Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63167R1
Full Title:	Microsurgical Skills of Establishing Permanent Jugular Vein Cannulation in Rats for Serial Blood Sampling of Orally Administered Drug
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TITLE:

Microsurgical Skills of Establishing Permanent Jugular Vein Cannulation in Rats for Serial Blood Sampling of Orally Administered Drug

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KEYWORDS:

JVC model, blood vessel, catheter implantation, blood collection, animal care, physiological monitoring, hematological test, pharmacokinetics, natural phenol

SUMMARY:

Detailed microsurgical techniques are demonstrated to establish a longer-term jugular vein cannulation rat model for sequential blood collection in the same animal. Physiological and hematological parameters have been monitored during the rat's recovery phase. This model has been applied to study pharmacokinetics of orally administered polyphenol without inducing animal stress.

ABSTRACT:

Blood sampling in small laboratory animals is necessary for pharmaceutical lead optimization but can cause great harm and stress to experimental animals, which could potentially affect results. The jugular vein cannulation (JVC) in rats is a widely used model for repeated blood collection but requires adequate training of surgery skills and animal care. This article details the microsurgical procedures for establishing and maintaining a permanent JVC rat model with specific focus on the placement and sealing of the jugular cannula. The importance of monitoring physiological (e.g., body weight, food, and water intake) and hematological parameters, was highlighted with results presented for 6 days post-surgery during the rat's recovery. The drug-plasma concentration-time profile of orally administered natural phenol ellagic acid was

determined in the JVC rat model.

INTRODUCTION:

Repeated acquisition of blood samples from small laboratory animals, such as rodents, guinea pigs, and rabbits, is an important aspect for pharmaceutical lead optimization and also for reducing the number of animals used in research[1,2]. The pipeline for developing new diagnostic tools and drug formulation (e.g., vaccine) requires access to different volumes of blood in order to evaluate their robustness and performance *in vivo*, such as pharmacokinetics (PK), toxicity, and sensitivity[3–5].

The laboratory approach to blood sample collection is broadly classified into two types, surgical and nonsurgical[6]. The nonsurgical approach is relatively easy to grasp for the researcher, which includes common techniques, such as cardiac puncture, orbital sinus puncture, and bleeding of the saphenous and tail vein. Multiple blood sampling is possible by some non-surgical methods, but the sample volume is small and can cause physical wound and psychological stress to the animals[1]. On the other hand, the surgical approach is a favorite alternative to repeated venipuncture, and it involves placement of a temporary or permanent cannula in the blood vessels of animals[7–9]. The large blood volume could be repeatedly withdrawn through the cannula in conscious rats while avoiding the stress and pain due to the handling technique, restrain, and anesthesia[7,8,10,11]. However, the cannula implantation requires an experienced researcher with adequate training in order to successfully collect the blood.

Blood collection through jugular vein cannulation (JVC) in rats is the most widely used method to study the drug PK[6,10,12,13]. Yet, establishment of the JVC rat model needs careful practice of microsurgical skills and knowledge of postsurgical care and maintenance. Especially, after the surgery, the rat requires administration of analgesics and sufficient recovery time to reach stable physiological condition for further experiments[13–15]. Although the body weight gain (i.e., >10 g) is a valid and commonly applied indicator for the rat's recovery, it is not uncommon that the rats have unexpected death postoperatively due to dehydration, infection, and inflammation, which could be subtle to notice at the early onset[14,15]. In addition, catheter obstruction in the JVC model remains to be an issue during the blood collection.

The present protocol has demonstrated in detail the microsurgical procedures for JVC in an anesthetized rat with specific focus on the identification, isolation, and cannulation of the jugular vein. The importance of physiological and hematological monitoring of the rats during the recovery phase is highlighted. Finally, serial blood samples were collected through the venous catheter to study the PK of the orally administered natural phenol ellagic acid with poor bioavailability (i.e., low systemic concentration) to verify the JVC rat model.

PROTOCOL:

The procedures described below were performed as part of a protocol approved by the Institutional Animal Care and Use Committee of Northwestern Polytechnical University (No. 202101117).

1. Preoperative preparation (the day before the surgery)

NOTE: Required solutions: normal saline (0.9% w/v sodium chloride), heparinized saline (1% w/v heparin sodium), catheter lock solution, non-steroidal anti-inflammatory drug (NSAID), and meloxicam solution (2 mg/mL).

1.1 Solution preparation

1.1.1. Aliquot 200 µL of pre-packaged catheter lock solution in a 1.5 mL sterile microcentrifuge tube.

NOTE: Catheter lock solution is composed of heparinized saline (0.4% v/v heparin sodium) mixed with glycerol (v/v,1:1).

1.1.2. Mix 1 g of heparin sodium in 100 mL of the normal saline to prepare 1% heparinized saline.

1.1.3. Dissolve meloxicam in normal saline to prepare a 2 mg/mL concentration solution for postoperative pain relief.

NOTE: Prepared heparinized saline and meloxicam solution are filtered through a 0.22 µm filter. All the solutions are sterilized and stored at 4 °C for future use.

1.2. Surgical instruments and materials

1.2.1. Pack all clean surgical tools in a pouch and tape it with a piece of autoclave sterilization tape. Refer to **Figure 1A** for the specific surgical instruments used.

1.2.2. Autoclave the surgical pouch at 121 °C for 30 min for the next day use.

1.3. Animal preparation

1.3.1. Prior to the surgery, house all male Sprague-Dawley (SD) rats in the standard Animal Room with controlled temperature at 22 ± 1 °C. Feed them with the standard laboratory food and water *ad libitum* for at least 7 days.

NOTE: Both male and female rats can be used for the JVC model, and their typical ages and body weights vary from 9–14 weeks and 294 ± 57 g, respectively.

