Chapter 10. Experimental approaches for assessing renal function

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Rationale for model development

One of the most obvious functions of the kidneys is the elimination of waste materials that are either ingested or produced by metabolism. Another function that is especially critical for the overall functioning of the organism is the control of the volume and composition of the body fluids. In the case of water and virtually all electrolytes in the body, the balance between the intake and output is mainly ensured by the kidney. These functions are performed by filtration of the plasma, followed by reabsorption and secretion of different substances from and into the urine, according to the body’s needs. Blood flow to the kidneys amounts to about 22% of the cardiac output and about 20% of the plasma flowing through the kidneys is filtered through the glomerular capillaries. All renal diseases, irrespective of the primary cause, ultimately affect these variables, with ensuing kidney dysfunction. The primary targets of all therapeutic approaches for chronic kidney disease are: maintenance or restoration of Glomerular Filtration Rate (GFR) and Renal Blood Flow (RBF). These variables are also the major means of evaluating the overall renal function in kidney disease. While surrogate measures of these variables have been widely used for patients, direct and accurate determination of kidney function is required for
experimental models of renal disease. Therefore the ability to study these processes is at the basis of our understanding of the physiology and pathology of the kidney.

Model implementation

The concept of renal clearance

The concept of renal clearance is based on the Fick’s principle, or the conservation of mass, whereby the mass of a substance entering the kidney by the blood equals the mass that leaves the kidney. The output mass can be calculated as the product of the concentration of the substance in the urine ($U_x$) and the volume of urine excreted per unit of time ($V_u$). The input mass similarly equals the product of the concentration of the substance in the plasma flowing through the kidneys ($P_x$) and the volume of plasma. Therefore, renal clearance ($C_x$) is the volume of plasma that is cleared of a certain substance by the kidneys, per unit of time.

$$C_x = \frac{U_x \times V_u}{P_x}$$

The renal clearance represents a measure of the kidney’s ability to remove a substance from the blood and eliminate it into the urine. It becomes obvious from the considerations above that in order to determine the precise amount of glomerular filtrate, for example, it is sufficient to measure the concentration of a certain substance dissolved in the plasma and the urine. These fluids are readily accessible for sampling and do not require direct access to the complex renal structures. In addition, this approach allows the evaluation of the overall renal function, without the limitation to a unique nephron, as in the case of renal micropuncture experiments.
Glomerular filtration rate
In order to determine the volume of plasma that is filtered through the glomerular capillary membranes, the clearance concept can be applied, with certain characteristics of the substance studied. Ideally, such a substance must be:

- Biologically inert
- Easily and precisely quantifiable in the plasma and urine
- Freely filtrated by the glomerulus
- Not reabsorbed or secreted by the renal tubules

Therefore, the clearance of such a substance directly yields the glomerular filtration rate, since the filtered quantity equals the quantity excreted in the urine. The most widely used substance that fulfills these requirements is Inulin, a fructose polymer mostly extracted from chicory. GFR can be calculated as follows, based on urine and plasma samples, and measurement of the concentration of Inulin:

\[ GFR = \frac{U_{ln} \times V_u}{P_{ln}} \]

The concentration of inulin can be measured using a variety of techniques, but given the very small sample size in the case of the laboratory rat or mouse, the techniques with the highest accuracy are preferred. Accuracy is usually achieved by labelling the Inulin molecule with either radioactive (most accurate) or fluorescent (more convenient) tags. Tritiated Inulin (³H-Inulin) is state-of-the-art, notwithstanding the cumbersome radioactive regulations, due to the ease and precision of measurement of radioactivity by liquid scintillation counting. Fluorescein isothiocyanate (FITC) – tagged Inulin is also available, but its use is somewhat limited by the accuracy of the method used to measure low amounts of fluorescence.
Experimental models in rodents

Renal blood flow
In the same context of the clearance concept, a substance that is so efficiently excreted by the kidneys so that virtually the whole amount that enters the kidney per unit of time is excreted into the urine, can be used to estimate the volume of plasma that enters the kidney in a given time interval. This represents the Renal Plasma Flow (RPF), which can then be corrected for Hematocrit to obtain Renal Blood Flow (RBF). Such a substance is the \textit{para-aminohippuric acid} (PAH), an organic acid that is not produced in the body. PAH is freely filtered by the glomeruli, but most importantly for its use as an indicator of RPF, it is also actively secreted by the tubules, once it reaches peritubular capillaries. Therefore, at moderate plasma concentrations, PAH is completely excreted into urine, once it reaches the kidneys via the renal artery. Its clearance equals RPF:

$$RPF = \frac{U_{PAH} \times V_u}{P_{PAH}}$$

Experimental setup for measurement of renal hemodynamics: GFR and RBF
In order to ensure accuracy, the experiment requires extensive surgical approaches and a long duration and is therefore a terminal procedure. The anesthetic of choice is usually a barbiturate, which confers a constant level of anesthesia for at least 120 min, and can be supplemented easily if the experiments require a longer time. Thiobutabarbital (Inactin\textsuperscript{®}) fulfills these criteria and can easily be used in the laboratory rat. It is administered by intraperitoneal injection (110 mg/kg) and anesthesia installs in about 20 min. Alternatively, gas anesthesia can be used, but requires relatively laborious administration for extended periods of time.
Once the animal is anesthetized, hair is removed from the neck, abdomen and posterior limbs and the areas prepared for surgical incisions. Sterile techniques are not a must since the procedure is terminal, but antiseptic techniques are recommended to prevent bacteriemia, with consequences upon the variables measured. The animal is placed in supine position on a heated surgical table or pad, and the temperature is monitored with a rectal probe. It is essential to maintain body temperature constant throughout the experiment, in order to obtain accurate measurements in the physiological state. Ideally, a self-regulating feedback-controlled heating pad can be used, so large variations in body temperature due to the surgical procedures and exposure of the abdominal cavity are not a concern for the investigator.

The first surgical step is to perform a tracheostomy (Fig. 1). This is necessary because respiratory distress due to accumulation of fluids in the upper respiratory tract is a frequent occurrence during this experiment. A midline incision in the neck region, extending from the gonion to approx. 0.5 cm above the sternum for a 250 g rat suffices to expose the neck muscles. The sternohyoideus muscle fibers are gently pulled apart using non-toothed forceps and the trachea underneath is exposed. A small incision (1-2 mm) is made between 2 cartilages and a large piece of tubing (external diameter similar to the trachea) is inserted downwards for about 0.5 cm. Care must be taken for the tube not to reach the tracheal carina. Flow of air is observed by fogging of a mirror placed at the cranial end of the cannula. The cannula is finally secured in place using 2-0 silk thread tied around the trachea distally to the incision and over the cannula.
The same incision can be extended transversally to expose the external jugular vein on either side. Alternatively, another paramedial incision is performed. The external jugular vein is apparent as a bulging dark blue vessel running cranially from the mid-clavicular region (Fig 2). After isolating the vein, a small incision in the vessel wall is made using iris scissors and a catheter is introduced caudally no more than 0.5 cm (care must be taken that the catheter does not reach the right atrium).
The catheter is constructed by fusing a larger diameter tube (PE-50) with a smaller diameter one (PE-10), using either heat or cyanoacrylate glue (Fig. 3). After insertion, the catheter is secured in place with a distal and a proximal suture.

Fig. 2. External jugular vein exposure (left); final aspect with jugular vein catheter and tracheostomy secured

Fig 3. General construction of dual-size polyethylene catheters for venous and arterial insertion in major vessels in the rat
Next, both the femoral artery and vein are cannulated in the same manner. The vessels are accessible through an inguinal incision and must be separated from the sciatic nerve and from each other using cotton-tipped swabs. The thinner part of catheter used for insertion in the artery must be longer than the venous one, in order to be advanced into the lower aorta, for accurate blood pressure recording.

Then the left kidney is exposed through a midline abdominal incision. The left ureter is identified starting from the renal pelvis running downward on top of the retroperitoneal fat. A magnifying glass of surgical microscope may be necessary at this step. The ureter is transparent and very thin and the only distinguishing feature is the peristaltic movement. The ureter is then cannulated using a thin catheter (they are rarely available in this small outer diameter; it can be constructed by heating and pulling a polyethylene PE-10 catheter). Care must be taken during manipulation of the catheter with the microsurgical forceps as excessive maneuvering will enhance peristaltism and even lead to powerful segmental contraction, which will make the lumen inaccessible for catheterization (Fig. 4).

