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Long-Term Continuous Measurement of Renal Blood Flow in Conscious Rats

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TITLE:
Long-Term Continuous Measurement of Renal Blood Flow in Conscious Rats

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SUMMARY:
The present protocol describes a long-term continuous measurement of renal blood flow in conscious rats and simultaneously recording blood pressure with implanted catheters (fluid-filled or by telemetry).

ABSTRACT:
The kidneys play a crucial role in maintaining the homeostasis of body fluids. The regulation of renal blood flow (RBF) is essential to the vital functions of filtration and metabolism in kidney function. Many acute studies have been carried out in anesthetized animals to measure RBF under various conditions to determine mechanisms responsible for the regulation of kidney perfusion. However, for technical reasons, it has not been possible to measure RBF continuously (24 h/day) in unrestrained unanesthetized rats over prolonged periods. These methods allow the continuous determination of RBF over many weeks while also simultaneously recording blood pressure (BP) with implanted catheters (fluid-filled or by telemetry). RBF monitoring is carried out with rats placed in a circular servo-controlled rat cage that enables the unrestrained movement of the rat throughout the study. At the same time, the tangling of cables from the flow probe and arterial catheters is prevented. Rats are first instrumented with an ultrasonic flow probe placement on the left renal artery and an arterial catheter implanted in the right femoral artery. These are routed subcutaneously to the nape of the neck, and connected to the flowmeter and pressure transducer, respectively, to measure RBF and BP. Following surgical implantation, rats are immediately placed in the cage to recover for at least one week and stabilize the ultrasonic probe recordings. Urine collection is also feasible in this system. The surgical and post-surgical procedures for continuous monitoring are demonstrated in this protocol.

INTRODUCTION:
The kidneys are only 0.5% of the bodyweight but rich in blood flow, receiving 20%-25% of the total cardiac output[1]. The regulation of renal blood flow (RBF) is central to kidney function, body fluid, and electrolyte homeostasis. The importance of blood flow regulation to the kidney is nicely

illustrated by the substantial increase of RBF in the remaining kidney after unilateral nephrectomy[2-4] and by the reductions of RBF that occur in kidney failure[5-7]. Whether such changes in RBF occur in response to alterations in kidney function or a decrease in function due to reduction of RBF has been challenging to ascertain in anesthetized surgically prepared animals or human subjects. Temporal studies are required in which the events can be determined before and following a defined change and observed in the same animal during the progression of events. In the animal and human studies, RBF has been estimated indirectly by the clearance of para-amino hippuric acid (PAH)[8-10] and in more recent time by imaging techniques such as ultrasound[9,11,12], MRI[4,13], and PET-CT[14,15] which give helpful snapshot images of each kidney and which can follow the progression of the disease. It is challenging to evaluate RBF in small animals by ultrasound or MRI scans without anesthesia. It has been impossible to continuously measure RBF under conscious conditions in the same rat over prolonged periods.

The present protocol, therefore, developed techniques that enable simultaneous continuous 24 h/day measurements of RBF, which has been combined with continuous blood pressure measurement methods for freely moving rats as described previously[16-21]. This technology allows for the temporal evaluation of RBF in various models of rats to study cause-effect relationships in various renal disorders in the future.

PROTOCOL:

The protocol is approved by the Medical College of Wisconsin Institutional Animal Care and Use. Dahl salt-sensitive rats (males and females), ~8 weeks of age, 200-350 g, were used for the experiments.

1. Animal preparation

1.1. Install a movement response caging system for the rat, a perivascular flow module, syringe pump, recording device, and software (see **Table of Materials**) in the animal room.

1.2. Place the rats in the cage to become familiar with the environment, food, and water system at least the week prior to surgery. Fast the rats from the day before the surgery because a high stomach content may interfere with the placement of the flow probe into the left renal artery and prevent tracheal aspiration.

1.3. Connect a 5 cm of polyurethane tubing (inner diameter 0.30 mm and outer diameter 0.64 mm) to the end of the 90 cm of polyurethane tubing (inner diameter 0.64 mm and outer diameter 1.02 mm) with PVC cement to make a femoral arterial catheter (see **Table of Materials**).