1.3.2. Anesthetize the rat with 3%–3.5% isoflurane mixed with oxygen in a pre-anesthesia chamber. Determine whether the rat becomes unconscious by the lack of response to foot pinch.

1.3.3. Gently take the rat out, place the rat's nose into an anesthetic nosepiece supplying 2%–2.5% isoflurane.

1.3.4. In the ventral and dorsal position, shave the fur thoroughly around its right shoulder and posterior areas of neck with a pet razor. Return the rat to the cage for surgery to be performed on the next day.

2. Before the surgery on the day

2.1. Prepare the aseptic workstation

2.1.1. Spray 75% medical alcohol to disinfect the operation area, and then place the heating pad covered with a clean cushion. Set the LED lamp with a cold light source beside the workstation.

2.1.2. Pre-warm the required solutions (step 1.1) to room temperature.

2.1.3. Fill 0.6 mL of heparinized saline and 0.15 mL of catheter lock solution in two sterile 1.0 mL blunt tipped syringes, respectively. Withdraw 2.5 mL of the normal saline using a sterile 5.0 mL syringe.

2.1.4. Soak the 6-0 sterile non-absorbable silk suture thread and cotton balls in 75% medical alcohol. Squeeze out excess ethanol before use.

2.1.5. Weigh and record the rat's body weight.

3. During the surgery

3.1. Surgical preparation

3.1.1. Wear the surgical coat, sterile gloves, and facial mask. Then open the sterilized surgical pouch, leave all surgical tools in 75% medical alcohol, and dry them before use.

3.2. Jugular vein isolation

NOTE: The estimated operation time for this part is 10 min.

3.2.1. Anesthetize the surgery-ready and shaved rat with 3%–3.5% isoflurane mixed with oxygen in the pre-anesthesia chamber and determine whether the rat becomes unconscious by the lack of response to foot pinch.

3.2.2. Place the rat's nose into the anesthetic nosepiece supplied with 2%–2.5% isoflurane for adequate anesthesia.

3.2.3. Using adhesive tape, restrain the rat's forearms in their ventral position to each side of the surgical platform.

3.2.4. Gently scrub the surgical area by alternating between cotton balls soaked in 75% medical alcohol and iodine-based scrub for a total of three times.

3.2.5. Carefully lift the skin near the clavicle on the right side of the midline of the neck with forceps and make an incision towards the chest about 1.5–2.0 cm in length with a pair of surgical scissors.

3.2.6. Blunt dissect the thin tissue cover with iris scissors to expose the underneath jugular vein. The proximal cephalic end of the external jugular vein consists of two branches, which can be visually identified.

NOTE: Depending on the age and sex of the rat, the soft tissue (e.g., salivary glands, lymphatic nodes, and fatty tissues) covering the jugular vein varies. Compared to the young rats, the old rats are fatter (e.g., BW > 300 g), and thus need more tissue separation before the jugular vein is visible.

3.2.7. Lift the jugular vein along with its connective membranous tissues to visualize the lymph gland attached to the jugular vein. Carefully separate the vein along the vascular direction from surrounding muscle, fat, and other tissues.

3.2.8. Nudge the forceps under the jugular vein without damaging the collateral blood vessels and pass two pieces of 6-0 suture under the vein to mark the two ends of the blood vessel individually.

3.2.9. Pull one piece of the suture as far as possible toward the rat head and ligate the vein cranially with 2–3 knots using forceps.

3.2.10. Place the second ligature on the caudal end of the vein with 1 loose knot.

3.3. Jugular vein cannulation

NOTE: The estimated operation time for this part is 15 min.

3.3.1. Open the package containing 11 cm polyurethane (PU) catheter (I.D. 0.6 mm x O.D. 0.9 mm, **Figure 1B**) and attach the catheter to the prepared blunt tipped syringe filled with the heparinized saline.

3.3.2. Slowly push the heparinized saline into the catheter to avoid air bubbles.

3.3.3. Nudge the non-tip flat side of the forceps under the jugular vein to exit on the other side. Make a small v-shaped cut on the vein near the cranial tie with a pair of castroviejo micro scissors and gently open the incision with the tip of the elbow vessel dilator forceps.

NOTE: Rinse the incision with pre-warmed normal saline (37 °C) if a small amount of blood gushes

out.

3.3.4. Cut out the oblique opening of the front end of the jugular vein catheter. Clamp the oblique end of the tube with forceps and slide it into the jugular vein.

NOTE: This step may need another person to facilitate the catheter sliding.

3.3.5. While advancing the catheter, slowly withdraw the elbow microsurgical forceps and clamp the outer surface of the vessel with forceps.

NOTE: If the right blood vessel is selected and the tip of the catheter is successfully slid into the blood vessel, the entire catheter insertion process should not feel any flow resistance.

3.3.6. Stop inserting the catheter upon hitting the first blue mark of the PU tube (**Figure 1B**), which is approximately 3.0 cm in length.

3.3.7. Secure the inserted catheter to the vein with both caudal and rostral ligatures using forceps.

3.3.8. Thread a 6-0 suture through the exposed tissue on the right side of the incision using a suture needle (1/2 curved cutting, 12 mm) and tie the ligature with a hemostat.

3.3.9. Bend the catheter at the second blue mark (**Figure 1B**) to bind with the same ligature (in step 3.3.8) and to avoid occluding the PU-tubing.

3.3.10. Snip all the extra suture thread and close the catheter by replacing the blunt tipped syringe with a 22 G stainless-steel plug.

3.4. Catheter exteriorization

NOTE: The estimated operation time for this part is 10 min.

3.4.1. Place the rat in the dorsal position and gently clean the area between the scapulae with the cotton ball soaked in 75% medical alcohol.