![Fig. 4. Catheterization of the left urether. Combined experiment using selective ligation of the lower polar artery (right)](image_url)

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In summary, the surgical preparation for the experimental measurement of renal function involves:

- Tracheal cannula
  - Monitor respiration during the procedure
- Jugular vein catheter
  - Will be connected to an automated infuser delivering $^3$H- Inulin in saline solution at a rate of 0.5 mL/h. The concentration of radioactive inulin will be adjusted according to the total activity of the supplied substance. PAH can be added to this solution for measurement of RBF
- Femoral vein catheter
  - Used to infuse artificial rat plasma, composed of Bovine serum albumin (2.5g/100mL) + Bovine Immunoglobulins (2.5g/100mL) in 0.9% NaCl. The rate of this infusion is 12.5mL/kg/h for the first 45 min, thereafter 1.5 mL/kg/h, throughout the experiment, in order to maintain euvolemia. If this solution is not used, the preparation will become hypovolemic due to liquid loss through evaporation from the large abdominal incision and the loss of upper respiratory humidification. The lost fluids cannot be simply replaced with saline because loss of plasma proteins in the extravascular space due to increased capillary permeability will osmotically attract further fluid from the circulatory system.
- Femoral artery catheter
  - Is connected to a pressure transducer for continuous monitoring of blood pressure, one of the major determinants of GFR.
- Urethral catheter
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- used to perform timed urine collections for measurement of urine Inulin and PAH concentrations.

An equilibration period of about 45 min is necessary before the samples required for calculation of GFR and RBF are taken. This equilibration period is necessary for the Inulin and PAH to reach constant plasma concentrations. As noted above, time invariability of the plasma concentrations of either substances for which clearance is calculated, is absolutely required throughout the experiment.

For the actual measurement of GFR and RBF, at least 2 timed clearance periods lasting 20 min must be used. At the beginning of each period, a fresh plastic tube is used to collect the urine flowing through the urethral catheter (Fig. 5). Given the low urine flow in the rat, and the status of the animal, the volume collected will normally not exceed 200 microliters so the volume should be estimated by the weight difference of the tube before and after collection, using a laboratory balance with precision of < 0.1 microgram. In the middle of each 20-min clearance periods, the arterial line is disconnected and blood is allowed to freely flow out into microhematocrit tubes (2/ measurement, approx. 20 microliters).

Finally, the concentrations of Inulin and PAH in the urine collected over each 20 min clearance periods are determined and the values used for calculation of GFR and RPF, using the formulas above. For estimation of RBF, the RPF values are multiplied by 1-Hematocrit (expressed in absolute value, e.g. 0.47 = Hematocrit of 47%).
Issues and troubleshooting

- If the experimental setup above is used to test the effects of different substances administered acutely to the animal, the duration of the experiment can exceed 2 hours (not including preparatory and equilibration steps). It is possible that euvolemia cannot be maintained throughout
this time, as manifested by a gradual reduction of arterial pressure. The rate of artificial rat plasma can be increased for short periods of time (~ 10%), and bolus 1-2 mL saline administered. However, if blood pressure is not constant throughout a whole 20 min clearance period, GFR values must be interpreted with caution.

- Anesthesia can be supplemented if signs of loss of anesthetic level are noticed: shallow breath, reaction to tail pinch or corneal reflex. Anesthetic supplements should be titrated in steps of 10-20% of the initial dose.
- If major differences are found in the plasma concentration of Inulin or PAH between the two clearance periods, another clearance experiment has to be performed. This means that the infusion rate did not match the renal excretion and the plasma values taken at a single time point do not reflect the concentration throughout the clearance period, thus confounding the results.

General experimental considerations

- The measurement of RPF using PAH is based on the assumption that all the PAH that enters the kidney per unit of time is completely excreted into the urine. However, due to the fact that not all blood that enters the kidney passes through peritubular capillaries to allow for active PAH secretion, a small amount (< 10%) will eventually return into the circulation via the renal vein. This means that the extraction ratio of PAH is slightly less than 1 and this method underestimates RPF by a factor of < 10%. To overcome this limitation, direct measurement of blood flow through the renal artery is possible using transit-time ultrasound flow probes placed around the renal artery (Figure 2)
The disadvantages of using the techniques described in this chapter mainly relate to the extensive surgical procedures involved and the difficulty to maintain the physiological status of the animal during the procedure. Based on the same clearance principles, the techniques can

**Fig. 2.** Upper panels: Transit-time perivascular flow probe for rat artery. (© Transonic Systems, Inc., www.transonic.com). Lower panel: Representative recording of blood pressure (upper trace) and Renal blood flow (lower trace) in a rat subject to clearance experiments.
be adapted so that urine sample collection is not required. This approach is based on the assumption that after the equilibration period, there is a precise and constant balance between the infusion rate of either inulin or PAH and its elimination in the urine. Therefore, urinary output of these substances can be replaced with the actual rate of infusion.

- When the above condition cannot be fulfilled, due to intra-experimental variations in volemia, blood pressure, etc., a single-injection technique can be used. This involves bolus injection of Inulin or PAH and timed blood sampling. The area of the curve under the decreasing concentrations of the substance over time can be used to interpolate its excretion rate.

References:
