IRI kidneys (**Figure 6**). RDNx significantly suppressed renal tubular injury, inflammation, and fibrosis progression in both UUO and IRI kidneys. This result was comparable to those of sham-operated kidneys (**Figure 3, Figure 4, Figure 5, Figure 6**), suggesting that renal nerve orchestrates renal tubular injury, inflammation and fibrosis in renal fibrosis models.

FIGURE AND TABLE LEGENDS:

Figure 1: Confirmation of renal denervation in UUO model. Renal denervation in left kidneys of male mice was carried out 2 days prior to UUO. The left ureters were obstructed for 0 h, 1 h, 3 h, 6 h, or 12 h at 1, 2, 3, or 10 days. (**A**) Paraffin-embedded kidney sections in intact or renal denervation (RDNx) without UUO were immunostained with anti-TH antibody (brown; n = 4). Hematoxylin was used to mark nuclei (blue). The arrow indicates TH-positive sympathetic nerve. (**B**) Western blot analysis with anti-TH antibody was performed to confirm the total expression of TH in intact or RDNx kidneys without UUO (n = 4). β-actin was used as a loading control. (**C**) Levels of kidney NE were measured by ELISA in UUO kidneys at indicated timepoints (n = 6). Scale bar = 50 μm. Data expressed as mean ± SD ($p < 0.05$ *vs.* intact; $^{###}p < 0.001$ *vs*. intact). This figure has been modified from a previous publication[15].

Figure 2: Confirmation of renal denervation in ischemic AKI to CKD model. Renal denervation in left kidneys of male mice was performed 2 days prior to IRI. Mice were subjected to 30 min ischemic periods of left kidneys and sacrificed at 0, 0.5, 1, 2, 4, 8, or 16 days post-IRI. (**A**) Western blot analysis with anti-TH antibody confirmed the total expression of TH in intact or RDNx kidneys with/without 16 days post-injury (n = 4). β-actin was used as a loading control. (**B**) Levels of kidney NE were measured by ELISA in IRI kidneys at indicated timepoints (n = 4). Data expressed as mean ± SD ($^{*}p < 0.05$ vs. intact in sham; $^{\#}p < 0.05$ vs. respective intact). This figure has been modified from a previous publication[16].

Figure 3: Effect of renal denervation in renal tubular injury and inflammation in UUO mice kidneys. Renal denervation in left kidneys of male mice was performed 2 days prior to UUO. The left ureters were obstructed for 0, 3, or 10 days. (**A**) Paraffin-embedded kidney sections were used for PAS staining. Histological damage score was measured in five (randomly chosen) fields per kidney at 10 days post-injury using PAS-stained kidney section (n = 4). (**B**) Paraffin-embedded kidney sections were used for immunohistochemistry with PMN (brown; neutrophil marker). (**C**) Number of PMN-positive neutrophils were evaluated in randomly chosen five fields per kidney at 3- and 10-days post-injury (n = 4–6). (**D**) Kidney level of ICAM-1, TNF-α, IL-1β, IL-6, and TLR4 in kidneys 10 days post-injury was evaluated by western blot analysis (n = 4–6). β-actin was used as a loading control. Scale bar = 50 μm. Data expressed as mean ± SD ($^{###}p < 0.001$ vs. respective intact). This figure has been modified from a previous publication[15].

Figure 4: Effect of renal denervation in renal tubular injury and inflammation in ischemic AKI

to CKD transition model Renal denervation in left kidneys of male mice was performed 2 days prior to IRI. Mice were subjected to 30 min ischemic periods of left kidneys and sacrificed at 0, 2, 4, 8, or 16 days post-IRI. (**A**) Paraffin-embedded kidney sections at post-injury 0, 2, 4, 8, or 16 days were used for PAS staining. Histological damage score was measured in randomly chosen five fields per kidney using PAS-stained kidney section at indicated time points (n = 4). (**B**) Paraffin-embedded kidney sections were used for TUNEL assay, and the number of apoptotic cells were evaluated in randomly chosen five fields per kidney at indicated timepoints (*n* = 5). (**C**) GFR was measured at indicated timepoints (*n* = 5). (**D**) Paraffin-embedded kidney sections were used for immunohistochemistry with PMN (brown; neutrophil marker). (**E**) Number of PMN-positive neutrophils were evaluated in randomly chosen five fields per kidney at indicated timepoints (n = 5). (**F**) Paraffin-embedded kidney sections were used for immunohistochemistry of F4/80 (brown; macrophage marker). (**G**) Number of F4/80-macrophages were evaluated in randomly chosen five fields per kidney at indicated timepoints (n = 5). Scale bar = 50 µm. Data expressed as mean ± SD ($^{\#}p < 0.05$ vs. respective intact). This figure has been modified from a previous publication[16].

Figure 5: Effect of renal denervation in renal fibrosis in UUO mice kidneys. Renal denervation in left kidneys of male mice was performed 2 days prior to UUO. The left ureters were obstructed for 0, 3, or 10 days. (**A**) Sirius red stain with paraffin-embedded kidney sections at 0, 3, or 10 days post-injury was used to evaluate total collagen level. (**B**) Sirius Red-positive area was evaluated in randomly chosen five fields per kidney at indicated timepoints (n = 4–6). (**C**) Expression of α-SMA, fibronectin, TGF-β, and p-Smad3 in kidneys 10 days post-injury was examined by western blot analysis using specific antibodies (n = 4–6). β-actin was used for as a loading control. Scale bar = 50 µm. Data expressed as mean ± SD ($^{\#}p < 0.05$ vs. respective intact; $^{\#\#\#}p < 0.001$ vs. respective intact). This figure has been modified from a previous publication[15].

Figure 6: Effect of renal denervation in renal fibrosis in ischemic AKI to CKD transition model: Renal denervation in left kidneys of male mice was performed days prior to IRI. Mice were subjected to 30 min ischemia of left kidney and sacrificed at 0, 2, 4, 8 or 16 days post-IRI. (**A**) Sirius Red Stain with paraffin-embedded kidney section at 0, 2, 4, 8, or 16 days post-injury was used to evaluate total collagen level. (**B**) Sirius Red-positive area was evaluated in randomly chosen five fields per kidney at indicated timepoints (n = 5). (**C**) Expression of α-SMA and p-Smad3 at indicated time points was examined by western blot analysis using specific antibodies (n = 4). β-actin was used for a loading control. Data expressed as mean ± SD ($^{\#}p < 0.05$ vs. respective intact). This figure has been modified from a previous publication[16].

DISCUSSION:

This protocol describes the detailed methods for renal nerve ablation in a mouse model. Further, the pivotal role of the renal nerve in triggering inflammatory and fibrotic responses to injury in CKD models is demonstrated. Complete separation of renal artery from connective tissue and veins is a critical step for successful RDNx that allows full exposure of renal nerves and complete nerve ablation. Since there is an overlap of the renal artery over the vein, the portion overlapped is not well-exposed to the alcohol, unless the artery and vein are completely separated, which

may result in incomplete ablation of renal nerve. During RDNx, ethanol may be exposed to connective tissues. Caution is needed to limit the exposure with tiny filter paper soaked in 95% ethanol.

In patients with uncontrolled hypertension, catheter-based ablation method has been used[11,12]. However, in animal models bigger than mice, surgical ablation can be used, because the renal nerve can be seen under a surgical microscope. Compared to the rat model, the renal nerve of a mouse is relatively small and harder to identify. Since all methods frequently used in RDNx have been successful in ablation of the renal nerve[11,15,23], use of a particular method depends on the animal model or which is most is accessible in a researcher's environment. On the other hand, whether additional surgery in the denervated animal is required is also critical to consider when choosing the appropriate method of RDNx. The outcome of catheter-based RDNx in human patients may be different from that of animal models, since catheter-based method could make incomplete RDNx.[12]

Expression of TH is used to confirm the success of sympathetic renal nerve ablation[15,16,24]. Norepinephrine-releasing neurons, as well as other catecholaminergic neurons, express TH[25]. Both sensory and sympathetic nerves are innervated into the kidney[24]. It should be noted that this RDNx method using 95% ethanol does not discriminate between the two types of nerves, and both are ablated[15,16], which is a limitation.

Based on previous reports[15,16], both sympathetic and sensory nerves are involved in renal inflammation and fibrogenesis. Calcitonin gene-related peptide (CGRP, a marker of afferent/sensory nerves) levels also markedly decreased in the kidneys with RDNx, whereas administration of $CGRP^{8-37}$ (an antagonist of CGRP) prevented kidney fibrosis and inflammation in both UUO and AKI to CKD models[15,16]. To investigate the precise role of the renal sensory nerve, capsaicin exposure can be used. A small piece of gauze or filter paper soaked in a capsaicin solution (33 mM in 5% ethanol, 5% Tween 80 and 90% normal saline) can be applied to the renal artery and vein for 15 min[26]. To avoid non-renal exposure of capsaicin, parafilm can be placed under the renal artery and vein[26]. After capsaicin exposure, removal of the sensory nerve can be evaluated by measurement of CGRP level[15,16,24,26].

Collectively, this method for RDNx is replicable to abolish sympathetic nerves and has been reproduced, suggesting that it is applicable to research investigating the mechanisms underlying renal inflammation and fibrogenesis in normotensive (as well as hypertensive) animal models. Although further research is required to better understand the mechanisms and the translatability of RDNx to clinical medicine, this protocol and our previous studies suggest that ablation of the renal nerve or intervention of its downstream signaling can serve as an option for prevention or treatment of renal tubular injury, inflammation, and fibrosis progression in diverse renal diseases.

ACKNOWLEDGMENTS:

This study is supported by NIH grants DK-116987, DK-120533 and American Heart Association (A.H.A.) Grant in Aid 15GRNT25080031 (B.J.P.), AHA postdoctoral fellowship Grant

15POST25130003 (H.S.J.), and grants (NRF-2016R1C1B2012080 and NRF-2019R1F1A1041410) from the National Research Foundation of Korea (J.K.).

DISCLOSURES:

The authors declare no competing financial interests.

REFERENCES:

1 Grams, M. E. et al. Predicting timing of clinical outcomes in patients with chronic kidney disease and severely decreased glomerular filtration rate. *Kidney International.* **93** (6), 1442-1451 (2018).

2 Coca, S. G., Singanamala, S., Parikh, C. R. Chronic kidney disease after acute kidney injury: a systematic review and meta-analysis. *Kidney International.* **81** (5), 442-448 (2012).

3 Hsu, R. K., Hsu, C. Y. The Role of Acute Kidney Injury in Chronic Kidney Disease. *Seminars in Nephrology.* **36** (4), 283-292 (2016).

4 Okusa, M. D., Rosin, D. L., Tracey, K. J. Targeting neural reflex circuits in immunity to treat kidney disease. *Nature Reviews in Nephrology.* **13** (11), 669-680 (2017).

5 Kanagy, N. L. Alpha(2)-adrenergic receptor signalling in hypertension. *Clinical Science (London).* **109** (5), 431-437 (2005).

6 Converse, R. L., Jr. et al. Sympathetic overactivity in patients with chronic renal failure. *New England Journal of Medicine.* **327** (27), 1912-1918 (1992).

7 Veelken, R. et al. Autonomic renal denervation ameliorates experimental glomerulonephritis. *Journal of American Society of Nephrology.* **19** (7), 1371-1378 (2008).

8 Veelken, R., Schmieder, R. E. Renal denervation--implications for chronic kidney disease. *Nature Reviews Nephrology.* **10** (6), 305-313 (2014).

9 Wei, F. F., Zhang, Z. Y., Huang, Q. F., Staessen, J. A. Diagnosis and management of resistant hypertension: state of the art. *Nature Reviews Nephrology.* **14** (7), 428-441 (2018).

10 Wyatt, C. M., Textor, S. C. Emerging evidence on renal denervation for the treatment of hypertension. *Kidney International.* **94** (4), 644-646 (2018).

11 Bhatt, D. L. et al. A controlled trial of renal denervation for resistant hypertension. *New England Journal of Medicine.* **370** (15), 1393-1401 (2014).

12 Jalil, F., White, W. B. A New Era of Renal Denervation Trials for Patients With Hypertension? *American Journal of Kidney Diseases.* **71** (5), 615-618 (2018).

13 Amann, K. et al. Glomerulosclerosis and progression: effect of subantihypertensive doses of alpha and beta blockers. *Kidney International.* **60** (4), 1309-1323 (2001).

14 Amann, K. et al. Effects of low dose sympathetic inhibition on glomerulosclerosis and albuminuria in subtotally nephrectomized rats. *Journal of American Society of Nephrology.* **11** (8), 1469-1478 (2000).

15 Kim, J., Padanilam, B. J. Renal nerves drive interstitial fibrogenesis in obstructive nephropathy. *Journal of American Society of Nephrology.* **24** (2), 229-242 (2013).

16 Kim, J., Padanilam, B. J. Renal denervation prevents long-term sequelae of ischemic renal injury. *Kidney International.* **87** (2), 350-358 (2015).

17 Eriguchi, M. et al. Renal denervation has blood pressure-independent protective effects on kidney and heart in a rat model of chronic kidney disease. *Kidney International.* **87** (1), 116-127 (2015).