1.3.1. Sterilize the catheters with an Ethylene oxide sterilizer, the flow probe with 2.5% glutaraldehyde, and the surgical instruments in a steam autoclave. Wipe surgical tables, microscopy, and lights with 1% sodium hypochlorite.

2. Surgery

2.1. Place the RBF probe following the steps below.

2.1.1. Anesthetize the rats with 2.0%-2.5% Isoflurane to the degree that the rats do not respond to the pain stimulus. Place it on the surgical table set at 37 °C and inject 0.09 mg/kg of buprenorphine and 15 mg/kg of cefazolin (see **Table of Materials**) before surgery.

2.1.2. Shave the entire abdomen with an electric clipper and a region on the nape of the neck around the 7th cervical vertebrae where the catheter and flow prove wires will exit.

2.1.3. After shaving, wipe the area with 70% ethanol, 10% povidone-iodine, and again with 70% ethanol.

2.1.4. Place the rat in the prone position. Make a 1 cm cut with the scissors on the nape of the neck and the left flank. Then, perform a blunt dissection with hemostatic forceps and clear a subcutaneous space from the flank incision to the back of the neck.

2.1.5. Pass the flow probe through this subcutaneous tunnel from the neck to the flank incision with hemostatic forceps.

2.1.6. Place the rat in the supine position. Make a 4-5 cm midline abdominal incision.

2.1.7. Dissect the area around the renal artery with curved tweezers to expose a space sufficient to place the flow probe (see **Table of Materials**). Then bluntly pierce the left quadratus lumborum muscle with the hemostatic forceps and pull the head of the flow probe into the abdominal cavity.

2.1.8. Hook the tip of the flow probe to the left renal artery and connect it to the flow meter (see **Table of Materials**). Add some gel around the probe tip, and the value of the flow rate will appear on the flow meter.

NOTE: Although it depends on the size of the rat, a flow of about 3-5 mL/min will be observed in a 230 g rat.

2.1.9. Glue the polyester fiber mesh attached to the probe with tissue adhesive to the abdominal wall and hold until dry and bonded (~1-2 min). Once the flow is in place, disconnect the flow probe from the flow meter and cover the abdomen with saline-soaked gauze and move on to the step of inserting the catheter.

2.2. Insert the femoral catheter following the steps below.

NOTE: The method for inserting a fluid-filled catheter is the same as regular telemetry installations. Although telemetry is preferred, the arterial catheter enables pressure monitoring and period blood sampling from the conscious rat.

2.2.1. First, fill the catheter with saline and clamp it with vascular forceps before making a 1 cm skin incision with scissors on the left thigh to dissect and expose the femoral artery. While blocking the flow at the proximal side of the femoral artery with a thread, insert the catheter.

2.2.2. Flush with a small amount of saline, plug with appropriate size stainless wire, and tie the catheter with a thread to fix it.

2.2.3. Once the ligature is tied around the catheter, create a subcutaneous tunnel by using a stainless-steel trocar from the thigh to the back of the neck to bring the catheter to the neck region. Secure it with 3-0 silk sutures placed in the trapezius muscle.

2.3. Suture the probe.

2.3.1. Turn the rat to the prone position and stitch the circular loop of the flow probe subcutaneously at the flank. Suture the incision at the flank and the neck with 4-0 surgical suture (see **Table of Materials**).

2.3.2. Attach a skin button to the flow probe and suture it with 3-0 silk at the back of the neck.

2.3.3. Connect the flow probe to the flow meter again, turn the rat back to the dorsal position to check RBF, and make final adjustments of the flow probe to optimize its position on the renal artery.

2.3.4. Finally, suture the muscle with 3-0 silk and the skin with 4-0 surgical suture.

3. Recovery of the animal

3.1. After careful observation, until the rats are fully recovered from the anesthesia, return the rats to a movement response caging system, connect the flow probe to the blood flow meter, and allow a recovery period of about a week to stabilize the probe and flow measurement.

NOTE: Recording doesn't have to be done during this period.

3.2. Infuse 3% heparinized saline continuously throughout the study from the arterial catheter at the rate of 100 µL/h to prevent the clotting.

3.3. When the flow stabilizes after 5-6 days, set the flowmeter calibration to measure blood flow at 0-20 mL/min and begin the continuous recording of RBF.

REPRESENTATIVE RESULTS:

The mean arterial pressure data (**Figure 1A**) and blood flow data (**Figure 1B**) from a representative male Dahl salt-sensitive rat are shown. The Dahl salt-sensitive rats are maintained in a colony and bred at the Medical College of Wisconsin. The surgery was done at the age of 8 weeks, and the bodyweight was 249 g at the time of surgery. Rats were fed with a 0.4% NaCl diet, and the diet was changed to a 4% NaCl diet at the age of 10 weeks. Measurements were continued for 3 weeks on a 4% NaCl diet, and the experiment was terminated at 13 weeks of age. The data is shown with a minute average. A clear diurnal difference was observed in mean arterial pressure and blood flow. While blood pressure increases with a high salt diet, blood flow tends to decrease rather than increase, suggesting increased renal vascular resistance.

FIGURE LEGEND:

Figure 1: Representative arterial pressure and blood flow data. Mean arterial pressure (mm Hg) (**A**) and renal blood flow (mL/min) (**B**) are shown with a minute average. LS: low salt (0.4% NaCl) diet, HS: High salt (4% NaCl) diet.

DISCUSSION:

The present protocol describes a technique that utilizes commercially available instrumentation to record RBF and arterial pressure continuously over many weeks. In addition, urine can be collected using the device described in step 1.1. It can also be used to evaluate metabolites in the urine and, when an arterial catheter is implanted, blood sampling for analysis.

Traditionally, RBF measurements have been obtained acutely in surgically prepared anesthetized animals or estimated by PAH clearance. However, it has been shown that various anesthetics and surgery[22,23] alter renal blood flow and arterial pressure. Studies in humans have reported that isoflurane reduced PAH clearance from 476.8-243.3 mL/min and inulin clearance from 88.0 to 55.7 mL/min, respectively[8]. Thiobarbital is an anesthetic that is widely used for critical studies of rats. Still, it is reported that H_2O_2 production in the mitochondria of the renal cortex increases 90 min after anesthesia with Thiobarbital[24], which might affect blood flow. Measurements in unanesthetized and unstressed animals would be far preferable for many experimental studies. The method of measuring RBF by implanting a flow probe has been demonstrated in dogs[25] and rats[26]. This work also has established a way of measuring RBF in rats in the laboratory.

Applying the techniques described in this presentation can address many questions related to the sequential events following a given stimulus. The unanesthetized instrumented rat model enables the determination of both immediate and chronic responses to drug and long-term consequences of various stimuli that may occur during the development of hypertension.

The surgery involves minimal blood loss with nearly 100% survival rates with some training. The flow probes can be reused after washing with protease contained anionic detergent and sterilization following a 4-week experiment. However, the plastic coating will gradually deteriorate and, after several uses, will require repair. The skin button in the next where the catheters exit represents the most significant potential problem as it is vulnerable to infection, irritation, and scratching if not carefully cleaned and disinfected. However, if this becomes loose, it can be quickly repaired under anesthesia.

The critical step of the procedure is the surgery, and it might take a while to master the technique. However, once achieved, unanesthetized chronic studies can be productively carried out with minimum problems. It is possible to operate on rats of 200-350 g regardless of strain or sex. Experiments on rats of different sizes and animals are also possible using flow probes of different sizes already prepared by manufacturers.

