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TITLE:

Massive Pontine Hemorrhage by Dual Injection of Autologous Blood

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KEYWORDS:

Pontine hemorrhage, rat, pons, model, massive, brainstem, posterior circulation

SUMMARY:

We present a protocol to establish a massive pontine hemorrhage model in a rat via dual injection of autologous blood.

ABSTRACT:

We provide a protocol to establish a massive pontine hemorrhage model in a rat. Rats weighing about 250 grams were used in this study. One hundred microliters of autologous blood was taken from the tail vein and stereotaxically injected into the pons. The injection process was divided into 2 steps: First, 10 µL of blood was injected into a specific location, anteroposterior position (AP) -9.0 mm; lateral (Lat) 0 mm; vertical (Vert) -9.2 mm, followed by a second injection of the residual blood located at AP -9.0 mm; Lat 0 mm; Vert -9.0 mm with a 20-minute interval. The balance beam test, limb placement test, and the modified Voestch neuroscore were used to evaluate neurological function. Magnetic Resonance Imaging (MRI) was used to assess the volume of hemorrhage in vivo. The symptoms of this model were in line with patients with massive pontine hemorrhage.

INTRODUCTION:

Intracerebral hemorrhage accounts for one-fifth of stroke patients. The prognosis of intracerebral hemorrhage depends on the speed, volume, and location of bleeding[1,2]. Compared to the forebrain hemorrhage, the brainstem hemorrhage has higher mortality and morbidity[3]. About 40% of brainstem hemorrhage occurs in the pons[4]. The etiology and

pathophysiology of pontine hemorrhage are quite different and less studied than forebrain hemorrhage[5].

There are two kinds of pontine hemorrhage animal models. One is spontaneous hemorrhage model induced by infusion of bacterial collagenase in the pons[6-8]. The biggest advantage of this model is that the bleeding is spontaneous. However, collagenase can only induce a small volume of pontine hemorrhage. Besides, collagenase might cause other injuries to the brain. The other model is induced by stereotactic injection of autologous blood into the pons[9]. The advantage of this model is that it is easy to master with a high success rate. Theoretically, researchers could inject any volume of blood into any location of pons. However, due to the back-leakage through the needle route, the injected volume is limited. Recently, the double-injection method has been promoted to reduce the back-leakage[9]. This method injects autologous blood twice with a 20-minute interval between the injections. The double-injection method is applied to induce mild (30 µL) and moderate (60 µL) pontine hemorrhage but not massive pontine hemorrhage. In the clinic, the majority of pontine hemorrhage patients with poor prognosis have massive hemorrhage (more than 10 mL).

In the previous study, we provided a protocol to establish a pontine ischemic stroke model in rat[10]. In this study, we modify the existing dual injection method, and provide a detailed protocol to induce massive pontine hemorrhage in a rat by dual injection of 100 µL autologous blood at two different locations in the pons.

PROTOCOL:

The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Guangzhou Medical University. Rats were provided by the Animal Center of Southern Medical University. The experimental design is shown in **Figure 1**.

1. Animal and instruments

1.1. Use 8-week-old male Sprague–Dawley rats weighing 250 ± 10 g.

1.2. House the rats for at least 7 days before surgery under controlled environmental conditions with an ambient temperature of 25 °C, relative humidity of 65%, and a 12/12-h light-dark cycle.

1.3. Provide food and water with no limit.

1.4. Prepare the instruments (**Figure 2A-E**).

2. Inject the blood in the pons

2.1. Weigh the rats again 3 days before surgery to select rats with suitable body weight for

the experiments.

2.2. During the 3 days before modeling, train the rats to walk on balance beam 3 times per day to ensure normal rats could pass the balance beam without pause.

2.3. Preheat the heating pad to 37 °C before anesthesia.

2.4. Attach a microdrill to the holder on the stereotaxic frame.

2.5. Inject the rats with 50 mg/kg ketamine and 5 mg/kg xylazine intraperitoneally. Wait until there is no toe-pinch response.