18 Jang, H. S., Padanilam, B. J. Simultaneous deletion of Bax and Bak is required to prevent apoptosis and interstitial fibrosis in obstructive nephropathy. *American Journal of Physiology-Renal Physiology.* **309** (6), F540-550 (2015).
19 Jang, H. S., Kim, J., Park, Y. K., Park, K. M. Infiltrated macrophages contribute to recovery after ischemic injury but not to ischemic preconditioning in kidneys. *Transplantation.* **85** (3), 447-455 (2008).
20 Han, S. J., Jang, H. S., Kim, J. I., Lipschutz, J. H., Park, K. M. Unilateral nephrectomy elongates primary cilia in the remaining kidney via reactive oxygen species. *Science Reports.* **6** 22281 (2016).
21 Scarfe, L. et al. Transdermal Measurement of Glomerular Filtration Rate in Mice. *Journal of Visualized Experiments.* 10.3791/58520 (140), (2018).
22 Jang, H. S., Kim, J. I., Han, S. J., Park, K. M. Recruitment and subsequent proliferation of bone marrow-derived cells in the postischemic kidney are important to the progression of fibrosis. *American Journal of Physiology-Renal Physiology.* **306** (12), F1451-1461 (2014).
23 Clayton, S. C., Haack, K. K., Zucker, I. H. Renal denervation modulates angiotensin receptor expression in the renal cortex of rabbits with chronic heart failure. *American Journal of Physiology-Renal Physiology.* **300** (1), F31-39 (2011).
24 Mulder, J., Hokfelt, T., Knuepfer, M. M., Kopp, U. C. Renal sensory and sympathetic nerves reinnervate the kidney in a similar time-dependent fashion after renal denervation in rats. *Am Journal of Physiology - Regulatory, Integrative and Comparative Physiology.* **304** (8), R675-682 (2013).
25 Weihe, E., Depboylu, C., Schutz, B., Schafer, M. K., Eiden, L. E. Three types of tyrosine hydroxylase-positive CNS neurons distinguished by dopa decarboxylase and VMAT2 co-expression. *Cell and Molecular Neurobiology.* **26** (4-6), 659-678 (2006).
26 Foss, J. D., Wainford, R. D., Engeland, W. C., Fink, G. D., Osborn, J. W. A novel method of selective ablation of afferent renal nerves by periaxonal application of capsaicin. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology.* **308** (2), R112-122 (2015).

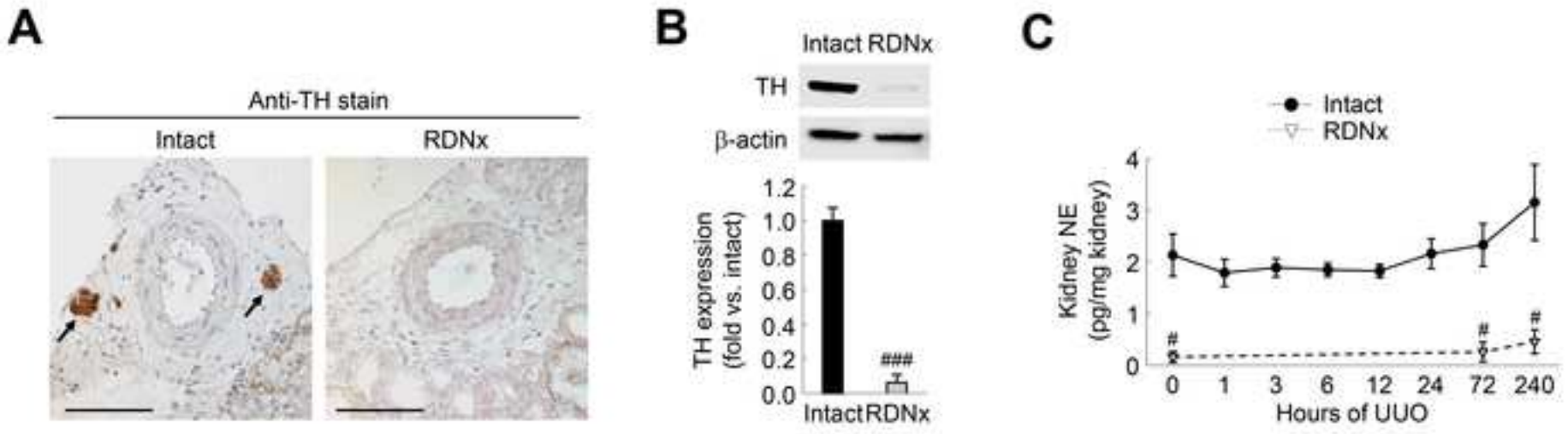

Figure 1

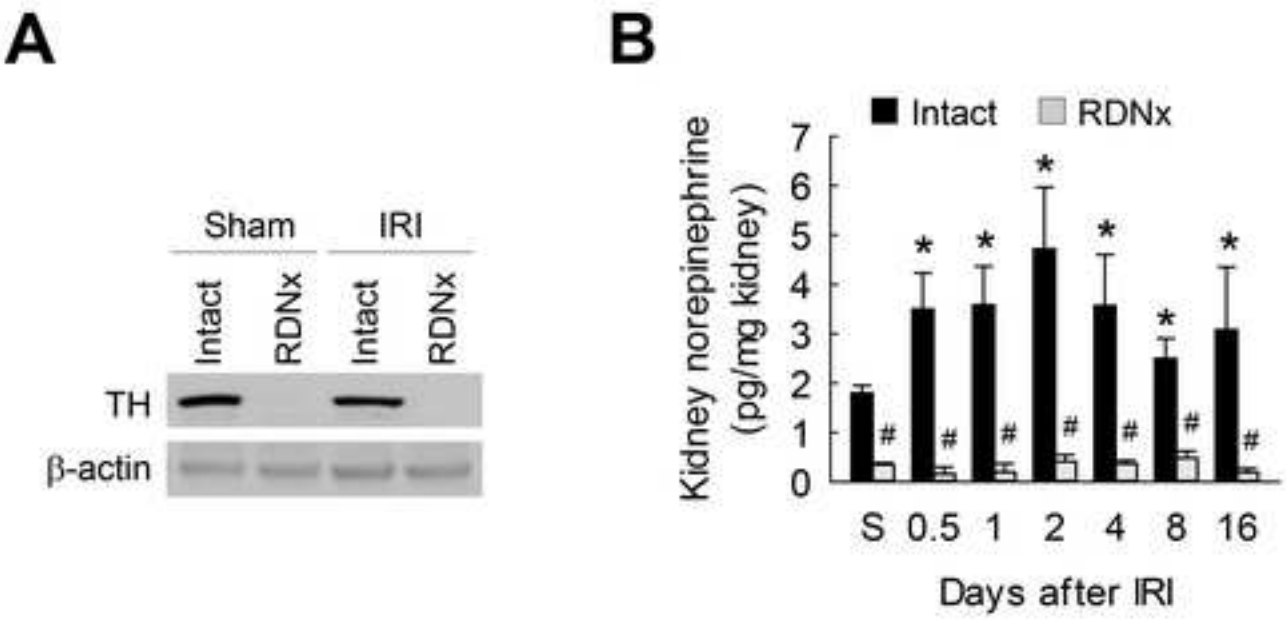

Figure 2

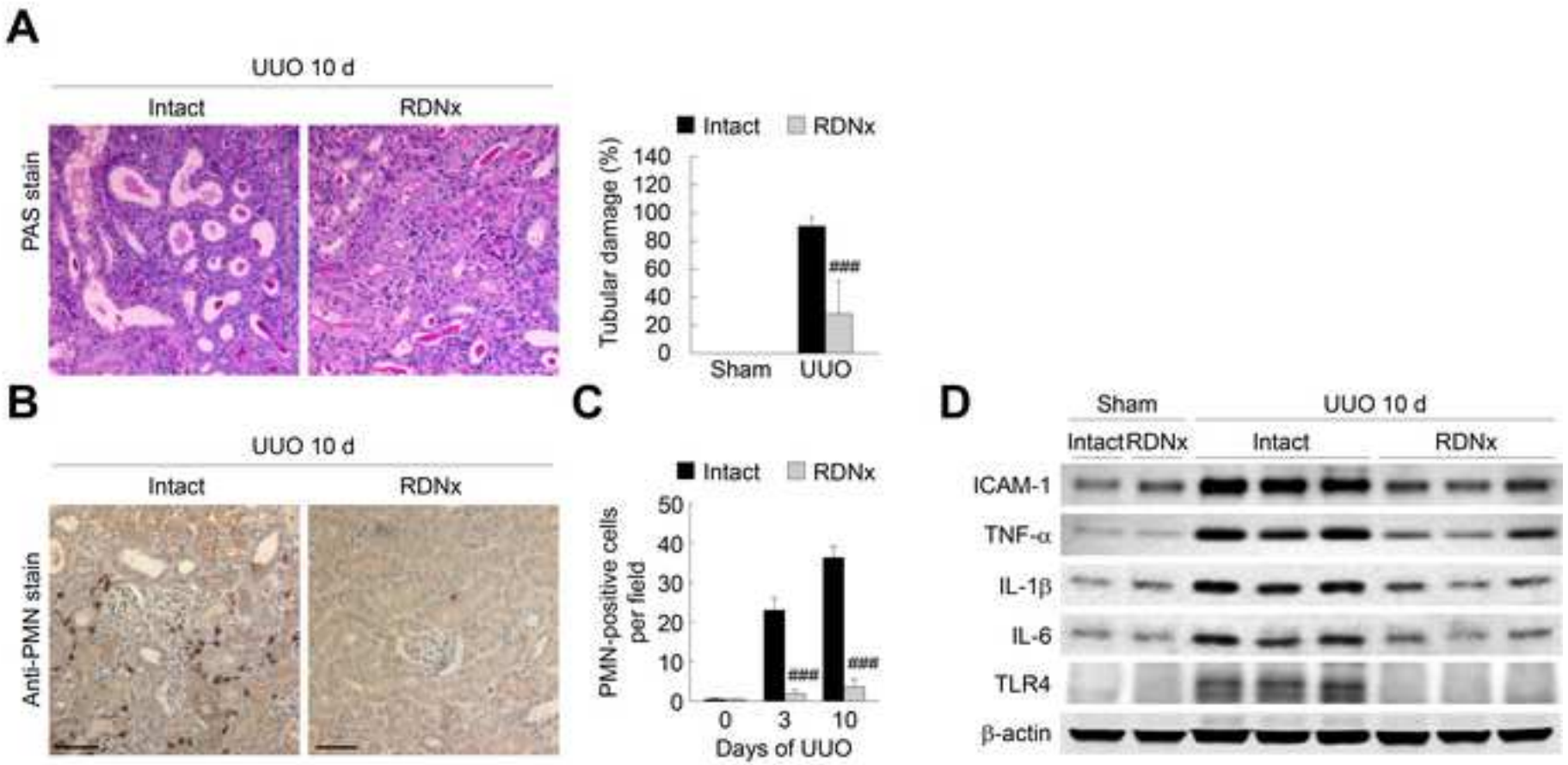

Figure 3

Figure 4

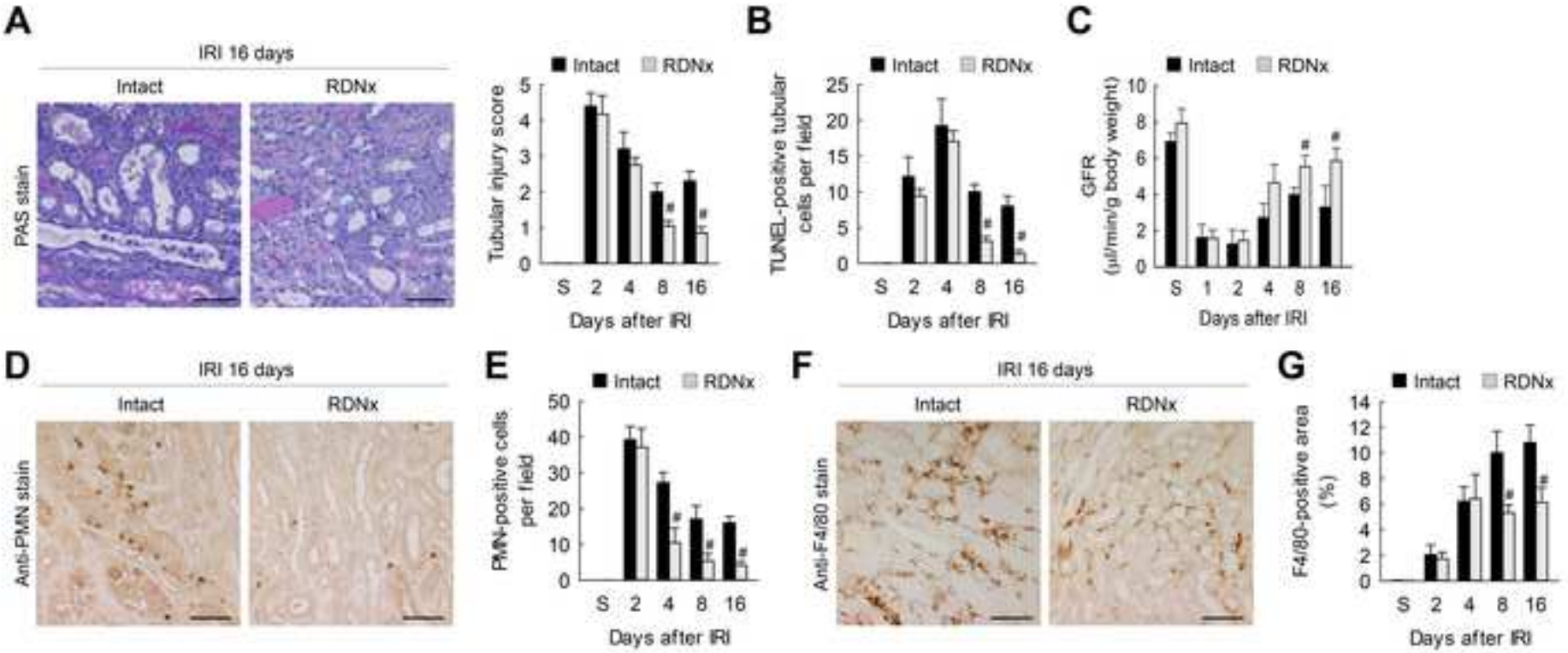

Figure 4

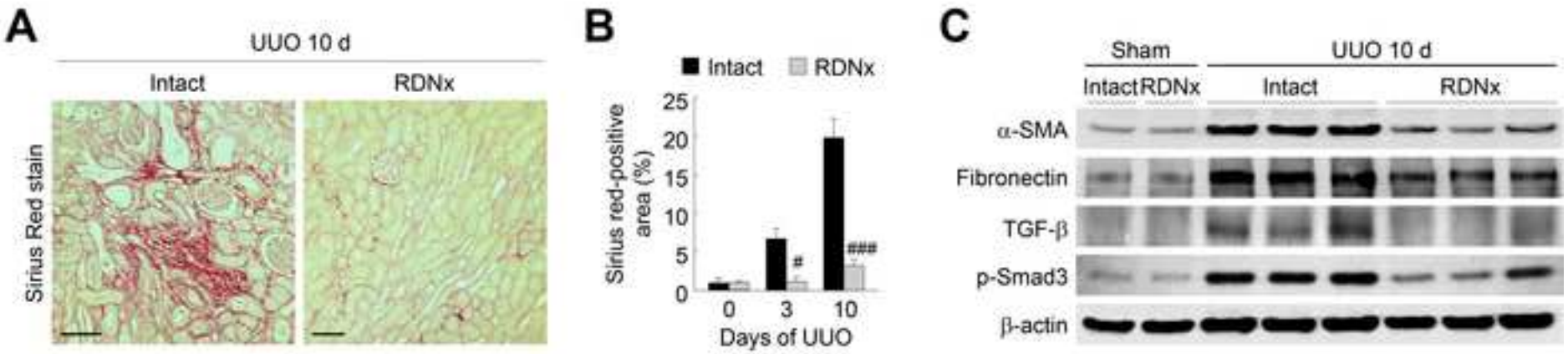

Figure 5

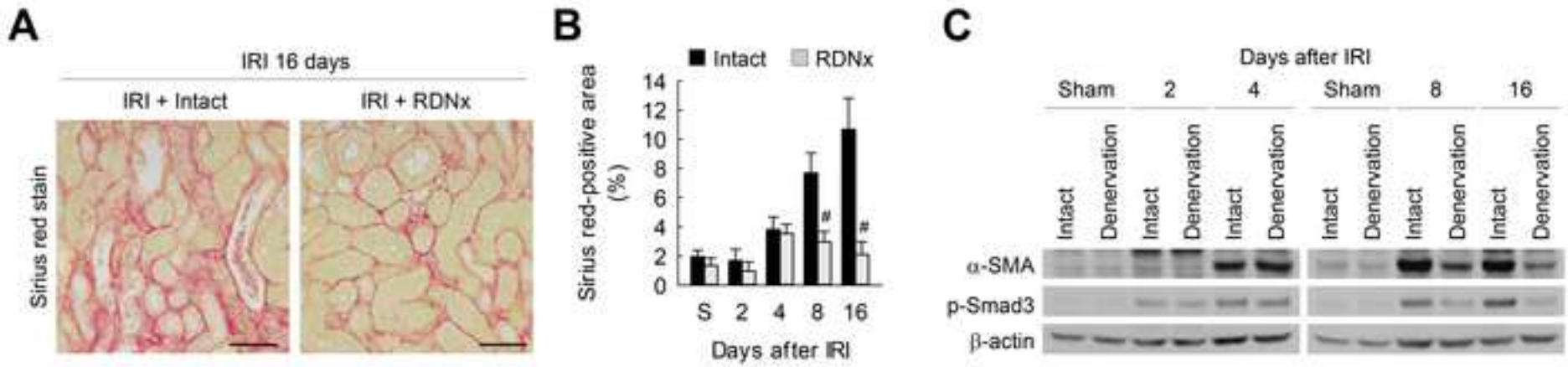

Figure 6

Table of Materials

Name	Company
129S1/SvlmJ	Jax Lab
0.1% SDS	BioRad
0.5% acetic acid (glacial)	Fisher Chemical
1mM EDTA	Sigma
1.3% Picric acid	Sigma
10 mM sodium citrate, pH6.0	Sigma
3% BSA	Sigma
3,3-diaminobenzidine (DAB)	Vector Lab
4% Paraformaldehyde	Electron Microscopy Sciences
4mM Sodium metabisulfite	Sigma
5% skim milk	BioRad
5-0 Silk	Oasis
70% Isopropyl alcohol	Fisher Chemical
95% Ethanol	Decon Labs
Anti-α-SMA antibody	Sigma
Anti-β-actin antibody	Sigma
Anti-F4/80 antibody	Proteintech
Anti-Fibronectin antibody	Cedarlane
Anti-ICAM-1 antibody	Santa Cruz
Anti-IL-1β antibody	Abcam
Anti-IL6 antibody	Abcam
Anti-Phospho-Smad3 antibody	Abcam
Anti-PMN antibody	Accurate
Anti-TGF-β antibody	Santa Cruz
Anti-TLR4 antibody	IMGENEX
Anti-TNF-α antibody	Abcam
Anti-Tyrosine Hydroxylase antibody	Abcam
Autoclave	Tuttnauer
Autoclip	MikRon
Bouin's Fixative	Polysciences
Coplin Jar	Grainger
Cotton tip	Midline
Creatinine Assay Kit	BioAssay Systems
DC Temperature Controller	FHC
Direct Red 80	Sigma
Filter paper	Whatman
FITC-conjugated sinistrin	MediBeacon
Heparinized capillary tube	Fisher Scientific
Heparinized tube	Terumo Medical Corp.
HRP-conjugated anti-rabbit antibody	Vector Lab
Insulin syringe	Becton Dickinson
Ketamine	Par Pharmaceutical
Lab Works analysis software	Ultra-Violet Products
Light microscope	Leica

Metabolic cage	Tecniplast
Microaneurysm clamp	Roboz
Microdissecting forcep	Roboz
Microplate reader	Tecan
Mounting medium	Fisher Scientific
Noepinephrine ELISA kit	ALPCO Diagnostics
PAGE gel of Any KD	BioRad
Phosphatase inhibitor	Sigma
Povidon-Iodine Prep Pad	Professional Disposables International
Protease	Calbiochem
Protein lysis buffer	Thermo Scientific
PVDF membrane	BioRad
Scalpel Handle	Roboz
Scissors	Roboz
Surgical blade	Bard-Parker
Surgical microscope	Nikon
Superblock	Thermo Scientific
Transcutaneous Measurement System	MediBeacon
Tris-Glycine buffer	BioRad
Tris-Glycine-SDS buffer	BioRad
TUNEL assay kit	Roche
Tweezers	Roboz
Western Lightning Chemiluminescence Substrate solution	PerkinElmer
Xylazine	Akorn Animal health
Xylene	HistoPrep

Catalog Number	Comments/decriptions
Stock #000090	
1610416	
BP1185	Sirius Red Stain
E6758	
P6744	Sirius Red Stain
C9999	
A7906	
SK-4100	
15710-S	
S9000	
1706404	
MV-682-V	Ureteral obstruction
A459	
2701	Removal of renal nerve
A5228	
A-5316	
18705-1-AP	
CL5495AP	
SC-1511-R	
ab9722	
ab83339	
ab51451	
AIAD51140	
SC-7892	
IMG-579A	
ab9739	
ab112	
EZ9PLUS	
205016	
16045-1	
3WEF1	
MDS202055	
DICT-500	
40-90-8D	
365548	Sirius Red Stain
3030917	Removal of renal nerve
N/A	GFR analysis
22-260-950	
Capiject	
PI-1000	
305500	
Ketalar	Anesthetic agent
N/A	Analysis of Western blot band density
Leica DMR	

3600M021	GFR analysis
RS-5422	Ischemia/reperfusion
RS-5069	
Infinite 200 PRO	
SP15-100	
17-NORHU-E01.1	
456-9034	
P5726	
C12400	
539134	
78510	
162-0176	
RS-9843	
RS-5882	
371110	
SMZ-745	
37535	
N/A	GFR analysis
1610771	
1610744	
11684795910	
RS-5137	
NEL10400	
139-236	Anesthetic agent
HC700	

Dear Dr. Padanilam,

Your manuscript, JoVE60833 "Renal denervation approach to prevent inflammation and fibrogenesis in chronic kidney disease," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **Dec 05, 2019**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Peer Review,
Peer Review
JoVE
617.674.1888
Follow us: Facebook | Twitter | LinkedIn
About JoVE

_____

Editorial comments:

We appreciate the reviewers for their insightful comments to our manuscript.

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.

Response: We have confirmed that in revised version.

2. Please include a single line space between each step, substep and note in the protocol section.

Response: We have confirmed that in revised version.

3. Please define all abbreviations during the first-time use.

Response: We have confirmed that in revised version.

4. 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: DC Temperature Controller; FHC, Bowdoin, 96 ME, USA, Capiject; Terumo Medical Corporation, Elkton, MD, USA, Cat. #22-260-950; Fisher 117 Scientific, Pittsburgh, PA, USA, MediBeacon; St. Louis, MO, USA, Cat. #15710-S; Electron Microscopy Sciences, 143 Hatfield, PA, USA, Cat. #16045-1; Polysciences, Warrington, PA, USA, ALPCO 152 Diagnostics, Windham, NH, USA, Cat. #ab112; Abcam, Cambridge, UK, Cat. #PI-1000; Vector, Burlingame, CA, USA, DAB, Cat. #SK-4100; Vector, Burlingame, CA, USA, Cat. #SP15-100; FisherScientific, Pittsburgh, PA, USA, T-PER Tissue Protein Extraction 179 Reagent (Cat. #78510; Thermo Scientific, Rockford, IL, USA, Cat. 180 #P5726; Sigma, St. Louis, MO, USA, Cat. #539134; Calbiochem, St. Louis, MO, USA, Cat. #456-9034; BioRad, Hercules, 183 CA, USA, (Cat. #1610744; BioRad, Hercules, CA, USA, Cat. #11684795910; Roche, Mannheim, Germany, QuantiChromTM 130 Creatinine Assay kit (Cat. #DICT-500; BioAssay Systems, Hayward, CA, USA

Response: We have done that in revised version.

5. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 81-90, 97-99, 105-106, 108-110, 204-211, 382-384.

Response: We have replaced these sentences with minimal overlaps from previous works in the revised version.

6. 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.” However, notes should be concise and used sparingly.

Response: We have done that in revised version.

7. The Protocol should contain only action items that direct the reader to do something.

Response: We have confirmed that in revised version.

8. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Response: We have modified in revised version.

9. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

Response: We have confirmed that in revised version.

10. 1.1. Any sex specific bias? Do you check for the depth of anesthesia prior to the start of the experiment?

Response: We have no data regarding sex difference, since we used only male mice in the study. We had checked the depth of anesthesia before starting the surgery, as well as during the surgery, and started the surgery when only mouse was in depth of anesthesia.

11. 1.2: How was the incision performed? How big? How was the isolation performed?

Response: We have added the details in Protocol Section.

12. 1.3: How is the painting performed?

Response: We have added the detail in Protocol Section.

13. 2.1.3: Please expand on how this was done?

Response: We have added the detail at several parts in Protocol Section.

14. 2: Please include post-operative procedures? How did you check for the generation of different models- results for any marker studies?

Response: We have added the detail in Protocol Section.

15. 3.1: How did you harvest the kidneys? Did you harvest both or one? How and how much blood samples were collected?

Response: We have added the detail in Protocol Section.

16. 3.2: Did you just let the tube sit for plasma separation? Did you perform any centrifugation- if yes what is the speed, temp, time? Can you store the blood or plasma prior to the experiment?

Response: We have added the detail in Protocol Section.

17. 4.1: How was this done?

Response: We have added the detail in Protocol Section.

18. 4.3: How was creatinine clearance or GFR estimated?

Response: We have added the detail in Protocol Section.

19. 4.4 -4.6: Please explain how this was done using the commercially available kit.

Response: We have added the detail in Protocol Section.