3.4.2. Make a very small incision at the center of the dorsal neck with surgical scissors. Through the dorsal incision, guide and gently push the trochar underneath the skin toward the ventral incision on the right side of the neck.

3.4.3. Put the venous catheter into the trochar and then pull out and guide the venous catheter toward the dorsal incision.

3.4.4. Secure the exteriorized catheter into the muscle layer in the same way with the suture (see the procedure in steps 3.3.8 and 3.3.9).

3.4.5. Close the skin layer of ventral and dorsal incisions with the suture needle (3/8 curved cutting, 17 mm). Swab all surgical incisions with iodophor.

NOTE: The wound-clips is an alternative method to close the skin incision.

3.4.6. Remove the catheter plug by clasping the catheter with fingertips. Place a new blunt tipped syringe and slowly draw back the syringe to test the blood flow.

NOTE: Since the rat is in the supine position, one may not be able to obtain blood samples. Blood samples could be obtained by changing to a side body position.

3.4.7. Hold the catheter again with fingertips and inject 0.1 mL of lock solution into the catheter using the blunt tipped syringe.

3.4.8. Hold the catheter with fingertips and replace the syringe with a stainless-steel plug. Unclamp the catheter and push the plug slightly in to ensure the tightness of the catheter.

4. Immediate post-surgical care

4.1. Subcutaneously inject (*s.q.*) meloxicam solution at a dose of 2 mg/kg immediately after the procedure in step 3.4.6 and terminate the anesthesia.

NOTE: Select analgesics which avoid potential drug-drug interactions according to the drug compound of interest used in the future study.

4.2. Recover the rat in the dorsal decubitus position by caging it individually with fresh corncob bedding. Often, provide a temperature-regulated heating pad to maintain the core body temperature.

NOTE: For animal welfare, leaving food and water on the bedding is an effective way to alleviate the pain caused by neck movements when eating and drinking.

4.3. Record the end time of the surgery and monitor the rat at 2 h intervals for at least 4 h. Provide additional analgesia for the recovery if the rat shows signs of pain or distress.

5. Physiological and hematological monitoring during recovery phase

5.1. Monitor the body weight and the food and water intake daily and record the data. For hematological test, collect a small volume of fresh blood at the same time period every day for 6 consecutive days.

5.2. Place the rat in a stainless-steel restrainer 1 h after the surgical intervention. Open the plug and insert the syringe into the venous PU catheter to ensure the catheter is not obstructed.

5.3. Discard the initial withdrawn blood, which contains a mixture of blood, heparinized saline, and catheter lock solution.

5.4. Use a new syringe to collect 150 µL of fresh blood sample and transfer the blood sample to the 0.5 mL tube containing K2EDTA (1.8 mg/mL blood) spray dried on the tube wall.

NOTE: If the catheter is blocked, 0.2 mL of heparinized saline can be injected into the catheter to flush the catheter a few minutes before the next blood collection time.

5.5. Inject sterile saline in the same volume to compensate for the withdrawn blood. Inject 150 µL of pre-warmed normal saline (37 °C) and infuse 0.2 mL sterile heparinized normal saline through the catheter.

5.6. Inject 100 µL of the lock solution into the catheter to ensure the sealing and sterility of the catheter before the next sample collection.

5.7. Analyze the blood samples within 2 h of collection using an automated blood cell counter.

6. Repeated blood sampling for pharmacokinetic studies of oral administered drug

NOTE: Rats with weight gain >10 g and stable hematological level are suggested to be enrolled for future study. Following the current protocol, the JVC rat model is ready to use 4–6 days post-surgery.

6.1. After 4–6 days of surgery, fast the rat for 12 h with free access to water.

NOTE: Depending on the experimental goal, fasting the animal is optional.

6.2. Orally gavage the fasted rat with natural phenol bioactive ellagic acid at a dose of 6 mg/kg with a straight gavage needle[16].

6.3. Collect 200 µL of blood samples in the heparinized tubes *via* the jugular vein cannula at pre-determined time points over 24 h post-oral administration. The blood collection process follows the procedure in step 5.5.

NOTE: The catheter does not need to be closed with the lock solution until the blood collection is completed.

6.4. Immediately centrifuge the blood sample at 3000 x *g* at 4 °C for 10 min.

6.5. Analyze the extracted plasma sample by liquid chromatography-mass spectroscopy[17,18].

REPRESENTATIVE RESULTS:

This protocol has thoroughly demonstrated how to establish a long-term JVC model using microsurgical skills for serial blood collection. **Figure 1A** shows the essential surgical instruments and materials used to conduct the surgery. The specification of PU catheter with three blue marks is also illustrated, which is helpful for guiding the researcher to place the vein cannula in step 3.3., how to use the marks on the PU catheter to guide the cannulation (**Figure 1B**). It is also important to be aware of the timeline required to establish the JVC rat model (**Figure 1C**). Although the operating time for the JVC is approximately 35 min, if the researcher is skillful, it takes 10–14 days (the adaptation and recovery phase) for the JVC rat model to be ready for use, compared to the non-surgical approach, such as the tail snipping or orbital sinus puncture, which can be used immediately with proper training.

The physiological and hematological conditions over 6 days postoperatively was also investigated (**Figure 2**). The rat's body weight gain, food and water intake, and complete blood cell count were variable during the recovery phase (**Figure 2A,B**). It was found that the majority of rats under the present study condition recover within 4–6 days post-surgery as evidenced by restored levels of some key features, such as body weight gain >10 g, regular diet intake, and selected blood components relating to infection, dehydration, and inflammation, including white blood cell count, red blood cell count, hemoglobin and platelet count (**Figure 2C–F**). It is worth noting that the amount of water intake in rats was relatively large on the first day post-operation, indicating dehydration.

Pharmacokinetics of the natural polyphenol, ellagic acid was studied in the established JVC rat model (**Figure 3**). The ellagic acid is characterized with poor drug bioavailability. When administered in a low dose (e.g., 6 mg/kg), a large volume of blood sample is required to detect its concentration in the plasma. **Figure 3** shows low plasma-concentration ellagic acid concentration in ng/mL over 24 h and its varied gastro-intestinal tract (GIT) absorption owing to its poor solubility and permeability.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the main surgical instruments and supplies used for JVC rat model establishment. (**A**) Top: a-d is normal saline, iodophor, plastic ware, spray bottle with 75% medical alcohol, respectively; Middle: e-o is 5.0 mL syringe, 1.0 mL syringe, blunt tipped syringe, sterile cannula, surgical scissors, iris scissors, half-curved forceps, vessel dilator balanced forceps, castroviejo micro scissors, stainless steel trochar, pet razor, respectively; bottom: p-w is cotton swabs, 6-0 sterile non-absorbable silk suture thread, cotton balls, two types of suture needle, stainless steel plug, curved hemostat, adhesive tape, anesthetic nosepiece, respectively. (**B**) Specification of the PU catheter used for cannulation of jugular vein in rats. The catheter is 11 mm in total length with O.D 0.6 mm x I.D 0.9 mm. The catheter has three blue marks to serve as an anchor point during the cannulation; (**C**) Suggested timeline of establishing JVC rat model. In this study, the rat's body weight, as well as the food and water intake, were recorded daily during the recovery phase, and blood samples were collected once daily for routine hematological monitoring.

Figure 2: Physiological and hematological monitoring of rats over 6 days post-operatively. (**A**)

Body weight change; (**B**) The change in water and food intake; (**C–F**) White blood cell count, red blood cell count, hemoglobin, and platelet count, respectively. The data represent the mean ± SEM with n = 6. The numeric values in blue represent the mean value.

Figure 3: Plasma ellagic acid concentration-time profiles of rats over 24 h after oral gavage. The data represent the mean ± SEM with n = 3. The values of PK parameters are obtained using add-in program PKSolver in a spreadsheet software (e.g., Microsoft Excel)[19]. Cmax: peak concentration, Tmax: time to reach Cmax; AUCinf: area under the plasma concentration-time curve from time zero to infinity.

DISCUSSION:

Mastering the technique of vessel cannulation requires significant practice and learning the lesson from each operation. Christakis et al. using cumulative sum (CUSUM) analysis, found that a researcher needs to practice 200 rats over a period of one year before being ready for the PK evaluation of drug candidates[20]. Yet, the operating time required for the vein cannulation can be significantly reduced by the number of rats performed[13,20]. Using our protocol, the success rate of effectively cannulating the jugular vein and collecting the blood sample increased from approximately 50% to above 80% (total rats performed were 15), and the initial operating time was reduced to 35 min from 2 h.

The demonstration of establishing a JVC rat model involves several critical steps. Firstly, the incision area around the neck is important for initially locating the jugular vein. If the right JVC is performed, the incision area is generally selected on the upper side of the clavicle along the right side of the neck midline (see section 3.2 jugular vein isolation). Secondly, JVC depends on preparation of a clean segment of the vein. Upon blunt dissection of soft tissue, the jugular vein is visible and identified by these two features: 1) two branches at the proximal end, and 2) a lymph node attached to it. Thirdly, while sliding the catheter into the jugular vein (see section 3.3 jugular vein cannulation), trimming the front end of the catheter, and supporting the blood vessel with steady external force could greatly improve the success rate of cannulation. Moreover, proper analgesia and heat must be provided for comforting the rat, as stress and pain can cause alterations in animal's behavior that may influence their post-operative recovery. Lastly, the duration of anesthesia, heat loss, and the complication can cause unexpected rat death; thus, it is important to closely monitor the rats during and after the surgery for at least 3 days. Evaluation of multiple health indicators, such as the body weight gain, diet, and drinking status, and hematological components of rats during the recovery period, could provide information that can be compared with reference values of interest of healthy SD rats in the database[21–24]. Most rats gain their body weight (e.g., >10 g) by day 3 post-surgery and thus, should be ready for use. Yet, for studies involving blood biomarkers evaluation (e.g., leukocyte, cytokines), it is recommended to enroll the rats by day 4–6 post-surgery, to ensure the normal hematological indexes for rats.

Despite its usefulness in PK study, depending on the catheter materials, not all drug candidates are suitable for the single cannulation. Gaud et al. found high log P compounds were bound to the PE catheter material, resulting in altered PK[25]. In addition, the analgesics (e.g., meloxicam) is

often applied to reduce the pain in rat post-surgery. Considering the elimination half-life of meloxicam is around 19–23 h[26,27], the single dose of meloxicam (2 mg/kg) injected *s.q.* is almost cleared out of the body after 24 h. Yet, potential drug-drug interactions can occur in use of meloxicam. It is likely that multiple doses of meloxicam for several consecutive days may cause liver injury and transiently elevate the aminotransferase[28]. Additionally, meloxicam can compete with other drugs for Cytochrome P450 metabolism[29,30]. Thus, the dose and type of analgesics selected should be screened depending on the drug chosen for the study.

In conclusion, this protocol has thoroughly demonstrated how to establish a long-term JVC rat model for blood collection at the laboratory setting and to investigate the physiological status of rats during the postsurgical recovery phase. The highlighted vital surgical steps and experiences could be helpful for the researcher to efficiently achieve the application of the cannulation model.

ACKNOWLEDGMENTS:

This work is supported by National Natural Science Foundation of China (No. 82003692) to R.X. Zhang; Top Academic Scholarship at Northwestern Polytechnical University to R. Miao.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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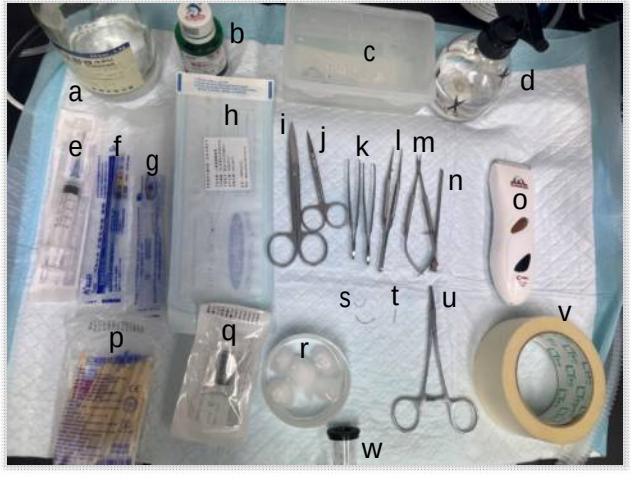
A



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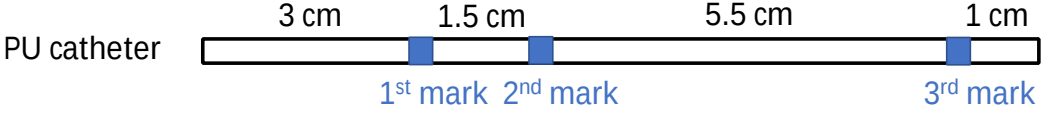

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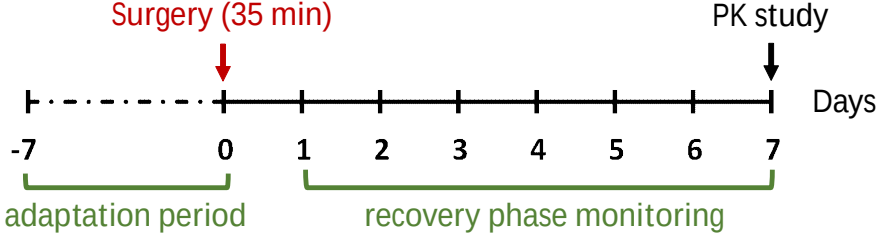

Figure 2

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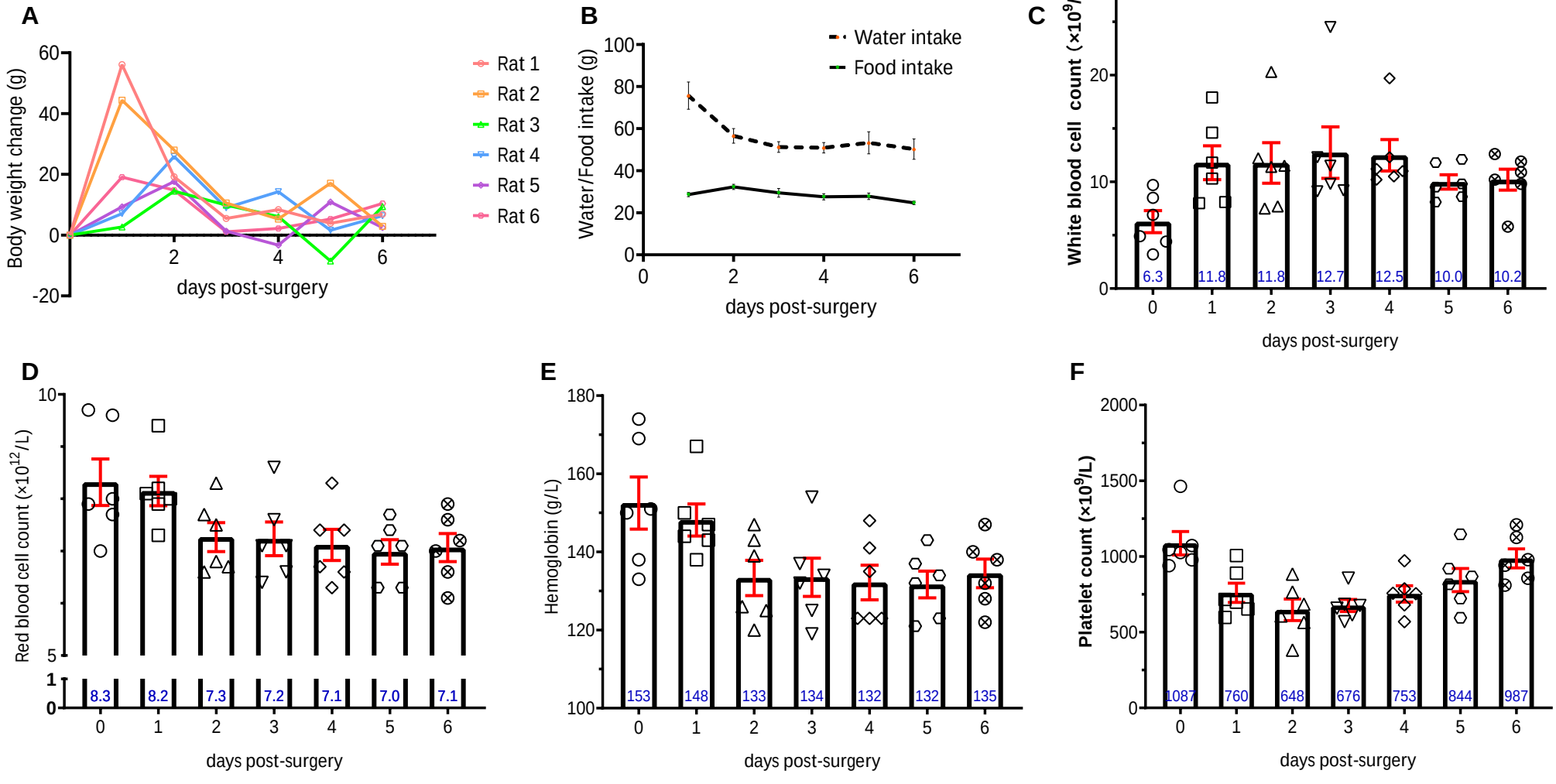

Figure 3

PK Parameters	Treatment
	Ellagic acid (6 mg/kg)
C_{max} (ng/mL)	29.207
T_{max} (h)	0.504
AUC.inf (ng/mL·h)	531.073

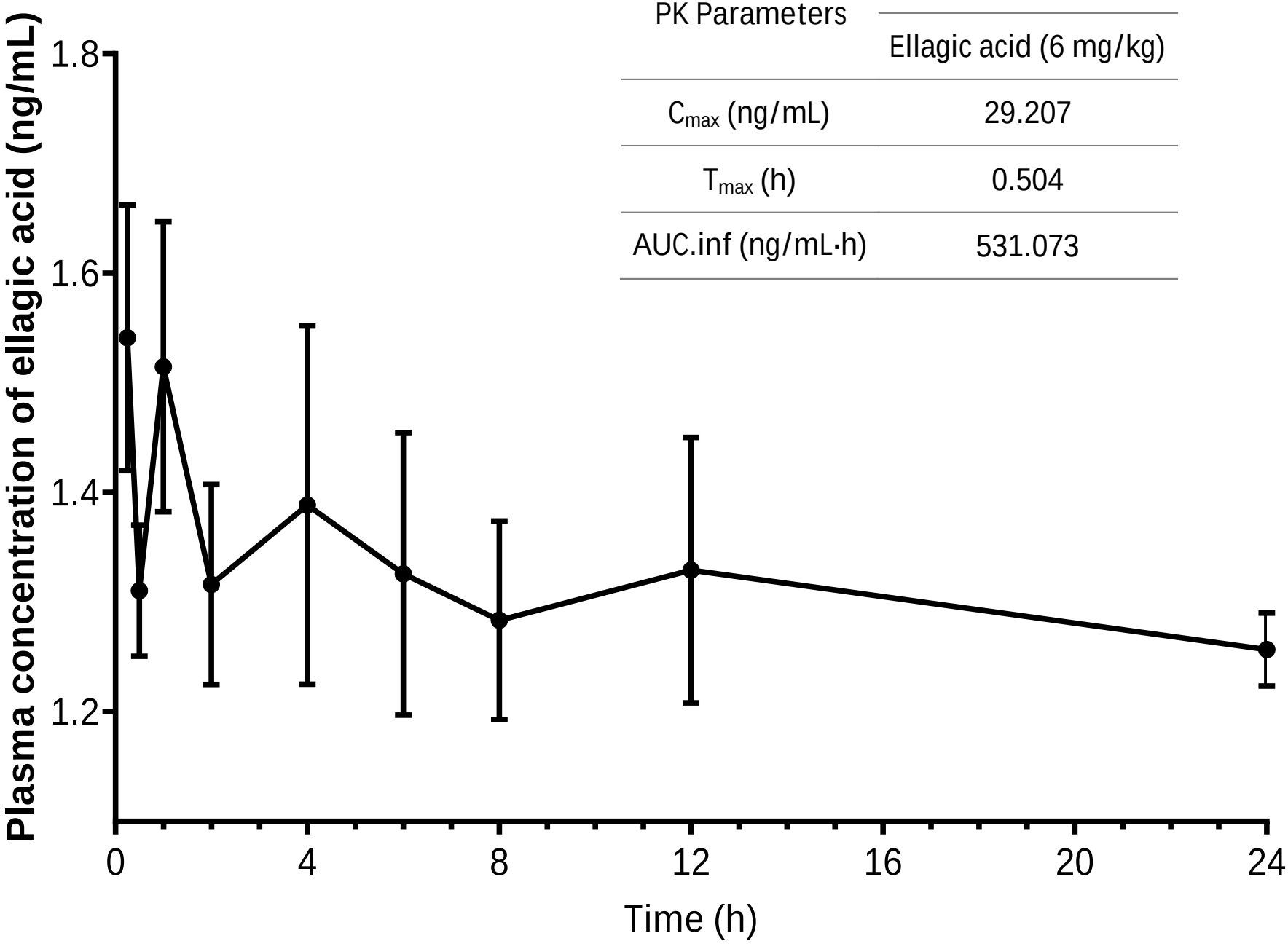

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Table of Materials

Table of Materials_revised.xls

Dear Dr. Amit Krishnan,

Thank you and reviewers for valuable and helpful comments on our manuscript (JoVE63167) entitled “Microsurgical Skills of Establishing Permanent Jugular Vein Cannulation in Rats for Serial Blood Sampling of Orally Administered Drug”.

We have thoroughly revised the manuscript and addressed editorial and reviewers’ comments using tract-and-change in the main text. We also prepared individual response in a point-to-point format in the rebuttal document below. We hope that you will find our revision satisfactory and grant acceptance of the revised manuscript for publication in Journal of Visualized Experiments.

Sincerely yours,

Zhang, Rui Xue (Vicki), Ph.D.

Response to Editorial Comments:

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

Response 1: We have thoroughly proofread the resubmitted manuscript.

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

Response 2: we have revised the text containing the personal pronouns.

3. 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. Please include all safety procedures and use of hoods, etc.

Response 3: We have carefully checked the protocol section to ensure all text is written in the imperative tense.

4. Please do not highlight any steps describing anesthesia and euthanasia.

Response 4: We have removed those highlighted protocol involving anesthesia and euthanasia.

5. Line 196: Please specify the size of the catheter used.

Response 5: Line 200, the size of catheter has been specified as follows: “Open the package of the 11-cm polyurethane (PU) catheter (I.D. 0.6 mm × O.D. 0.9 mm, **Figure 1B**) …”.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step (e.g., step 3.4.3).

Response 6: Thank you for the suggestion. We have checked the manuscript and divided the large paragraph into multiple performable steps (Line 252, Line 255).

7. Please ensure that the highlighted content is less than 3 pages.

Response 7: The highlighted content is within 3 pages.

8. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

Response 8: The forma of references has been fully revised.

9. Figure 2: Please specify in the figure legends whether the values represented in blue (panels C, D, E, F) represent the mean.

Response 9: Thanks for pointing this out. In Figure 2 legend, Line 390, “The numeric values in blue represent the mean”.

10. Figure 3: Please include the unit of time in X-axis within parenthesis.

Response 10: We have included the unit of time in X-axis within parenthesis in Figure 3.

Response to Reviewer #1's Comments:

The article reports the procedure for jugular vein cannulation in rats, a technique very important for researchers who work with animal models, especially in toxicological and pharmacokinetic studies that need the collection blood samples at regular time intervals. The manuscript is well written and detailed.

The experimental protocol has been analyzed and approved by an IACUC in China.

I did not have access to videos, and the images that were attached to the manuscript were of low resolution, so that I could not analyze them properly nor the content of legends.

Response: Thank you for the positive comment and the thorough review! The video is asked to submit upon manuscript acceptance. By then, JoVE's video production team will help us with the video filming.

Thanks for letting us know of image resolution, which was probably compressed due to exported file format (.tiff). We have improved the figure quality by changing the format to pdf in the revised manuscript. We hope it will be better for you to review.

Minor Concerns:

1. What is the composition of catheter lock solution?

Response 1: The composition of catheter lock solution was added in line 97: "Catheter lock solution is composed of heparinized saline (0.4% v/v heparin sodium) mixed with glycerol (v/v,1:1).".

2. Item 3.1 (lines 151-152): if surgical instruments were autoclaved, why they should be immersed in 75% ethanol and dried thereafter?

Response 2: Thank you for the question. The set of surgical instruments were autoclaved for each animal before the surgery. Since some of instruments were repeatedly used at various protocol steps, 75% ethanol and drying help keep them sterile and clean before the next use.

3. Line 177 and line 185 - do the authors mean salivary glands and lymph nodes?

Response 3: Thank you for the correction. We have revised the Line 181 to "NOTE: Depending on the age and sex of the rat, the soft tissue (e.g., salivary glands and

lymphatic nodes and fatty tissues) can cover the jugular vein varies.".

4. Line 196 - what is the length and the diameter of polyurethane catheter?

Response 4: We have specified the dimension of the catheter in Line 200, "Open the package of the 11-cm polyurethane (PU) catheter (I.D. 0.6 mm × O.D. 0.9 mm, **Figure 1B**) …".

5. Suggestion to include in item 4.3 - To decrease the pain caused by raising movements after catheter implantation to obtain water and food in the cage, food and water should be left on the bedding, and not at the top of the cage.

Response 5: Thanks for your helpful suggestion! We have implemented this method to improve the postoperative care of animals obtaining. *Per* your suggestion, it has been included in Line 286-287: "NOTE: For animal welfare, leave food and water on the bedding is an effective way to alleviate the pain caused by neck movements when eating and drinking.".

6. Line 309 - fasting is really necessary? Is not causing distress to rats?

Response 6: In our study, fasting animal is necessary as the food can influence the pharmacokinetics of orally administered natural compound. Fasting has also been adopted by many other pharmacokinetic studies. In other situations, fasting may not be necessary. We have modified the step 6.1 in Line 328 to: "NOTE: Depending on the experimental goal, fasting the animal is optional".

In the current protocol, we did not observe any sign of stress in rat. Besides, fasting procedure was implemented in recovered healthy rats and the duration is short. In addition, rats were free access to water.

Response to Reviewer #2's comments:

The authors have very nicely provided the details of jugular vein cannulation in rat. I just want to mention that details of JVC also covered in "Jugular vein catheterization for repeated blood sampling in the unrestrained conscious rat. Thrivikraman KV, et al. Brain Res Brain Res Protoc. 2002. PMID: 12431707"

Response: Thank you for the positive comments and suggesting this important reference.

we positively cited Thrivikraman et al.,2002. Brain Res Brain Res Protoc in Line 58-62 and as reference [8] in the previous version of manuscript. Our current study details the specific procedures of jugular vein cannulation in both text and video. Moreover, physiological and blood test indexes of rats post-operation were investigated, as the recovery status of animals can directly affect the accuracy of follow-up experimental data. We believe that this technique is very important for researchers working in the field of pharmaceutics, toxicology and pharmacokinetics that need series blood samples for *in vivo* compound evaluation.

Major Concerns:

1. Line 91. Catheter lock solution: contents and percentages of lock solution to be provided

Response 1: The composition of catheter lock solution was added in line 97: "Catheter lock solution is composed of heparinized saline (0.4% v/v heparin sodium) mixed with glycerol (v/v,1:1).".

2. Line 265: Meloxicam is used to relieve pain at 2mg/kg, SC: Scientists will use this model to understand the PK of new compounds. Information regarding whether it affects the PK of other compounds is to be commented in Discussion section. Example meloxicam's half-life, and its effect on different liver enzymes etc.