2.6. Transport the rat into the stereotaxic frame in a prone position. Put the ear bars above the ear canal to secure the head. Fix the skull in a horizontal position to avoid skewing of the injection (**Figure 2F**).

2.7. Maintain anesthesia by isoflurane (97.5% oxygen and 2.5% isoflurane) through a stereotaxic nose cone with an inlet and outlet port.

2.8. Use eye ointment to keep the cornea moist.

2.9. Shave the hair above the skull with a micro shaver.

2.10. Draw a 3 cm mid-line with a marker pen in the skull from the line of the bilateral lateral canthus to 0.5 cm behind the posterior fontanelle, as shown in **Figure 2F**.

2.11. Apply Entoiodine surgical scrub in a circular fashion, starting at the middle of the marked mid-line and rotating outward.

2.12. Place the surgical drape.

2.13. Make an incision with a scalpel along the marked mid-line.

2.14. Use a cotton swab to remove any potential blood.

2.15. Place a piece of forceps on each side of the scalp flap to expose the skull (**Figure 2G**).

2.16. Dip a cotton swab in 0.9% saline and gently remove the connective tissues from the skull bone to avoid the connective tissues getting caught in the microdrill.

2.17. Mark the central point of the bregma as the origin point via a marker pen.

2.18. Perform a craniotomy (1 mm in diameter) using a microdrill at AP-9.0 mm, Lat 0 mm (**Figure 1B** and **Table 1**). Proceed carefully because this point is very close to the venous

sinus (**Figure 2G**).

2.19. Remove the microdrill from the stereotaxic frame.

2.20. Put a 100 µL Hamilton syringe into the stereotaxic holder (**Figure 2K**). Turn on the injection pump switch, click the **Rapid Inhalation** button, aspirate heparin solution (12500 U diluted in 100 mL of saline) to 100 µL and then drain it completely to prevent blood clotting too fast.

2.21. Apply chlorhexidine and 75% alcohol surgical scrub to the whole tail from root to tip at least 3 times to disinfect the skin, soften the horniness, and dilate the tail vein to increase the success rate of injection.

2.22. Attach a scalp acupuncture to a 1 mL syringe.

2.23. Insert the scalp acupuncture into a lateral tail vein 3 cm from the tail tip and take 150 µL of blood (**Figure 2H**).

2.24. Remove the scalp acupuncture from the 1 mL syringe.

2.25. Transfer the blood into a tube (**Figure 2I**).

NOTE: Transfer quickly to prevent blood clotting.

2.26. Change the surgical gloves.

2.27. Choose **Withdraw** mode, set the volume to 100 µL, set the speed at 200 µL/min, click the RUN button, aspirate 100 µL of blood into the Hamilton syringe (**Figure 2J**).

2.28. Choose **Infuse** mode, set the speed at 1 µL/min.

2.29. Advance the syringe until the tip reaches 9.2 mm below the surface of the brain (**Figure 1C** and **Figure 2L**).

2.30. Click the **Run** button, inject the first 10 µL of blood at a speed of 1 µL/min (**Table 1**).

2.31. Stop the injection and leave the syringe in position for 20 min to prevent blood from flowing into the subarachnoid space.

2.32. Retract the syringe until the tip arrives at 9.0 mm below the surface of the brain.

2.33. Restart injection at the same speed of 1 µL/min until the residual blood has been injected completely.

2.34. Leave the syringe in position for 10 min to avoid blood backflow.

2.35. Remove the syringe from the brain slowly.

2.36. Use bone cement to cover the craniotomy hole.

2.37. When the cement dries, suture the wound with 4-0 polyamide suture filament. After sewing 3 or 4 stiches, tie 2-1-1 standard surgical knots.

2.38. To prevent infection, inject the rat with penicillin (0.25 mL, 80 IU diluted in 4 mL saline) intraperitoneally.

2.39. Inject the rats subcutaneously with Butorphanol tartrate (2.5 mg/kg) and repeat the injection every 2 hours for relieving postoperative pain until 24 hours after surgery.

2.40. Observe the rat every 15 min until it fully recovers from anesthesia. Return it to the original cage, with a heating pad underneath the cage. Provide the rat free access to food and water until sacrifice.

3. Behavioral tests

NOTE: Perform behavioral tests on Day 1, Day 3, Day 7, and Day 14 after modeling, including the balance beam test, limb placement test, and the modified Voetsch neuroscore.

3.1. Balance beam test[11].

NOTE: This is a test specifically for examining the sensorimotor function of the hindlimb.

3.1.1. Prepared the apparatus to ensure that the beam (3 cm wide × 70 cm long) is 20 cm above the floor.

3.1.2. Put a dark box at the back end of the beam with a narrow entryway.

3.1.3. Place a white noise generator and a bright light source, which are used to motivate the rat to traverse the beam and enter the goal box, at the head end of the beam.

3.1.4. Stop the noise and light when the animal enters the goal box. Record the latency from head end to the goal box (in seconds) and the performance of hindlimb during traversing the beam.

3.1.5. Record the performance score as following: 0, balances with steady posture; 1, grips side of beam; 2, snuggles the beam with 1 hindlimb falling off; 3, snuggles the beam with 2 limbs falling off, or spinning around the beam for more than 60 s; 4, attempts to balance on beam >40 s, but falls off; 5, attempts to balance on beam >20 s, but falls off; and 6, falls off,

with no attempt to balance or balancing on beam <20 s.

3.2. Limb placement test

NOTE: The limb placement test examines 3 independent stimuli of vision, touch, and proprioception with 6 parameters, to evaluate the sensorimotor function of rat. The total neurological function score ranged from 0 to 12. Before the test, rat should be habituated to handling.

3.2.1. Hold the rat by the back and place it on the table slowly from a height of 10 cm. Normally, the rat would stretch forelimbs and place them on the table.

3.2.2. Grab the rat by the back and hold it facing the edge of the table. The reaction of forelimbs is observed. A normal rat would place the forelimbs on the table.

3.2.3. Let the rat grasp the edge of the table. A normal rat would use the chin to prevent the nose and nose hair from touching the table edge.

3.2.4. Put the rat on the table, push the rat with a gentle lateral pressure behind the rat's shoulder toward the edge of the table, and observe the placement of the forelimbs and hindlimbs. A normal rat would grasp the edge with its forelimbs and hindlimbs.

3.2.5. Place the rat on the table with the face toward the edge, and gently push it from the back to the edge. A normal rat would grasp the edge with its forelimbs.

3.2.6. Place the rat on the table with its back to the edge and push it by the back to the edge of the table. Observe the reaction of the hindlimbs. A normal rat would grasp the edge with its hindlimbs.

3.2.7. Assess the placement of the forelimbs or hindlimbs to the table edge. Record the scores as follows: 0, no placement; 1, unfinished and/or delayed placement; 2, immediate, complete placement.

3.3. The modified Voetsch neuroscore[8]

NOTE: The modified Voetsch neuroscore is a vertebrobasilar scale score of sensorimotor ability, containing 14 parameters: head movement, activity, hearing, pain reflex, corneal reflex, proprioception, neck sensation, exploration, circling, axial torso sensation, 4-limb movement, forelimb movement, climbing, and beam walk. The score for each parameter ranges from 0 (complete neurological deficit) to 3 (no neurological deficit). The total neurological function score ranges from 0 to 42.

3.3.1. Place the rat on the table (50 cm long × 35 cm wide) and allow it to move around for five minutes. Observe the spontaneous movement of its head: moves in all dimensions, 3;

prefers one side, 2; only movement to 1 side, 1; flexed to unilateral side, 0.