However, there are limitations and specific issues that one must pay attention to. First, surgery must be conducted using sterilized instruments, catheters, and flow probes to the extent possible to minimize post-surgical infections. Second, as the surgery is extensive and requires over an hour, a sufficiently long recovery period must be provided before obtaining "control" measurements for the study. This period in our laboratory generally extends from 7-10 days. Third, ileus (an occlusion or paralysis of the gut) has been a problem in some cases representing a postoperative complication. This can be prevented by avoiding exposure of the intestine (e.g., keep wrapped in moist gauze) during the procedure and avoiding closing the abdominal incision until the bond was well dried. It is essential to avoid exposing the intestine to the renal artery during surgery and ensure the intestine is not twisted when suturing. Fourth, it should be recognized that RBF will increase proportionally with increasing kidney weight. This must be considered in studies in which renal hypertrophy occurs following removal of the contralateral kidney. Fifth, we only have experience measuring RBF for up to a month and have not tried to extend measurements beyond this period. Since things were working well throughout this period in nearly all cases, studies could likely be extended many weeks beyond. Finally, a brief word about parallel arterial pressure measurements: the implanted fluid-filled catheters with dilute heparin to maintain 24 h/day patency and implanted telemetry devices are utilized. Each has advantages and disadvantages depending on the experimental design and needs. For example, blood sampling is possible from the arterial catheter if the catheter method is chosen, and heparinization is not required for the telemetry method. However, both have served us well during long-term measurements of RBF and BP.

ACKNOWLEDGMENTS:
This study was supported by grants for scientific research (P01 HL116264, RO1 HL137748). The authors would like to thank Theresa Kurth for her advice and help in maintaining the experimental environment as the lab manager.

DISCLOSURES:
The authors have nothing to disclose.

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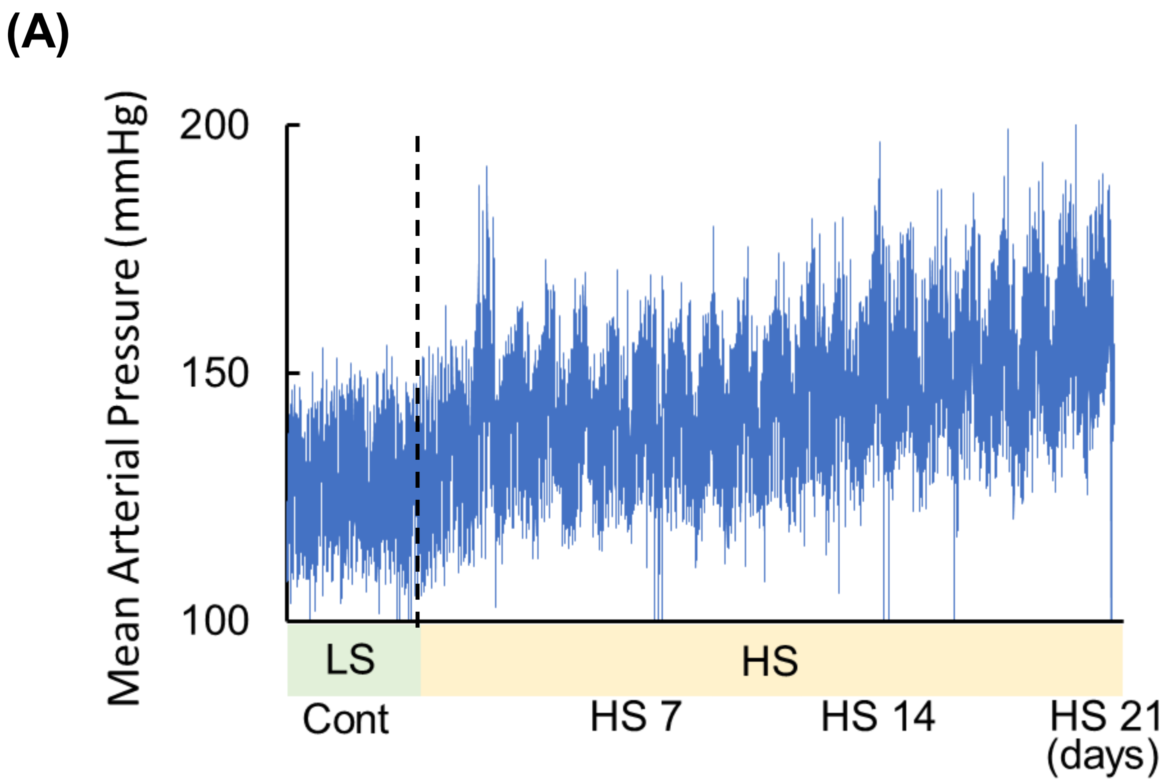
(A)
Mean Arterial Pressure (mmHg)
200
150
100
LS
HS
Cont
HS 7
HS 14
HS 21
(days)

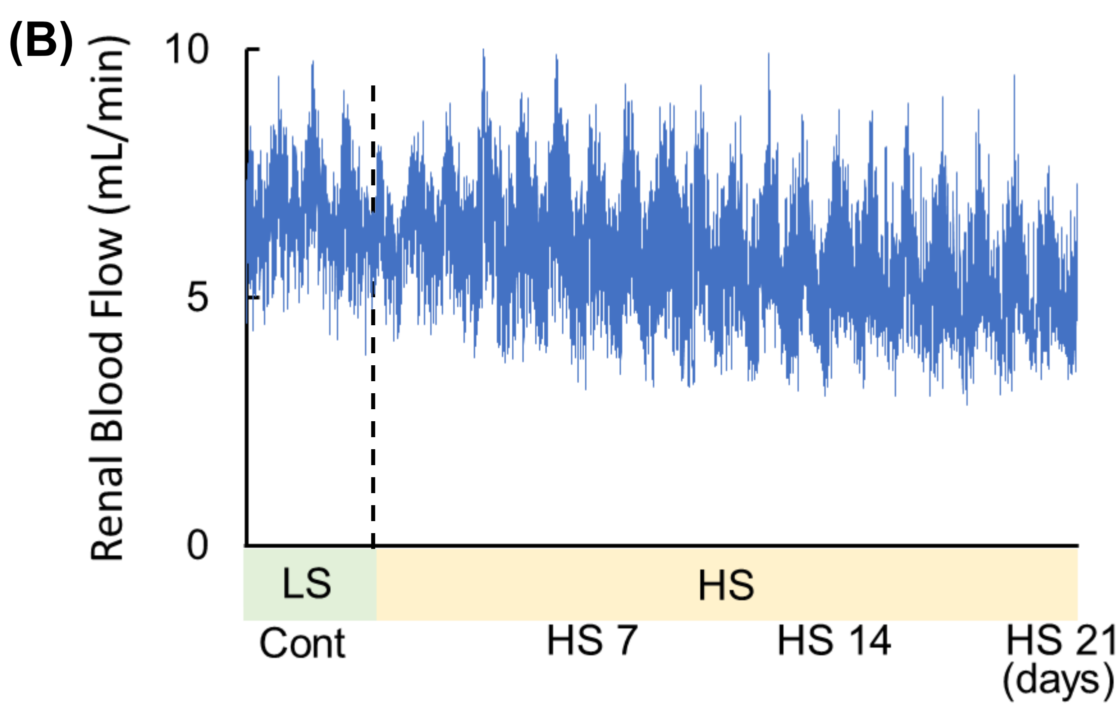
(B)
Renal Blood Flow (mL/min)
10
5
0
LS
HS
Cont
HS 7
HS 14
HS 21
(days)

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Table of Materials

63560_R1_Table of Materials_FINAL.xlsx

We wish to express our appreciation to the Editor and Reviewers for their insightful comments, which have helped us significantly improve the paper.

Editorial comments:

Editorial Changes

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.
2. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We added the statement. (Line 76-77)

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We removed personal pronouns from the protocol.

4. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We revised accordingly.

5. 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.

We revised all of the sentence to imperative form.

6. Please use SI units as much as possible and abbreviate all units: L, mL, µL, cm, kg, h, min, s, etc. Please include a space between all numbers and the corresponding unit: 50 mg, 100 mL, 37 °C, etc.

We did revision accordingly.

7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? The protocol text should provide a detailed description to enable the accurate replication of the

presented method by both experts and researchers new to the field.
Please specify all instrument used for performing dissection, incision, etc. E.g., scissors, scalpel, etc.
What the age, sex of the rat used for the study.

We did revision accordingly.

Step 1.1: Please provide the cage specification and how were the various systems installed and mounted in the cage.

The information is provided in the material tables. BASi Raturn includes cage and a movement response system.

Step 2.1.1: Is 200 to 350 weight of the rat? The statement is confusing. Also, if this is the rat weight, please provide the associated unit.

Yes, we clarified accordingly. (Line 97)

What is the percentage of isoflurane used? Please mention how proper anesthetization is confirmed. we clarified accordingly. (Line 98)

Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia. We don't use it.

What was the rat's position for surgery? How did you secure the rat in this position? we clarified the position. Since the position have to be changed frequently during the surgery, the position of the rat is not fixed. (Line 108)
Step 2.1.2: What region was shaved in the nape of the neck?

We clarified accordingly (Line 103)
Step 2.1.7: The probe was connected to the renal artery? If yes, how was this done? What gel was added? How was the flow rate measured?

We clarified the procedure and provided the information of the gel on the material file. (Line 121-124)
Step 2.2.2: How was the catheter plugging and fixing done? Please provide all associated steps.

We clarified the procedure (Line 139-140)
Step 3.1: During the recovery period, how often were the recordings done?

Recording don't have to be done during this period. We usually don't record it. (Measurement could be done without recording) (Line 161)
Step 3.2: How often is the heparinized saline infused in the rat body?

Heparin infusion is the continuous infusion throughout the study at the rate of 100 uL/hr. We clarified it. (Line 162)

8. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

We added it. (Line 158)

9. Line 163: Please provide reference for using the specific rat strain mentioned.

We clarified it (Line 169-170)

10. Please highlight up to 3 pages 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We highlighted the surgery procedure.

11. As we are a methods journal, please also discuss the critical steps within the protocol in the Discussion.

We added it. (Line 214-216)

12. Figure 1: Please use the unit mL/min instead of ml/min.

We revised it.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The method titled "A method to measure renal blood flow in rats chronically and continuously under the consciousness condition", by Satoshi et. al., is well described and optimized method for continuous monitoring of kidney blood flow in rats along with cardiovascular activity measurements for a long period of time. The method will be useful for several types of kidney related studies in future. I found this method attractive except the need of continuous heparin infusion in rats, which would make this method unsuitable for several types of studies including septic shock.

Thank you very much for the comment. Heparinization is required for the blood pressure measurement by catheter, but it is not required if the telemetry used. We clarified it. (Line 239-240)

For example, blood sampling is possible from the arterial catheter if we choose catheter method, and heparinization is not required for telemetry method.

Minor Concerns:
I would like to suggest following minor corrections in the manuscript.
Title suggestion - Since "consciousness" means conscious condition, I kindly suggest changing the title of the manuscript. Some of the suggested title are as follows –
1. A method to measure renal blood flow in rats chronically and continuously under conscious condition.
2. A method to measure renal blood flow chronically and continuously in conscious rats.

We revised the title as "A novel method of long-term continuous measurement of renal blood flow in conscious rats.".

Line 3 . Sentence suggestion for " Many acute studies have been carried in anesthetized…." - "Many acute studies have been carried out in anesthetized…" We revised it.

References are required for the sentence in the line 62-65.

We added references.
Line 104 - sentence suggestion for "After shaving, wipe the area with 70% ethanol, ….." - "After shaving, the area is wiped with 70% ethanol,……". Similarly, please check the sentences of line 107 to 126 and throughout the manuscript.

We revised all of the sentence to imperative form.
Thank you.

Reviewer #2:
Manuscript Summary:The authors developed an useful methodology to perform a continuous 24hr/day measurement of renal blood flow. In my opinion this manuscript is interesting and with high scientific value.

Minor Concerns:

I am of the opinion that the authors should discuss the age of the animal/size best suited for performing this procedure. I also think it is important to address the anatomical differences that exist between males and females in the discussion with regard to surgical approach.

Thank you very much for the comment. We added the description (Line 216-219)

It is possible to operate on rats of 200g to 350g regardless of strain or sex, and experiments on rats of different sizes and different animals should also be possible by using flow probes of different sizes that are already prepared by manufacturers.