Response 2: Thank you for pointing out the potential drug-drug interaction. The elimination half-life ($t_{1/2\beta}$) of meloxicam is around 19 h - 23 h (Drug Research 47 (3): 253–58. PMID: 9105543; European Journal of Pharmaceutics and Biopharmaceutics

70 (3): 889–94. PMID: 18715548.). To reduce the pain of animal, one-time, a single dose (2mg/kg) of meloxicam was applied *s.q.* on the day post-operation. After 3-4 days recovery, the analgesics should be cleared out of the body. However, it is possible that multiple doses of meloxicam may lead to toxicity in the liver and gastrointestinal tract (Toxicology and Industrial Health 32 (6): 980–86. PMID: 24958741).

We have discussed the caution in using meloxicam as the painkiller in terms of potential drug-drug interaction in Line 435-438. The "NOTE" is also added in Lin 278, "NOTE: Select analgesics should avoid potential drug-drug interactions according to the drug compound of interest in the future study.".

3. Line 289: EDTA was used as anticoagulant: percentage of ETDA used to be provided

Response 3: We used 0.5 mL K2EDTA tube, for which EDTA is spray-dried on the wall. We have specified the EDTA tube in Line 304-306: "Use the new syringe to collect 150 μL of fresh blood sample and transfer the blood sample to the test tube containing EDTA anticoagulant0.5 mL tube containing K2EDTA (1.8 mg/mL blood) spray dried on the tube wall.

4. Line 339: It is mentioned that majority of animals recover in 4 - 6 days. As cannulations are done for screening purpose, can the animals be used earlier than 4 - 6 days based on physiological and hematological data?

Response 4: According to our physiological and hematological data, the JVC rat model is ready for use 4-6 days post-surgery. Other studies set 3-4 days to enroll the rats by monitoring their body weight change. (Brain Research Protocols 10 (2): 84–94. PMID: 12431707; Journal of Visualized Experiments. no. 95. PMID: 25741606.). For studies involving toxicology or drug efficacy, blood cells or substances in the blood, such as oxygen transport of red blood cells or leukocyte related inflammatory factors, are often evaluated Adequate postoperative recovery (at least 4 days) reduces the recovery variation in rats. We have added the discussion in Lines 421-424.

5. Figure 3: Ellagic acid was dosed orally at 6 mg/kg: The profile is not representative -of a compound after oral administration. Ellagic acid PK profile is given in 'Pharmacokinetic study of ellagic acid in rat after oral administration of

pomegranate leaf extract' F. Lei et al. / J. Chromatogr. B 796 (2003) 189-194. Can the authors give different example with better profile?

Response 5: The pharmacokinetic profile of ellagic acid is the application of establishing the JVC rat model.

Ellagic acids is classified as biopharmaceutical classification system class IV drug with poor solubility, poor permeability and erratic absorption. Particularly, at the low dose (6 mg/kg), the pharmacokinetics of EA can be varied, which is similar to the reported human observation (Journal of Functional Foods, 19: 225–35. DOI: 10.1016/J.JFF.2015.09.019.).

Using the same JVC model, our unpublished data showed new formulation of ellagic acid improved the pharmacokinetics compared to free suspension.

We have added PK parameters of Ellagic acid in suspension in Figure 3, by fitting the concentration profiles into the PKsolver. Although the administered dose is different (6mg/kg vs 800 mg/kg), the values of C_{max} and T_{max} are in the range of previous report [Journal of Chromatography B 796 (1): 189–94. PMID: 14552830.]

Minor Concerns:

1. Figure 2: X and Y axis titles are not clear

Response 1: We have improved the resolution of figure 2.

Dear Dr. Mondal,

Thank you for inviting us to share our laboratory techniques with the scientific field. We are now submitting our manuscript entitled "Microsurgical Skills of Establishing Permanent Jugular Vein Cannulation in Rat for Serial Blood Sampling of Orally Administered Drugs", for publication as a research method paper in *Journal of Visualized Experiments*.

Sequential blood collection from small laboratory animals (e.g., rodents) is necessary for evaluation of the therapeutic index during pharmaceutical lead optimization. Non-surgical approaches to multiple blood sampling, such as orbital sinus puncture and tail snipping, are possible, but the sample volume is small and the procedure can cause harm and stress to the animals, which could potentially affect the experimental results. The surgical cannulation approach, on the other hand, is a preferred alternative to repeated venipuncture because of several advantages of replacing the lost fluid volume, controlling blood volume collection, and reducing animals' pain and stress. Particularly, blood sampling through the jugular vein catheterization (JVC) rat model is a widely applied method for studying drug pharmacokinetics and effects. Despite of its usefulness, successful cannula implantation in jugular vein requires careful practice of microsurgical skills and knowledge of postsurgical care and maintenance.

Therefore, we aim to thoroughly demonstrate how to establish a long-term JVC rat model for blood collection at the laboratory setting and to investigate the physiological status of rats during the postsurgical recovery phase. In the manuscript, we detail the microsurgical procedures for the catheter implantation in the jugular vein of rat with specific focus on the placement and sealing of jugular cannula. We highlight the importance of monitoring postoperative physiological and hematological indicators of the rats to ensure their recovery for use. The established JVC model is applied to determine the time-plasma drug concentration of the orally administered natural phenol antioxidant ellagic acid.

We trust that this study provides informative protocol of establishing and maintaining a long-term blood collection rat model, which will be of great interest to researchers in the biological and medical fields. We appreciate you and reviewers in advance for the kind consideration and valuable comments.

Sincerely Yours,

Rui Xue Zhang, Ph.D., Associate Professor
Northwestern Polytechnical University, Xi'an, Shaanxi, China

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Author(s): **Weijia Lu, Ruimin Miao, Sijuan Hu, Junhong Liu, Fanqi Jin, Rui Xue Zhang**

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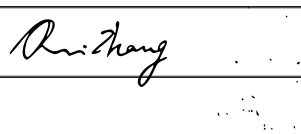
Signature:	
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