3.3.2. Place the rat on the table (50 cm long × 35 cm wide) and allow it to move around for five minutes. Activity is defined as: fully responsive, 3; moderately responsive, 2; minimally responsive, 1; coma, 0.

3.3.3. Observe the craniocaudal circling: turns bilaterally, 3; prefers 1 side, 2; only to 1 side, 1; fallen to 1 side, 0.

3.3.4. Evaluate the forelimbs movement: equal and bilateral movement, 3; slight asymmetry, 2; great asymmetry, 1; paresis, 0.

3.3.5. Evaluate the 4-limbs movement: equal and bilateral movement, 3; slight asymmetry, 2; great asymmetry, 1; paresis, 0.

3.3.6. When the rat is stationary, perform an ear pinch to stimulate the auricles and test the pain reflex: moves away quickly and symmetrically from stimulus, 3; moves away slowly or asymmetrically from stimulus, 2; shows some movement in response to pain, 1; no reaction, 0.

3.3.7. When the rat is stationary, make a noise on either side of the rat's body to test hearing: fingers rubbing, 3; snap of fingers, 2; loud clapping, 1 and no startling, 0.

3.3.8. To check the proprioception, touch the rat's vibrissae on both sides respectively with a blunt stick and observe its response to the stimulus: react on touch, 3; diminished reaction on one side, 2; diminished on both sides, 1; absent, 0.

3.3.9. To evaluate the torso axial sensation, place a blunt stick on either side of the rat's body and observe its response to the stimulus: brisk and symmetrical reaction to stimuli, 3; slightly diminished or asymmetrical reaction, 2; greatly diminished and asymmetrical reaction, 1 and no reaction, 0.

3.3.10. To test the corneal reflex, hold the rat and quickly touch the cornea on both sides with a cotton swab: both eyes close quickly, 3; diminished reflex on one side, 2; diminished on both sides, 1; absent, 0.

3.3.11. To check the sensation of the neck, hold the rat and use a blunt stick to touch the neck: reacts to touch actively, 3; slow reaction to touch, 2; greatly diminished reaction, 1; no reaction, 0.

3.3.12. To observe exploration, use a light dark box.

NOTE: Two thirds of the box should be open and lit, while the rest of it is covered and dark. A 7 cm door connects the two compartments.

3.3.13. Place the rat in the light compartment for 5 min and then the dark compartment for another 5 min, allow it to move around and record the rat's activities. When 4 limbs are placed in one room, record it as an entrance. Record scores as follows: reaches the light and dark compartments actively, 3; reaches 1 compartment, 2; moves slowly only after stimulus, 1; and no movement, 0.

3.3.14. To check the climbing ability, place the rat at the bottom of a 20 cm wide, 70 cm long plane with a 30-degree angle to the horizontal ground. Record scores as follows: able to climb to top, 3; impaired climbing, 2; stationary grip, 1; and falls immediately, 0.

3.3.15. To examine the beam walk, put the rat on one end of a 3 cm wide, 70 cm long beam which is 20 cm above the ground, record scores as following: explores the entire beam, 3; explores part of the beam, 2; some movement and falls, 1; no movement, 0.

3.3.16. Calculate the points.

4. Hemorrhage confirmation by MRI

4.1. Perform the MRI scan at 24 h post-surgery.

4.2. Anesthetize the rat with isoflurane (5% for induction, 1%- 1.5% for maintenance).

4.3. Secure the rat's head in the rat brain array coil combined with a transmit-only volume coil.

4.4. Place the coil together with the rat in the MRI scanner. Secure the rat within the cradle via the tooth and ear bars.

4.5. Use a closed-circuit thermal jacket to maintain the rat's body temperature at 37 ± 0.5 °C during MRI scanning.

4.6. Perform a pilot sequence to ensure correct geometry.

4.7. Apply a fast-spin echo sequence to collect T2-weighted scans. Set the parameters as follows: echo time (TE), 132 ms; repetition time (TR), 2,500 ms; acquisition matrix, 148 × 148; field of view, 100 mm × 100 mm; 12 slices; 1.5 mm thick.