20. For all the steps marked for filming purpose, please explain the actions performed, how this step was performed in brief.

Response: 1) For denervation, we will prepare mouse anesthetized, shave the mouse back, make an incision on the back and then do renal denervation in both IRI and UUO models. 2) For confirmation of renal denervation, we will prepare both kidney lysate and kidney section and measure NE level by commercial Elisa kit using microplate reader or do immunohistochemistry with anti-tyrosine hydroxylase antibody. 3) For analysis of renal inflammation, we will prepare kidney section and do immunohistochemistry with anti-PMN (neutrophil) and –F4/80 (macrophage) antibodies. 4) For analyzing renal fibrosis, kidney section and Sirius Red Solution will be prepared for Sirius red stain and then the stain will be done and image will be taken from the cortical region.

21. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 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.

Response: We have confirmed that.

22. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. How did you first check the generation of the model and then confirmation of renal denervation in different model system and effect of renal denervation in different model system.

Response: We have described the results in terms of the manuscript title and generation of renal denervation animal models.

23. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. Presently the information which is uploaded does not show that the permission is granted.

Response: We have uploaded the complete copyright permissions for this publication.

24. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Response: All figure legends were modified by the sentence with respective reference.

25. As we are a methods journal, please ensure the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response: The discussion Section seems that all these details are being included.

26. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.

Response: We have confirmed that.

27. Please upload each figure separately to your Editorial Manager account.

Response: We have uploaded each figure separately.

28. Please sort the materials table in alphabetical order.

Response: We have confirmed that.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript describes method of renal denervation, a potential therapy for kidney disease.

Major Concerns:
None

Minor Concerns:
Page 1 line 84. The authors should specifically state that they isolated artery and vein to expose nerves as this seems to be critical in exposing nerves that would otherwise be hidden between artery and vein. This is discussed in Discussion Section line 364.
Response: Thank you for the detailed comment. We have done the renal denervation in renal artery

separated from renal vein and surrounding tissues as applying with small piece of filter paper soaked in 95% ethanol for 2 min. We have modified the protocol in detail.

Page 4 line 106. The authors should state specifically that they clamped both renal artery and vein.

Response: We have added the description for clamping both artery and vein in Protocol Section of the revised version.

Reviewer #2:

Manuscript Summary:

This is an interesting article about the role of renal Denervation in Chronic Kidney Disease (CKD). More discussion is needed to explain the mechanisms that may result in prevention of inflammation and fibrogenesis in chronic kidney disease.

Major Concerns:

a. the results cannot apply to humans because renal denervation in humans is performed with catheter-ablation and usually results in partial denervation.

Response: We agree with your comment. There may be difference between catheter- and chemical-based denervation. We have added this limitation in revised manuscript.

b. the model of this study was about transition of acute kidney injury(AKI) to CKD. You didn't study an established CKD and its progression to End stage renal disease.

Response: We used two established models of CKD" in our studies. This study describing the method of renal denervation is beyond the scope of establishing CKD progression to ESRD model.

Minor Concerns:

a) The protocol is not clear. Many measurements, many results but timing is unclear. Are all the measurements the same time from the same mouse?

Response: We have added the time points in figure legends. Samples with different time points were derived from different mice.

b) You didn't study the percentage of glomerulosclerosis and the pathology of the glomerulus. The findings are only from the tubules. You must refer to the glomerulus lesions as well.

Response: This study is focused on tubular injury and its subsequent consequence. Study regarding glomerular pathology is beyond our scope.

c) You must explain why the denervation is done first and then the induce of the lesion.

Response: Our data are focused that renal nerve-derived signaling initiates and develops kidney inflammation and fibrosis in renal fibrosis models. Further studies to define the role of renal nerve in the kidney with pre-existing injury that would be associated with its translatability to clinical medicine, are warranted. We have added a related sentence in the first paragraph of Results Section.

Reviewer #3:

Manuscript Summary:

This manuscript from Jang and colleagues evaluates the role of renal nerves in the development of renal inflammation in a preclinical model of chronic kidney disease. The inflammation and fibrosis in this model, induced by unilateral ureter complete ligation, is prevented by renal denervation prior to ureter obstruction. While these data do not advance the area, since this observation was previously reported by this group, the methodology could prove useful in the field for others. I have only 3 concerns to be addressed:

Major Concerns:

1) One concern I have is regarding the denervation confirmation. While NE content and TH expression are useful in determining denervation efficacy, this only confirms sympathetic/efferent denervation. Please consider adding substance P or CGRP content to these methods.

Response: This manuscript were focused on renal sympathetic nerve signaling, but sensory nerve also contribute to kidney fibrosis and inflammation as we described in Discussion Section. In addition, by the page limitation, we have just added the sentence regarding the results and the related reference in Discussion Section of the revised manuscript.

2) The authors do not use parafilm in their denervation technique, like that used in Ref 26 that the authors address in the discussion. How are the effects of ethanol exposure then limited to only the kidneys? This should be addressed.

Response: Thank you for the comment in detail. We used a small piece of filter paper soaked in 95% ethanol for denervation that is very limited to the artery other than other tissues. We have added the caution for the potential limitation in Discussion Section of the revised manuscript.

3) Though GFR measurements are described, the authors do not include any data on GFR or creatinine clearance. Please consider including in figures.

Response: Sorry for this missing. We have added the data in the revised version.

Minor Concerns:

N/A

TITLE

Renal denervation approach to prevent inflammation and fibrogenesis in chronic kidney disease

AUTHORSAND AFFILIATIONS

Hee-Seong Jang[1], Mi Ra Noh[1], Jinu Kim[1,2,3] and Babu J. Padanilam[1,4]

[1]Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, NE, USA
[2]Department of Anatomy, Jeju National University School of Medicine, Jeju, South Korea
[3]Interdisciplinary Graduate Program in Advanced Convergence Technology & Science, Jeju National University, Jeju, South Korea
[4]Internal Medicine, Section of Nephrology, University of Nebraska Medical Center, Omaha, NE, USA

Corresponding author:
Babu J. Padanilam (bpadanilam@unmc.edu)

Email addresses of co-authors:
Hee-Seong Jang (heeseong.jang@unmc.edu)
Mi Ra Noh (mira.noh@unmc.edu)
Jinu Kim (jinu.kim@jejunu.ac.kr)

KEYWORDS

Denervation, norepinephrine, adrenergic receptor, ischemia/reperfusion, ureteral obstruction, inflammation, fibrosis, chronic kidney disease

SUMMARY

Here, we describe a protocol for renal denervation that is useful to define the role of renal nerve-derived signaling, focused on sympathetic nerve-mediated signaling, in persistent renal tubular injury, inflammation and fibrogenesis.

ABSTRACT

Chronic kidney disease (CKD) is increasing in the world with no effective treatment strategy. Sympathetic nerve activation has been recognized as an important factor for development and progression of cardiovascular diseases, hypertension and CKD. Catheter-guided renal denervation is useful to control blood pressure (BP) in patients with resistant hypertension and CKD. Sympathetic nerve-derived norepinephrine (NE) has been implicated in tissue homeostasis and in progression of various diseases, including CKD. The molecular mechanisms and signaling pathways triggered by sympathetic nerve activation, that drive renal inflammation and fibrogenesis, in CKD progression remain undefined. Here we present detailed methodology on how to carry out renal denervation (RDNx) in experimental models of CKD. Our published results showed that this method effectively ablates renal nerve, as evinced by loss of tyrosine hydroxylase immunoreactivity and levels of kidney NE, resulting in suppression of renal tubular injury, inflammation and fibrogenesis in CKD models. Competence of surgeons performing

surgical procedures to denervate the kidney is a requirement to achieve consistent results. RDNx can be utilized to study roles of renal nerve, nerve-derived neurotransmitters and factors as well as to unveil their downstream signaling pathways. Defining the molecular mechanisms and function can lead to designing of novel therapeutic intervention of CKD regardless of its etiology, including diabetes, hypertension, and cardiovascular complications.

INTRODUCTION

Chronic kidney disease (CKD), characterized by tubular injury, persistent renal inflammation and fibrosis, ultimately leads to end stage kidney disease (ESKD)[1-3]. Sympathetic nervous system governs both normal and pathological functions of diverse organ systems, including that in kidney[4]. One of the catecholamines, norepinephrine (NE, noradrenaline), from sympathetic neurons is an effector of sympathetic nervous system[5]. In both patients and experimental models, increased sympathetic nerve activity and tissue level of NE, are hallmarks of CKD progression[6-8].

Renal denervation (RDNx) is being used as a therapeutic option for the treatment of drug-resistant hypertension[9-12] but the detailed molecular mechanism remains to be fully understood. Moreover, studies[13,14] defining the role of renal nerve in CKD progression using chemical denervation or antagonist/agonist of sympathetic nerve-derived neurotransmitters or its receptors is limited due to their systemic effects that may not be specific to the kidney. As we reported and later confirmed by others[15-17], RDNx can overcome this limitation and was successful in defining the in vivo role of renal nerve, renal nerve-derived factors and their downstream signaling to elicit renal inflammation and fibrosis.

Here we provide detailed methodology and representative results of RDNx on renal tubular injury, inflammation and fibrosis in mouse models of CKD.

PROTOCOL

Mice were cared before and during the experimental procedures in accordance with the policies of the Institutional Animal Care and Use Committee (IACUC), University of Nebraska Medical Center (UNMC), and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All protocols had received prior approval from the UNMC-IACUC.

1. Renal denervation

1.1. Use male (129S1/SvImJ) mice aged 8 - 10 weeks from Jackson Laboratories.

1.2. Anesthetize mice using a cocktail containing ketamine (200 mg/kg body weight) and xylazine (16 mg/kg body weight), intraperitoneally[16,18].

1.3. Prior to the start of surgery, check for the lack of response to the toe pinch.

1.3. Make an incision of 1.5 cm long using surgical blade on the back 5 mm lateral to the spine.

Commented [A1]: For the protocol section, please include all the steps as if you are describing someone how to do your experiment providing all specific details in a stepwise manner.

R: We have added the detailed protocols as you mentioned.

Commented [A2]: Included here. Please check.
Do you shave the skin? Do you sterilize the surgical site with iodine and alcohol? Please include all details.

R: We have added the detailed protocols about shaving and sterilization as you mentioned.

1.4. Expose the left kidney artery and vein and then separate the artery and vein carefully from the surrounding connective tissues using micro dissecting forceps under surgical microscope.

1.5. For stripping the nerves, paint the kidney vessels with filter paper soaked in 95% ethanol for 2 min[15,16].

NOTE: Ensure that the color of vessels turns to white under the microscope.

1.5. Wash the kidney vessels for 2 min with PBS.

1.6. Do sham-operation with the same surgical procedure except stripping the kidney nerves.

2. Renal fibrosis models

2.1. Unilateral ureteral obstruction (UUO) model:

2.1.1. Anesthetize male mice (129S1/SvImJ) aged 8-10 weeks as above.

2.1.2. Maintain body temperature at 36-38 °C on heating pad with temperature controller.

2.1.3. Expose the left kidney using autoclaved cotton swab through left flank incision on the back 1 cm lateral to the spine. Ligate the left ureter completely near the kidney pelvis using a 5-0 silk tie[15,18].

2.1.5. Do sham-operation with the same surgical procedure except ligation of ureter.

2.2. Ischemic acute kidney injury (AKI) to CKD transition model:

2.2.1. Denervate kidneys were denervated as discribed in step 1. Two days after the denervation, anesthetize male 129S1/SvImJ mice aged 8-10 weeks as above.

2.2.2. Maintain the body temperature at 36-38 °C on the heating pad with temperature controller.

2.2.3. Expose the left kidney using autoclaved cotton swab through left flank incision on the back 1 cm lateral to the spine.

2.2.4. Subject the mice to 30 min of left renal ischemia. To do so, clamp the renal pedicle with both artery and vein with nontraumatic microaneurysm clamps to induce ischemia as described previously.[16,19] Close the incisions temporarily during ischemia period using clamps.

2.2.6. Once the ischemic period is over, remove the clamps for reperfusion.

Commented [A3]: How do you visually identify?

R: We can identify the artery and vein under surgical microscope, since the vessels are connected to the kidney. We have changed the detail in the revised version.

Commented [A4]: What do you do after this- post operative procedure? Please include all specific details in the protocol section.

R: We have added the detailed protocols, including skin suture, as you mentioned.

Commented [A5]: Is this the same mice as the one used in 1.6? After how many days of renal denervation do you perform this step?

R: We have done the IRI or UUO surgery post-denervation 2 days. We added this point in the revised manuscript.

Commented [A6]: How do you check the depth of anesthesia before starting the experiment? Do you shave the animal prior to surgery?

R: We checked the depth of anesthesia by toe pinch. We have added the detail in the revised version.

Commented [A7]: How is this done?

R: We made left flank incision by surgical blade. The detail has included in the revised manuscript.

Commented [A8]: How do you check the depth of anesthesia?

R: We checked the depth of anesthesia by toe pinch. We have added the detail in the revised version.

Commented [A9]: Do you sterilize the skin prior to the surgery? Do you shave hair etc? Do you cover the animal with sterile drape?

R: We have added the detailed protocols about shaving and sterilization as you commented. We did not used the surgical drape.

2.2.7. Confirm reperfusion of the kidneys visually. The color of kidney will change from dark red to bright red upon reperfusion.

2.2.8. Perform sham-operation with the same surgical procedure except clamping the renal pedicle.

3. Harvesting the Kidney and Blood

3.1. Collect 100 µL blood samples from the retroocular vein plexus or 500 µL from vena cava.

3.2. For harvesting kidneys, expose the operated kidney by abdominal incision. Cut renal vessels and surrounding tissues with surgical blade and then remove the capsules.

NOTE: Ensure that UUO kidneys have urine filled in the pelvis to confirm induction of UUO.

3.3. Collect plasma from blood by centrifugation at 8000 *x g* for 3 min, room temperature (RT), using heparinized capillary tube and store at -20 °C until use.

3.4. For biochemical assays, snap freeze kidney, using liquid nitrogen immediately after removal from mouse, and store at -80 °C deep freezer until use.

4. Analysis of Renal Function

4.1. Anesthetize mice 15 days post-ischemia/reperfusion injury (IRI) as above.

4.2. Expose the right kidney using autoclaved cotton swab, through a right flank incision on the back 1 cm lateral to the spine.

4.3. Remove the kidney (nephrectomy) from renal vessels and ureter using scissors, after ligation of renal vessels and ureter[16,20].

4.4. Take blood samples from the retroocular vein plexus at 6 h prior to placing mouse in metabolic cage.

4.5. To obtain urine samples, place mice in mouse-specific metabolic cages for 18 h for the analysis of glomerular filtration rate (GFR) by creatinine clearance[16].

4.6. Measure urinary and plasma concentrations of creatinine using a commercially available kit (see **Table of Materials**). QuantiChromTM Creatinine Assay kit. In brief, add 30 µL plasma to a mixture of Reagent A and B of 100 µL each and then read optical density both immediately and 5 min post-incubation at 510 nm, room temperature (RT), using microplate reader. For urine, use a mixture with 50 µL Reagent A, 50 µL Reagent B, and 100 µL water for 5 µL urine.

4.7. Calculate GFR as follows: GFR = urinary concentration of creatinine x urine volume/plasma

Commented [A10]: What do you do after reperfusion? Do you euthanize the animal? Do you let the animal survive? How do you perform the surgical site closure? etc.

R: The mice with IRI or UUO survive until the time point indicated at Methods Section 3.

Post-operative procedures?

R: The operated mice take 0.1 µg/kg BW buprenorphine once for 3 days for pain relief. We have added this point in the revised version.

Commented [A11]: After how many days ?

R: We have added the details about the harvested day in the revised version.

Commented [A12]: How?

R: We have added the details about blood collection in the revised version.

Commented [A13]: After how many days of 2.2.8 do you collect the kidney and blood?

R: That was various. We have added the details when the kidney and blood harvested in the revised version.

Commented [A14]: Please remove the commercial name from her and move to the table of materials.

R: We have removed all commercial name in the revised version.

concentration of creatinine/time of urine collection/body weight.

4.8. Alternatively, measure GFR by transcutaneous measurement system at 24 h post-Nx, followed by the protocol published in J Vis Exp.[21] In brief, under anesthesia with isoflurane, place a miniaturized fluorescence detector on the back of mice and inject fluorescein-isothiocyanate (FITC) conjugated sinistrin (an inulin analog) (0.15mg/g BW) to mice through retroocular vein plexus. In conscious and freely moving mice, data for half-life of FITC-sinistrin was collected for 1 hr and then analyzed by MPD studio software.

5. Confirmation of renal denervation

5.1. Evaluation of kidney NE level

5.1.1. Homogenize kidneys using 1 mM EDTA and 4 mM sodium metabisulfite in 0.1 N HCl.[15,16]

5.1.2. After centrifugation of the homogenates at 13600 *x g*, determine norepinephrine concentration in the supernatants by enzyme-linked immunosorbent assay (ELISA) kit as recommended by the manufacturer.

5.2. Immunohistochemistry for tyrosine hydroxylase:

5.2.1. Fix kidneys in 4% paraformaldehyde or Bouin's Fixative and processed for paraffin embedding.

5.2.1. Deparaffinize paraffin-embedded kidney sections by xylene and rehydrated by 100%, 95%, 90% ethanol sequentially 2 times at each step.

5.2.2. Permeabilize the kidney sections in 0.1% SDS for 5 min and then wash with PBS three times at RT.

5.2.3. Autoclave the permeabilized kidney sections in 10 mM sodium citrate at 120 °C for 10 min for antigen retrieval.[16,22]

5.2.4. Cool down the autoclaved kidney sections for 30 min at RT and then wash with PBS three times at RT.

5.2.5. Incubate the kidneys with 3% BSA in PBS for 30 min at RT to prevent non-specific binding.

5.2.6. After removal of BSA by suction, incubate the kidneys with antibody (1:1000) against tyrosine hydroxylase overnight at 4 °C.[15]

5.2.7. Next day, wash the kidney sections with PBS for three times at RT and incubated with HRP-conjugated anti-rabbit antibody for 1 hour at RT.

Commented [A15]: Are these freshly isolated or frozen… also after which step do you isolate the kidney. Please include the details.

R: We used frozen kidneys for the experiment. We have added the detail in the revised version.

Commented [A16]: Volume?

R: We used 400 µl. We have added the detail in the revised version.

Commented [A17]: Since this step is highlighted, please briefly describe how this is done to show the actions in the video.

R: We have added the detail in the revised version.

Commented [A18]: Moved here – as this step is performed before paraffin embedding. Please check.

R: The fixed kidneys were processed for paraffin embedding. We have changed the detail in the revised version.

5.2.8. After washing with PBS three times at RT, apply to the kidney sections with 3, 3 – diaminobenzidine (DAB) and stain for up to 2 minutes.

5.2.9. After washing with PBS three times at RT, dehydrate the kidney sections with 90%, 95%, 100% ethanol and xylene sequentially for 5 min each, and mount cover slips with a 1:1 mixture of mounting medium and xylene.

5.3. Western blot analysis for tyrosine hydroxylase:

5.3.1. Isolate whole kidney proteins using lysis buffer with phosphatase inhibitor and protease.[16,18]

5.3.2. Run 30 µg of kidney protein on PAGE gel in Tris-Glycine-SDS buffer at 80 V for 1 h.

5.3.3. Transfer the protein to PVDF membrane from gel in Tris-Glycine buffer at 80 volt for 100 min at 4°C.

5.3.4. Block non-specific proteins on the membrane by 5% skim milk for 30 min at RT.

5.3.5. Incubate membranes with diluted antibody (1: 2,000) against tyrosine hydroxylase in 10 mL Superblock.[15,16]

5.3.6. Use anti-β-actin antibody for loading controls on stripped membranes.

5.3.7. Expose membranes to Western Lightning Chemiluminescence Substrate solution. The bands were quantified using an analysis software.

7. Analysis of tubular injury:

7.1. Tubular damage: Section paraffin-embedded kidney into 3 µm thick sections using microtome and stain kidney sections using Periodic Acid Schiff (PAS).

7.1.1. Select five fields from each PAS-stained kidney randomly in a blinded manner and photograph using light microscopy.

7.1.2. Score damaged tubule with flattened lumen by loss of brush border, proteinaceous cast, loss of tubular nucleus, and dilation as follows: grade 0, normal; grade 1, >10%; grade 2, 10-25%; grade 3, 26-50%; grade 4, 51-75%; grade 5, < 75%.[15,16,18]

7.2. Apoptotic cell death: Analyze apoptotic cell death in paraffin-sectioned kidney samples with TUNEL assay using a commercially available kit (see **Table of Materials**), following the manufacturer's protocol.

8. Inflammation:

Commented [A19]: Please do not use commercial terms in the manuscript.

Commercially available blocking solution.

R: We have removed all commercial name in the revised version.

Commented [A20]: How is this done? Please include steps or provide a citation.

R: We have added the detail in the revised version.

8.1. Analysis of neutrophil:

8.1.1. Following the Methods in step 5.2. protocol, prepare kidney sections and incubate with antibodies against polymorphonuclear neutrophil overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibody.[15,16]

8.1.2. Perform DAB stain and dehydration as described in 5.2.8 and 5.2.9.

8.1.3. Take photographic images from cortical or outer medullary region in a blinded manner.

8.2. Analysis of macrophage:

8.2.1. Following the protocol of Methods Section 5.2., prepare kidney sections.

8.2.2. After that, incubate the kidneys with antibodies against F4/80 overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody.[15,16]

8.2.3. Take the images from cortical or outer medullary region in a blinded manner.

8.3. Analysis of inflammatory cytokine level:

8.3.1. Following the protocol of Methods Section 5.3., prepare kidney samples.

8.3.2. After that, incubate the kidney samples with antibodies against ICAM-1, TNF-α, IL-1β, IL-6, and TLR4 overnight at 4 °C, followed by incubation with corresponding secondary antibodies.[15]

9. Evaluation of fibrosis:

9.1. Sirius red stain:

9.1.1. Following the protocol in Methods Section 5.2., rehydrate the kidney sections.

9.1.2. Stain the kidney sections with Sirius red solution [0.5 g Direct Red 80 plus 1.3% Picric acid in 500 ml distilled water] for 30 min.[15,16,18]

9.1.3. After washing with acidified water [0.5% acetic acid (glacial)] twice, remove the water from the slides physically.

9.1.4. After mounting (Methods section 6.2.), take the images from cortical or outer medullary region in a blinded manner.

9.2. Evaluation of fibroblast activation and fibrogenic factors:

9.2.1. Following the protocol in Section 6.3., prepare kidney samples.

9.2.2. After that, incubate the kidney samples with antibodies against alpha-SMA, fibronectin, TGF-β, phospho-Smad3 overnight at 4°C, followed by incubation with corresponding secondary antibodies.[15]

9.2.3. Use anti-β-actin antibody for loading controls on stripped membranes.

9.2.4. Quantify the bands using an analysis software.

REPRESENTATIVE RESULTS

Removal of renal nerve by renal denervation surgery

Renal denervation (RDNx) was carried out two days prior to IRI or UUO to define whether renal nerve contributes to initiation and development of kidney fibrosis and inflammation. Mice were subjected to either 30 min IRI followed by reperfusion for 1, 2, 4, 8 or 16 days or UUO for 3 or 10 days. To confirm whether RDNx was successfully applied, we checked tyrosine hydroxylase (TH)-positive sympathetic nerve fibers in adventitia of intrarenal arteries at 10 days post-UUO by immunohistochemistry (**Figure 1A**). Further, RDNx markedly reduced TH expression (**Figure 1B**) in the kidney. To determine the level of norepinephrine (NE), which is released from the sympathetic nerve fiber terminus, ELISA method was used. During UUO injury, intrarenal level of NE continued at the same level in both sham and UUO kidneys, but RDNx markedly suppressed its level (**Figure 1C**). Similarly, RDNx inhibited TH expression almost completely in both sham and IRI kidneys (**Figure 2A**). In IRI kidneys, NE level was increased in earlier time points and continued over time, at least, up to 16 days post-IRI (**Figure 2B**). These results show successful removal of sympathetic nerve fibers and subsequent blockage of NE release by RDNx.

Effect of renal denervation in renal tubular injury, inflammation and fibrosis.

We analyzed renal tubular injury, inflammation and fibrosis to confirm the effect of RDNx in established renal fibrosis models. Since renal tubular injury is an initial factor of renal inflammation and fibrosis, we checked tubular necrosis and apoptosis in UUO- and IRI-kidneys. Tubular damage was evaluated by using Periodic Acid Schiff (PAS) stained kidney section, while tubular apoptosis was by TUNEL assay. UUO-kidneys showed severe necrotic cell death in all renal tubules (**Figure 3A**) but was limited in proximal tubule of outer medulla (**Figure 4A**) in IRI kidney. Tubular apoptosis was similar with those of necrotic cell death (**Figure 4B**). Kidney function was preserved in IRI-kidneys with RDNx, compared to those of non-RDNx (**Figure 4C**). Kidney inflammation was analyzed by immunohistochemistry of PMN for neutrophil and F4/80 for macrophage, as well as Western blotting for proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α. Expressions of neutrophil, macrophage and proinflammatory cytokines were highly upregulated in both IRI and UUO-kidneys (**Figure 3B-D and 4D-G**). To determine fibrosis progression, Sirius red stain and Western blotting for profibrotic cytokines (p-Smad3 and TGF- β), myofibroblast activation (α-SMA) and extracellular matrix (fibronectin) were assessed. These markers of fibrosis were markedly increased in both UUO (**Figure 5**) and IRI-kidneys (**Figure 6**). RDNx significantly suppressed renal tubular injury, inflammation, and fibrosis progression in both UUO and IRI-kidneys and were comparable with those of sham-operated (**Figure 3-6**), suggesting

Commented [A21]: Please include this timeline in the protocol to bring out clarity.

R: We have added the detail at the protocol in the revised version.

that renal nerve orchestrates renal tubular injury, inflammation and fibrosis in renal fibrosis models.

FIGURE AND TABLE LEGENDS

Figure 1. Confirmation of renal denervation in UUO model: Renal denervation in left kidneys of male mice was carried out 2 days prior to UUO. The left ureters were obstructed for 0, 1, 3, 6 or 12hrs, 1, 2, 3 or 10 days. (A) Paraffin-embedded kidney sections in intact or renal denervation (RDNx) without UUO were immunostained with anti-tyrosine hydroxylase (TH) antibody (Brown color; n=4). Hematoxylin was used to mark nucleus (blue color). Arrow indicates TH-positive sympathetic nerve. (B) Western blot analysis with anti-TH antibody was performed to confirm total expression of TH in intact or RDNx kidneys without UUO (n=4). β-actin was used for an equal loading marker. (C) Levels of kidney NE were measured by ELISA in UUO kidneys at indicated time points (n=6). Scale bar, 50 μm. RDNx, renal denervation; TH, tyrosine hydroxylase. Data are expressed as means ± SD. $^{\#}p< 0.05$ vs. intact; $^{\#\#\#}p< 0.001$ vs. intact. This figure has been modified from J Am Soc Nephrol.[15]

Figure 2. Confirmation of renal denervation in ischemic AKI to CKD model: Renal denervation in left kidneys of male mice was carried out 2 days prior to IRI. Mice were subjected to 30 min ischemia of left kidney and sacrificed at 0, 0.5, 1, 2, 4, 8 or 16 days post-IRI. (A) Western blot analysis with anti-TH antibody was performed to confirm total expression of TH in intact or RDNx kidneys with/without 16 days post-injury (n=4). β-actin was used for a loading control. (B) Levels of kidney NE were measured by ELISA in IRI kidneys at indicated time points (n=4). RDNx, renal denervation; TH, tyrosine hydroxylase. Data are expressed as means ± SD. $^{*}p< 0.05$ vs. intact in Sham; $^{\#}p< 0.05$ vs. respective intact. This figure has been modified from Kidney Int.[16]

Figure 3. Effect of renal denervation in renal tubular injury and inflammation in UUO mice kidneys: Renal denervation in left kidneys of male mice was carried out 2 days prior to UUO. The left ureters were obstructed for 0, 3, or 10 days. (A) Paraffin-embedded kidney section was used for PAS staining. Histological damage score was measured in randomly chosen five fields per kidney at 10 days post-injury using PAS-stained kidney section (n=4). (B) Paraffin-embedded kidney section was used for immunohistochemistry of PMN (brown color; neutrophil marker). (C) Number of PMN-positive neutrophil was evaluated in randomly chosen five fields per kidney at 3- and 10-days post-injury (n=4-6). (D) Kidney level of ICAM-1, TNF-α, IL-1β, IL-6, and TLR4 in kidneys 10 days post-injury was evaluated by Western blot analysis (n=4-6). β-actin was used as an equal loading marker. Scale bar, 50 μm. RDNx, renal denervation; TH, tyrosine hydroxylase. Data are expressed as means ± SD. $^{\#\#\#}p< 0.001$ vs. respective intact. This figure has been modified from J Am Soc Nephrol.[15]

Figure 4. Effect of renal denervation in renal tubular injury and inflammation in ischemic AKI to CKD transition model: Renal denervation in left kidneys of male mice was carried out 2 days prior to IRI. Mice were subjected to 30 min ischemia of left kidney and sacrificed at 0, 2, 4, 8 or 16 days post-IRI. (A) Paraffin-embedded kidney section at post-injury 0, 2, 4, 8 or 16 days was used for PAS staining. Histological damage score was measured in randomly chosen five fields per kidney using PAS-stained kidney section at indicated time points (n=4). (B) Paraffin-

embedded kidney sections were used for TUNEL assay and number of apoptotic cell was evaluated in randomly chosen five fields per kidney at indicated time points (*n*=5). (C) GFR was measured as described in Protocol section at indicated time points (*n*=5). (D) Paraffin-embedded kidney sections were used for immunohistochemistry of PMN (brown color; neutrophil marker). (E) Number of PMN-positive neutrophil was evaluated in randomly chosen five fields per kidney at indicated time points (n=5). (F) Paraffin-embedded kidney sections were used for immunohistochemistry of F4/80 (brown color; macrophage marker). (G) Number of F4/80-macrophage was evaluated in randomly chosen five fields per kidney at indicated time points (n=5). Scale bar, 50 μm. RDNx, renal denervation; TH, tyrosine hydroxylase. Data are expressed as means ± SD. $^{\#}p< 0.05$ vs. respective intact. This figure has been modified from Kidney Int.[16]

Figure 5. Effect of renal denervation in renal fibrosis in UUO mice kidneys: Renal denervation in left kidneys of male mice was carried out 2 days prior to UUO. The left ureters were obstructed for 0, 3, or 10 days. (A) Sirius red stain with paraffin-embedded kidney section at post-injury 0, 3 or 10 days was used to evaluate total collagen level. (B) Sirius red-positive area was evaluated in randomly chosen five fields per kidney at indicated time points (n=4-6). (C) Expression of α-SMA, fibronectin, TGF-β, and p-Smad3 in kidneys 10 days post-injury was examined by Western blot analysis using specific antibodies (n=4-6). β-actin was used for a loading control. Scale bar, 50 μm. RDNx, renal denervation; TH, tyrosine hydroxylase. Data are expressed as means ± SD. $^{\#}p< 0.05$ vs. respective intact; $^{\#\#\#}p< 0.001$ vs. respective intact. This figure has been modified from J Am Soc Nephrol.[15]

Figure 6. Effect of renal denervation in renal fibrosis in ischemic AKI to CKD transition model: Renal denervation in left kidneys of male mice was carried out 2 days prior to IRI. Mice were subjected to 30 min ischemia of left kidney and sacrificed at 0, 2, 4, 8 or 16 days post-IRI. (A) Sirius red stain with paraffin-embedded kidney section at post-injury 0, 2, 4, 8 or 16 days was used to evaluate total collagen level. (B) Sirius red-positive area was evaluated in randomly chosen five fields per kidney at indicated time points (n=5). (C) Expression of α-SMA and p-Smad3 at indicated time points was examined by Western blot analysis using specific antibodies (n=4). β-actin was used for a loading control. Scale bar, 50 μm. RDNx, renal denervation; TH, tyrosine hydroxylase. Data are expressed as means ± SD. $^{\#}p< 0.05$ vs. respective intact. This figure has been modified from Kidney Int.[16]

DISCUSSION

We provide the detailed method on how renal nerve is ablated in the mouse model. Further, the pivotal role of the renal nerve in triggering inflammatory and fibrotic responses to injury in CKD models are demonstrated. Complete separation of renal artery from connective tissue and vein is a critical step for successful RDNx that allows full exposure of renal nerves to 95% ethanol that result in complete nerve ablation. Since there is overlap of the renal artery over the vein, the portion overlapped would not be exposed to the alcohol well, unless the artery and vein are completely separated, which could result in incomplete ablation of renal nerve. During the operation of RDNx, ethanol can be exposed to connective tissues. Precaution is needed to limit the exposure to minimal region with tiny filter paper soaked in 95% ethanol. In patients who have uncontrolled hypertension, catheter-based ablation method has been used.[11,12] However, in

animal models bigger than mouse, surgical ablation can be used because the renal nerve can be seen under the surgical microscope. Compared to rat model, renal nerve of mouse is relatively small and is hard to identify. Since all methods frequently used in RDNx seem to be successful in ablation of renal nerve,[11,15,23] usage of a particular method depends on the animal model or how simple of method is accessible in researcher's environment. On the other hand, whether additional surgery or procedure in the denervated animal is required or not will be also critical point to consider when choosing the appropriate method of RDNx. The outcome of catheter-based RDNx in human patients may be different from that of animal models since catheter-based method could make incomplete RDNx.[12]

Expression of tyrosine hydroxylase (TH) is a marker used to confirm the success of sympathetic renal nerve ablation.[15,16,24] Norepinephrine-releasing neurons, as well as those of other catecholaminergic neurons, express TH.[25] Both sensory and sympathetic nerves are innervated into the kidney.[24] It should be pointed out that our RDNx method using 95% ethanol does not discriminate between the two types of nerves and both are ablated [15,16] and is a limitation of our method of RDNx. Based on our previous reports,[15,16] both sympathetic and sensory nerves are involved in renal inflammation and fibrogenesis. Calcitonin gene-related peptide (CGRP; a marker of afferent/sensory nerve) level also markedly decreased in the kidneys with RDNx, whereas administration of $CGRP^{8-37}$, an antagonist of CGRP, prevents kidney fibrosis and inflammation in both UUO and AKI to CKD models.[15,16] To investigate a precise role of the renal sensory nerve, capsaicin exposure can be used. A small piece of gauze or filter paper soaked in a capsaicin solution (33 mM in 5% ethanol, 5% Tween 80 and 90% normal saline) is applied to the renal artery and vein for 15 min.[26] To avoid non-renal exposure of capsaicin, parafilm can be placed under renal artery and vein.[26] After capsaicin exposure, removal of the sensory nerve can be evaluated by measurement of the level of CGRP.[15,16,24,26]

Collectively, our method for RDNx is replicable to abolish sympathetic nerve and is reproduced by others, suggesting that the method is applicable in research delving into the mechanisms of renal inflammation and fibrogenesis in normotensive, as well as hypertensive, animal models. Although further research is required to better understand the mechanism and its translatability of RDNx to clinical medicine, our studies suggest a high potential for ablation of renal nerve or intervention of its downstream signaling as an option for prevention or treatment of renal tubular injury, inflammation and fibrosis progression in diverse renal diseases.

ACKNOWLEDGMENTS

This study is supported by NIH grants DK-116987, DK-120533 and American Heart Association (AHA) Grant in Aid 15GRNT25080031 (BJP), AHA postdoctoral fellowship Grant 15POST25130003 (HSJ), and grants (NRF-2016R1C1B2012080 and NRF-2019R1F1A1041410) from the National Research Foundation of Korea (JK).

DISCLOSURES

The authors declare no competing financial interests.

REFERENCES

1 Grams, M. E. *et al.* Predicting timing of clinical outcomes in patients with chronic kidney disease and severely decreased glomerular filtration rate. *Kidney Int.* **93** (6), 1442-1451, (2018).
2 Coca, S. G., Singanamala, S. & Parikh, C. R. Chronic kidney disease after acute kidney injury: a systematic review and meta-analysis. *Kidney Int.* **81** (5), 442-448, (2012).
3 Hsu, R. K. & Hsu, C. Y. The Role of Acute Kidney Injury in Chronic Kidney Disease. *Semin Nephrol.* **36** (4), 283-292, (2016).
4 Okusa, M. D., Rosin, D. L. & Tracey, K. J. Targeting neural reflex circuits in immunity to treat kidney disease. *Nat Rev Nephrol.* **13** (11), 669-680, (2017).
5 Kanagy, N. L. Alpha(2)-adrenergic receptor signalling in hypertension. *Clin Sci (Lond).* **109** (5), 431-437, (2005).
6 Converse, R. L., Jr. *et al.* Sympathetic overactivity in patients with chronic renal failure. *N Engl J Med.* **327** (27), 1912-1918, (1992).
7 Veelken, R. *et al.* Autonomic renal denervation ameliorates experimental glomerulonephritis. *J Am Soc Nephrol.* **19** (7), 1371-1378, (2008).
8 Veelken, R. & Schmieder, R. E. Renal denervation--implications for chronic kidney disease. *Nat Rev Nephrol.* **10** (6), 305-313, (2014).
9 Wei, F. F., Zhang, Z. Y., Huang, Q. F. & Staessen, J. A. Diagnosis and management of resistant hypertension: state of the art. *Nat Rev Nephrol.* **14** (7), 428-441, (2018).
10 Wyatt, C. M. & Textor, S. C. Emerging evidence on renal denervation for the treatment of hypertension. *Kidney Int.* **94** (4), 644-646, (2018).
11 Bhatt, D. L. *et al.* A controlled trial of renal denervation for resistant hypertension. *N Engl J Med.* **370** (15), 1393-1401, (2014).
12 Jalil, F. & White, W. B. A New Era of Renal Denervation Trials for Patients With Hypertension? *Am J Kidney Dis.* **71** (5), 615-618, (2018).
13 Amann, K. *et al.* Glomerulosclerosis and progression: effect of subantihypertensive doses of alpha and beta blockers. *Kidney Int.* **60** (4), 1309-1323, (2001).
14 Amann, K. *et al.* Effects of low dose sympathetic inhibition on glomerulosclerosis and albuminuria in subtotally nephrectomized rats. *J Am Soc Nephrol.* **11** (8), 1469-1478, (2000).
15 Kim, J. & Padanilam, B. J. Renal nerves drive interstitial fibrogenesis in obstructive nephropathy. *J Am Soc Nephrol.* **24** (2), 229-242, (2013).
16 Kim, J. & Padanilam, B. J. Renal denervation prevents long-term sequelae of ischemic renal injury. *Kidney Int.* **87** (2), 350-358, (2015).
17 Eriguchi, M. *et al.* Renal denervation has blood pressure-independent protective effects on kidney and heart in a rat model of chronic kidney disease. *Kidney Int.* **87** (1), 116-127, (2015).
18 Jang, H. S. & Padanilam, B. J. Simultaneous deletion of Bax and Bak is required to prevent apoptosis and interstitial fibrosis in obstructive nephropathy. *Am J Physiol Renal Physiol.* **309** (6), F540-550, (2015).
19 Jang, H. S., Kim, J., Park, Y. K. & Park, K. M. Infiltrated macrophages contribute to recovery after ischemic injury but not to ischemic preconditioning in kidneys. *Transplantation.* **85** (3), 447-455, (2008).
20 Han, S. J., Jang, H. S., Kim, J. I., Lipschutz, J. H. & Park, K. M. Unilateral nephrectomy elongates primary cilia in the remaining kidney via reactive oxygen species. *Sci Rep.* **6** 22281, (2016).
21 Scarfe, L. *et al.* Transdermal Measurement of Glomerular Filtration Rate in Mice. *J Vis Exp.*

10.3791/58520 (140), (2018).
22 Jang, H. S., Kim, J. I., Han, S. J. & Park, K. M. Recruitment and subsequent proliferation of bone marrow-derived cells in the postischemic kidney are important to the progression of fibrosis. *Am J Physiol Renal Physiol.* **306** (12), F1451-1461, (2014).
23 Clayton, S. C., Haack, K. K. & Zucker, I. H. Renal denervation modulates angiotensin receptor expression in the renal cortex of rabbits with chronic heart failure. *Am J Physiol Renal Physiol.* **300** (1), F31-39, (2011).
24 Mulder, J., Hokfelt, T., Knuepfer, M. M. & Kopp, U. C. Renal sensory and sympathetic nerves reinnervate the kidney in a similar time-dependent fashion after renal denervation in rats. *Am J Physiol Regul Integr Comp Physiol.* **304** (8), R675-682, (2013).
25 Weihe, E., Depboylu, C., Schutz, B., Schafer, M. K. & Eiden, L. E. Three types of tyrosine hydroxylase-positive CNS neurons distinguished by dopa decarboxylase and VMAT2 co-expression. *Cell Mol Neurobiol.* **26** (4-6), 659-678, (2006).
26 Foss, J. D., Wainford, R. D., Engeland, W. C., Fink, G. D. & Osborn, J. W. A novel method of selective ablation of afferent renal nerves by periaxonal application of capsaicin. *Am J Physiol Regul Integr Comp Physiol.* **308** (2), R112-122, (2015).

American Society of Nephrology LICENSE
TERMS AND CONDITIONS

Nov 25, 2019

This is a License Agreement between Hee-Seong Jang ("You") and American Society of Nephrology ("American Society of Nephrology") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by American Society of Nephrology, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	4665581507301
License date	Sep 10, 2019
Licensed content publisher	American Society of Nephrology
Licensed content title	Journal of the American Society of Nephrology : JASN
Licensed content date	Jan 1, 1990
Type of Use	Journal/Magazine
Requestor type	Publisher, not-for-profit
Format	Electronic
Portion	chart/graph/table/figure
Number of charts/graphs/tables/figures	2
The requesting person/organization is:	Babu Padanilam

Title or numeric reference of the portion(s)	Renal denervation approach to prevent inflammation and fibrogenesis in chronic kidney disease
Title of the article or chapter the portion is from	Renal denervation approach to prevent inflammation and fibrogenesis in chronic kidney disease
Editor of portion(s)	N/A
Author of portion(s)	N/A
Volume of serial or monograph.	N/A
Issue, if republishing an article from a serial	N/A
Page range of the portion	N/A
Publication date of portion	N/A
Rights for	Main product
Duration of use	Life of current and all future editions
Creation of copies for the disabled	no
With minor editing privileges	no
For distribution to	Worldwide
In the following language(s)	Original language of publication
With incidental promotional use	no
The lifetime unit quantity of new product	Up to 499

Title of new article	Renal denervation approach to prevent inflammation and fibrogenesis in chronic kidney disease
Lead author	Hee-Seong Jang
Title of targeted journal	Journal of Visualized Experiments
Publisher	MyJove Corp.
Expected publication date	Dec 2019
Billing Type	Invoice
Billing Address	Hee-Seong Jang 985850 Nebraska Medical Center Omaha, NE 68198 United States Attn: Hee-Seong Jang
Total (may include CCC user fee)	203.50 USD
Terms and Conditions	

TERMS AND CONDITIONS

The following terms are individual to this publisher:

None

Other Terms and Conditions:

STANDARD TERMS AND CONDITIONS

1. Description of Service; Defined Terms. This Republication License enables the User to obtain licenses for republication of one or more copyrighted works as described in detail on the relevant Order Confirmation (the "Work(s)"). Copyright Clearance Center, Inc. ("CCC") grants licenses through the Service on behalf of the rightsholder identified on the Order Confirmation (the "Rightsholder"). "Republication", as used herein, generally means the inclusion of a Work, in whole or in part, in a new work or works, also as described on the Order Confirmation. "User", as used herein, means the person or entity making such republication.

2. The terms set forth in the relevant Order Confirmation, and any terms set by the Rightsholder with respect to a particular Work, govern the terms of use of Works in connection with the Service. By using the Service, the person transacting for a republication license on behalf of the User represents and warrants that he/she/it (a) has been duly authorized by the User to accept, and hereby does accept, all such terms and conditions on behalf of User, and (b) shall inform User of all such terms and conditions. In the event such person is a "freelancer" or other third party independent of User and CCC, such party shall be deemed jointly a "User" for purposes of these terms and conditions. In any event, User shall be deemed to have accepted and agreed to all such terms and conditions if User republishes the Work in any fashion.

3. Scope of License; Limitations and Obligations.

3.1 All Works and all rights therein, including copyright rights, remain the sole and exclusive property of the Rightsholder. The license created by the exchange of an Order Confirmation (and/or any invoice) and payment by User of the full amount set forth on that document includes only those rights expressly set forth in the Order Confirmation and in these terms and conditions, and conveys no other rights in the Work(s) to User. All rights not expressly granted are hereby reserved.

3.2 General Payment Terms: You may pay by credit card or through an account with us payable at the end of the month. If you and we agree that you may establish a standing account with CCC, then the following terms apply: Remit Payment to: Copyright Clearance Center, 29118 Network Place, Chicago, IL 60673-1291. Payments Due: Invoices are payable upon their delivery to you (or upon our notice to you that they are available to you for downloading). After 30 days, outstanding amounts will be subject to a service charge of 1-1/2% per month or, if less, the maximum rate allowed by applicable law. Unless otherwise specifically set forth in the Order Confirmation or in a separate written agreement signed by CCC, invoices are due and payable on "net 30" terms. While User may exercise the rights licensed immediately upon issuance of the Order Confirmation, the license is automatically revoked and is null and void, as if it had never been issued, if complete payment for the license is not received on a timely basis either from User directly or through a payment agent, such as a credit card company.

3.3 Unless otherwise provided in the Order Confirmation, any grant of rights to User (i) is "one-time" (including the editions and product family specified in the license), (ii) is non-exclusive and non-transferable and (iii) is subject to any and all limitations and restrictions (such as, but not limited to, limitations on duration of use or circulation) included in the Order Confirmation or invoice and/or in these terms and conditions. Upon completion of the licensed use, User shall either secure a new permission for further use of the Work(s) or immediately cease any new use of the Work(s) and shall render inaccessible (such as by deleting or by removing or severing links or other locators) any further copies of the Work (except for copies printed on paper in accordance with this license and still in User's stock at the end of such period).

3.4 In the event that the material for which a republication license is sought includes third party materials (such as photographs, illustrations, graphs, inserts and similar materials) which are identified in such material as having been used by permission, User is responsible for identifying, and seeking separate licenses (under this Service or otherwise) for, any of such third party materials; without a separate license, such third party materials may not be used.

3.5 Use of proper copyright notice for a Work is required as a condition of any license granted under the Service. Unless otherwise provided in the Order Confirmation, a proper copyright notice will read substantially as follows: "Republished with permission of

[Rightsholder's name], from [Work's title, author, volume, edition number and year of copyright]; permission conveyed through Copyright Clearance Center, Inc. " Such notice must be provided in a reasonably legible font size and must be placed either immediately adjacent to the Work as used (for example, as part of a by-line or footnote but not as a separate electronic link) or in the place where substantially all other credits or notices for the new work containing the republished Work are located. Failure to include the required notice results in loss to the Rightsholder and CCC, and the User shall be liable to pay liquidated damages for each such failure equal to twice the use fee specified in the Order Confirmation, in addition to the use fee itself and any other fees and charges specified.

3.6 User may only make alterations to the Work if and as expressly set forth in the Order Confirmation. No Work may be used in any way that is defamatory, violates the rights of third parties (including such third parties' rights of copyright, privacy, publicity, or other tangible or intangible property), or is otherwise illegal, sexually explicit or obscene. In addition, User may not conjoin a Work with any other material that may result in damage to the reputation of the Rightsholder. User agrees to inform CCC if it becomes aware of any infringement of any rights in a Work and to cooperate with any reasonable request of CCC or the Rightsholder in connection therewith.

4. Indemnity. User hereby indemnifies and agrees to defend the Rightsholder and CCC, and their respective employees and directors, against all claims, liability, damages, costs and expenses, including legal fees and expenses, arising out of any use of a Work beyond the scope of the rights granted herein, or any use of a Work which has been altered in any unauthorized way by User, including claims of defamation or infringement of rights of copyright, publicity, privacy or other tangible or intangible property.

5. Limitation of Liability. UNDER NO CIRCUMSTANCES WILL CCC OR THE RIGHTSHOLDER BE LIABLE FOR ANY DIRECT, INDIRECT, CONSEQUENTIAL OR INCIDENTAL DAMAGES (INCLUDING WITHOUT LIMITATION DAMAGES FOR LOSS OF BUSINESS PROFITS OR INFORMATION, OR FOR BUSINESS INTERRUPTION) ARISING OUT OF THE USE OR INABILITY TO USE A WORK, EVEN IF ONE OF THEM HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. In any event, the total liability of the Rightsholder and CCC (including their respective employees and directors) shall not exceed the total amount actually paid by User for this license. User assumes full liability for the actions and omissions of its principals, employees, agents, affiliates, successors and assigns.

6. Limited Warranties. THE WORK(S) AND RIGHT(S) ARE PROVIDED "AS IS". CCC HAS THE RIGHT TO GRANT TO USER THE RIGHTS GRANTED IN THE ORDER CONFIRMATION DOCUMENT. CCC AND THE RIGHTSHOLDER DISCLAIM ALL OTHER WARRANTIES RELATING TO THE WORK(S) AND RIGHT(S), EITHER EXPRESS OR IMPLIED, INCLUDING WITHOUT LIMITATION IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. ADDITIONAL RIGHTS MAY BE REQUIRED TO USE ILLUSTRATIONS, GRAPHS, PHOTOGRAPHS, ABSTRACTS, INSERTS OR OTHER PORTIONS OF THE WORK (AS OPPOSED TO THE ENTIRE WORK) IN A MANNER CONTEMPLATED BY USER; USER UNDERSTANDS AND AGREES THAT NEITHER CCC NOR THE RIGHTSHOLDER MAY HAVE SUCH ADDITIONAL RIGHTS TO GRANT.

7. Effect of Breach. Any failure by User to pay any amount when due, or any use by User of a Work beyond the scope of the license set forth in the Order Confirmation and/or these terms and conditions, shall be a material breach of the license created by the Order Confirmation and these terms and conditions. Any breach not cured within 30 days of written notice thereof shall result in immediate termination of such license without further notice. Any unauthorized (but licensable) use of a Work that is terminated immediately upon

notice thereof may be liquidated by payment of the Rightsholder's ordinary license price therefor; any unauthorized (and unlicensable) use that is not terminated immediately for any reason (including, for example, because materials containing the Work cannot reasonably be recalled) will be subject to all remedies available at law or in equity, but in no event to a payment of less than three times the Rightsholder's ordinary license price for the most closely analogous licensable use plus Rightsholder's and/or CCC's costs and expenses incurred in collecting such payment.

8. **Miscellaneous.**

8.1 User acknowledges that CCC may, from time to time, make changes or additions to the Service or to these terms and conditions, and CCC reserves the right to send notice to the User by electronic mail or otherwise for the purposes of notifying User of such changes or additions; provided that any such changes or additions shall not apply to permissions already secured and paid for.

8.2 Use of User-related information collected through the Service is governed by CCC's privacy policy, available online here: http://www.copyright.com/content/cc3/en/tools/footer/privacypolicy.html.

8.3 The licensing transaction described in the Order Confirmation is personal to User. Therefore, User may not assign or transfer to any other person (whether a natural person or an organization of any kind) the license created by the Order Confirmation and these terms and conditions or any rights granted hereunder; provided, however, that User may assign such license in its entirety on written notice to CCC in the event of a transfer of all or substantially all of User's rights in the new material which includes the Work(s) licensed under this Service.

8.4 No amendment or waiver of any terms is binding unless set forth in writing and signed by the parties. The Rightsholder and CCC hereby object to any terms contained in any writing prepared by the User or its principals, employees, agents or affiliates and purporting to govern or otherwise relate to the licensing transaction described in the Order Confirmation, which terms are in any way inconsistent with any terms set forth in the Order Confirmation and/or in these terms and conditions or CCC's standard operating procedures, whether such writing is prepared prior to, simultaneously with or subsequent to the Order Confirmation, and whether such writing appears on a copy of the Order Confirmation or in a separate instrument.

8.5 The licensing transaction described in the Order Confirmation document shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such licensing transaction shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.If you have any comments or questions about the Service or Copyright Clearance Center, please contact us at 978-750-8400 or send an e-mail to info@copyright.com.

v 1.1

You will be invoiced within 48 hours of this transaction date. You may pay your invoice by credit card upon receipt of the invoice for this transaction. Please follow instructions provided at that time.

To pay for this transaction now; please remit a copy of this document along with your payment. Payment should be in the form of a check or money order referencing your account number and this invoice number COPYRIGHT.COM503264899.

Make payments to "COPYRIGHT CLEARANCE CENTER" and send to:

Copyright Clearance Center
29118 Network Place
Chicago, IL 60673-1291
Please disregard electronic and mailed copies if you remit payment in advance

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Elsevier Science and Technology Journals LICENSE
TERMS AND CONDITIONS

Nov 25, 2019

This is a License Agreement between Hee-Seong Jang ("You") and Elsevier Science and Technology Journals ("Elsevier Science and Technology Journals") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier Science and Technology Journals, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	4674950245847
License date	Sep 10, 2019
Licensed content publisher	Elsevier Science and Technology Journals
Licensed content title	Kidney international
Licensed content date	Jan 1, 1972
Type of Use	Journal/Magazine
Requestor type	Publisher, not-for-profit
Format	Electronic
Portion	chart/graph/table/figure
Number of charts/graphs/tables/figures	3
The requesting	Babu Padanilam

person/organization is:

Title or numeric reference of the portion(s)	Renal denervation approach to prevent inflammation and fibrogenesis in chronic kidney disease
Title of the article or chapter the portion is from	Renal denervation approach to prevent inflammation and fibrogenesis in chronic kidney disease
Editor of portion(s)	N/A
Author of portion(s)	N/A
Volume of serial or monograph.	N/A
Issue, if republishing an article from a serial	N/A
Page range of the portion	N/A
Publication date of portion	N/A
Rights for	Main product
Duration of use	Life of current edition
Creation of copies for the disabled	no
With minor editing privileges	no
For distribution to	Worldwide
In the following language(s)	Original language of publication
With incidental promotional use	no

The lifetime unit quantity of new product	Up to 499
Title of new article	Renal denervation approach to prevent inflammation and fibrogenesis in chronic kidney disease
Lead author	Hee-Seong Jang
Title of targeted journal	Journal of Visualized Experiments
Publisher	MyJove Corp.
Expected publication date	Dec 2019
Billing Type	Credit Card
Credit card info	Visa ending in 2356
Credit card expiration	03/2022
Total (may include CCC user fee)	289.94 USD

Terms and Conditions

TERMS AND CONDITIONS

The following terms are individual to this publisher:

None

Other Terms and Conditions:

STANDARD TERMS AND CONDITIONS

1. Description of Service; Defined Terms. This Republication License enables the User to obtain licenses for republication of one or more copyrighted works as described in detail on the relevant Order Confirmation (the “Work(s)”). Copyright Clearance Center, Inc. (“CCC”) grants licenses through the Service on behalf of the rightsholder identified on the Order Confirmation (the “Rightsholder”). “Republication”, as used herein, generally means the inclusion of a Work, in whole or in part, in a new work or works, also as described on the Order Confirmation. “User”, as used herein, means the person or entity making such republication.

2. The terms set forth in the relevant Order Confirmation, and any terms set by the Rightsholder with respect to a particular Work, govern the terms of use of Works in connection with the Service. By using the Service, the person transacting for a republication license on behalf of the User represents and warrants that he/she/it (a) has been duly authorized by the User to accept, and hereby does accept, all such terms and conditions on behalf of User, and (b) shall inform User of all such terms and conditions. In the event such person is a "freelancer" or other third party independent of User and CCC, such party shall be deemed jointly a "User" for purposes of these terms and conditions. In any event, User shall be deemed to have accepted and agreed to all such terms and conditions if User republishes the Work in any fashion.

3. Scope of License; Limitations and Obligations.

3.1 All Works and all rights therein, including copyright rights, remain the sole and exclusive property of the Rightsholder. The license created by the exchange of an Order Confirmation (and/or any invoice) and payment by User of the full amount set forth on that document includes only those rights expressly set forth in the Order Confirmation and in these terms and conditions, and conveys no other rights in the Work(s) to User. All rights not expressly granted are hereby reserved.

3.2 General Payment Terms: You may pay by credit card or through an account with us payable at the end of the month. If you and we agree that you may establish a standing account with CCC, then the following terms apply: Remit Payment to: Copyright Clearance Center, 29118 Network Place, Chicago, IL 60673-1291. Payments Due: Invoices are payable upon their delivery to you (or upon our notice to you that they are available to you for downloading). After 30 days, outstanding amounts will be subject to a service charge of 1-1/2% per month or, if less, the maximum rate allowed by applicable law. Unless otherwise specifically set forth in the Order Confirmation or in a separate written agreement signed by CCC, invoices are due and payable on "net 30" terms. While User may exercise the rights licensed immediately upon issuance of the Order Confirmation, the license is automatically revoked and is null and void, as if it had never been issued, if complete payment for the license is not received on a timely basis either from User directly or through a payment agent, such as a credit card company.

3.3 Unless otherwise provided in the Order Confirmation, any grant of rights to User (i) is "one-time" (including the editions and product family specified in the license), (ii) is non-exclusive and non-transferable and (iii) is subject to any and all limitations and restrictions (such as, but not limited to, limitations on duration of use or circulation) included in the Order Confirmation or invoice and/or in these terms and conditions. Upon completion of the licensed use, User shall either secure a new permission for further use of the Work(s) or immediately cease any new use of the Work(s) and shall render inaccessible (such as by deleting or by removing or severing links or other locators) any further copies of the Work (except for copies printed on paper in accordance with this license and still in User's stock at the end of such period).

3.4 In the event that the material for which a republication license is sought includes third party materials (such as photographs, illustrations, graphs, inserts and similar materials) which are identified in such material as having been used by permission, User is responsible for identifying, and seeking separate licenses (under this Service or otherwise) for, any of such third party materials; without a separate license, such third party materials may not be used.

3.5 Use of proper copyright notice for a Work is required as a condition of any license granted under the Service. Unless otherwise provided in the Order Confirmation, a proper copyright notice will read substantially as follows: "Republished with permission of

[Rightsholder's name], from [Work's title, author, volume, edition number and year of copyright]; permission conveyed through Copyright Clearance Center, Inc. " Such notice must be provided in a reasonably legible font size and must be placed either immediately adjacent to the Work as used (for example, as part of a by-line or footnote but not as a separate electronic link) or in the place where substantially all other credits or notices for the new work containing the republished Work are located. Failure to include the required notice results in loss to the Rightsholder and CCC, and the User shall be liable to pay liquidated damages for each such failure equal to twice the use fee specified in the Order Confirmation, in addition to the use fee itself and any other fees and charges specified.

3.6 User may only make alterations to the Work if and as expressly set forth in the Order Confirmation. No Work may be used in any way that is defamatory, violates the rights of third parties (including such third parties' rights of copyright, privacy, publicity, or other tangible or intangible property), or is otherwise illegal, sexually explicit or obscene. In addition, User may not conjoin a Work with any other material that may result in damage to the reputation of the Rightsholder. User agrees to inform CCC if it becomes aware of any infringement of any rights in a Work and to cooperate with any reasonable request of CCC or the Rightsholder in connection therewith.

4. Indemnity. User hereby indemnifies and agrees to defend the Rightsholder and CCC, and their respective employees and directors, against all claims, liability, damages, costs and expenses, including legal fees and expenses, arising out of any use of a Work beyond the scope of the rights granted herein, or any use of a Work which has been altered in any unauthorized way by User, including claims of defamation or infringement of rights of copyright, publicity, privacy or other tangible or intangible property.

5. Limitation of Liability. UNDER NO CIRCUMSTANCES WILL CCC OR THE RIGHTSHOLDER BE LIABLE FOR ANY DIRECT, INDIRECT, CONSEQUENTIAL OR INCIDENTAL DAMAGES (INCLUDING WITHOUT LIMITATION DAMAGES FOR LOSS OF BUSINESS PROFITS OR INFORMATION, OR FOR BUSINESS INTERRUPTION) ARISING OUT OF THE USE OR INABILITY TO USE A WORK, EVEN IF ONE OF THEM HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. In any event, the total liability of the Rightsholder and CCC (including their respective employees and directors) shall not exceed the total amount actually paid by User for this license. User assumes full liability for the actions and omissions of its principals, employees, agents, affiliates, successors and assigns.

6. Limited Warranties. THE WORK(S) AND RIGHT(S) ARE PROVIDED "AS IS". CCC HAS THE RIGHT TO GRANT TO USER THE RIGHTS GRANTED IN THE ORDER CONFIRMATION DOCUMENT. CCC AND THE RIGHTSHOLDER DISCLAIM ALL OTHER WARRANTIES RELATING TO THE WORK(S) AND RIGHT(S), EITHER EXPRESS OR IMPLIED, INCLUDING WITHOUT LIMITATION IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. ADDITIONAL RIGHTS MAY BE REQUIRED TO USE ILLUSTRATIONS, GRAPHS, PHOTOGRAPHS, ABSTRACTS, INSERTS OR OTHER PORTIONS OF THE WORK (AS OPPOSED TO THE ENTIRE WORK) IN A MANNER CONTEMPLATED BY USER; USER UNDERSTANDS AND AGREES THAT NEITHER CCC NOR THE RIGHTSHOLDER MAY HAVE SUCH ADDITIONAL RIGHTS TO GRANT.

7. Effect of Breach. Any failure by User to pay any amount when due, or any use by User of a Work beyond the scope of the license set forth in the Order Confirmation and/or these terms and conditions, shall be a material breach of the license created by the Order Confirmation and these terms and conditions. Any breach not cured within 30 days of written notice thereof shall result in immediate termination of such license without further notice. Any unauthorized (but licensable) use of a Work that is terminated immediately upon

notice thereof may be liquidated by payment of the Rightsholder's ordinary license price therefor; any unauthorized (and unlicensable) use that is not terminated immediately for any reason (including, for example, because materials containing the Work cannot reasonably be recalled) will be subject to all remedies available at law or in equity, but in no event to a payment of less than three times the Rightsholder's ordinary license price for the most closely analogous licensable use plus Rightsholder's and/or CCC's costs and expenses incurred in collecting such payment.

8. **Miscellaneous.**

8.1 User acknowledges that CCC may, from time to time, make changes or additions to the Service or to these terms and conditions, and CCC reserves the right to send notice to the User by electronic mail or otherwise for the purposes of notifying User of such changes or additions; provided that any such changes or additions shall not apply to permissions already secured and paid for.

8.2 Use of User-related information collected through the Service is governed by CCC's privacy policy, available online here: http://www.copyright.com/content/cc3/en/tools/footer/privacypolicy.html.

8.3 The licensing transaction described in the Order Confirmation is personal to User. Therefore, User may not assign or transfer to any other person (whether a natural person or an organization of any kind) the license created by the Order Confirmation and these terms and conditions or any rights granted hereunder; provided, however, that User may assign such license in its entirety on written notice to CCC in the event of a transfer of all or substantially all of User's rights in the new material which includes the Work(s) licensed under this Service.

8.4 No amendment or waiver of any terms is binding unless set forth in writing and signed by the parties. The Rightsholder and CCC hereby object to any terms contained in any writing prepared by the User or its principals, employees, agents or affiliates and purporting to govern or otherwise relate to the licensing transaction described in the Order Confirmation, which terms are in any way inconsistent with any terms set forth in the Order Confirmation and/or in these terms and conditions or CCC's standard operating procedures, whether such writing is prepared prior to, simultaneously with or subsequent to the Order Confirmation, and whether such writing appears on a copy of the Order Confirmation or in a separate instrument.

8.5 The licensing transaction described in the Order Confirmation document shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such licensing transaction shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.If you have any comments or questions about the Service or Copyright Clearance Center, please contact us at 978-750-8400 or send an e-mail to info@copyright.com.

v 1.1

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Renal denervation approach to prevent inflammation and fibrogenesis in chronic kidney disease
Author(s):	Hee-Seong Jang, Mi Ra Noh, Jinu Kim, and Babu J. Padanilam.

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Babu J. Padanilam
Department:	Cellular and Integrative Physiology
Institution:	University of Nebraska Medical Center
Title:	Professor

Signature: Babu Padanilam Date: 09/30/2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Signature Certificate

Document Ref.: PBREG-FWHJX-QSMBM-GCRIP

Document signed by:

babu Padanilam

Verified E-mail:
bpadanil@yahoo.com

IP: 192.94.102.4 | Date: 30 Sep 2019 20:48:10 UTC

babu Padanilam

Document completed by all parties on:
30 Sep 2019 20:48:10 UTC