4.8. Return the rat to the cage.

5. Hemorrhage confirmation by gross anatomy

NOTE: Sacrifice the rats at the designated timepoint, 24 h and 14 d after surgery (**Figure 1D**).

5.1. Anesthetize the rat with 5% isoflurane until loss of consciousness. Then, euthanize it with carbon dioxide (CO_2) (20-30% of the volume of the cage per minute).

5.2. Confirm death using the following signs: no chest rising and falling, no palpable heartbeat, no response to toe pinch, poor mucosa color, color change, or opacity in eyes.

5.3. Perform cervical dislocation.

5.4. Secure the rat by taping the paws on a sterile platform. Create a midline incision from the cervix to expose the hypogastrium to thorax and liver. Make a lateral incision from the upper sternal margin along the clavicle to the far left and another lateral cut from the xiphoid along the diaphragm to the far left, to expose heart. Lift the ribcage flap and fix it to the platform with a pin.

5.5. Connect the end of a needle (27 G) to a perfusion pump containing 4 °C saline. Advance the needle tip into the heart along the left edge of the left ventricle to avoid entering the atrium. Turn on the perfusion pump to ensure the tip is in the left ventricle, then make a cut in the right atrium.

5.6. Turn off the perfusion pump when the liquid drained out of the right atrium turns colorless and the liver turns white. This procedure needs approximately 100 mL 4 °C saline.

5.7. Decapitate the rat and harvest the whole brain using scissors and forceps. Remove any moisture from the brain surface with blotting paper.

5.8. Keep the whole brain at -80 °C for 1 min.

NOTE: This step could be skipped if the brain can be cut without freezing.

5.9. Lay the brain into the coronal rat brain matrix with the dorsal side up.

5.10. Insert a 0.21 mm thick stainless-steel blade into the hemorrhage center according to the hole in the surface of the brain.

5.11. Insert other blades into the brain at an interval of 2 mm.

5.12. Immerse the brain sections in 10 mL of 4% paraformaldehyde (PFA) solution for 24 h at 4 °C. Rinse them with 0.01 mmol/L phosphate buffered saline (PBS).

5.13. Organize the sections from rostral to caudal and image them.

6. Paraffin section and hematoxylin and eosin (HE) staining

6.1. Fix the brain with 4% PFA solution for at least 24 h at room temperature. The volume of

PFA should be 5-10 times of brain volume.

6.2. Cut the brain from the hemorrhage center into two pieces via blade and coronal rat brain matrix.

6.3. Make paraffin-embedded tissue blocks.

6.4. Section the paraffin-embedded tissue block coronally in 40 μm thickness slides on a microtome, cut 4 sections in succession, starting from hemorrhage center, and float them in a 40 °C water bath.

6.5. Mount the 40 μm sections (8 slides in total for one brain) onto clean glass slides and air dry overnight at room temperature.

6.6. Bake the slides at 56 °C for 1 h.

6.7. Wash for 3 min in xylene, 3 times.

6.8. Dip sections 30 times in 100% ethanol, 30 more times in 100% ethanol, 30 times in 95% ethanol, and 30 more times in 95% ethanol.

6.9. Rinse in tap water until clear.

6.10. Immerse the sections in Hematoxylin for about 10 min.

6.11. Rinse in the tap water until clear.

6.12. Immerse sections in eosin stain for 30 s.

6.13. Dehydrate slides with 3-4 dips 95% ethanol, 3-4 dips in 100% ethanol, 100% Ethanol for 1 minute, and 10 dips in 100% ethanol + xylene (1:1).

6.14. Clean sections with xylene for 1 min, 2 times.

6.15. Mount using non-aqueous mounting medium and a coverslip.

6.16. Dry the sections in the air overnight at room temperature, then san them.

7. Statistics

7.1. Use GraphPad Prism 6.0 to calculate Student's t-test or Mann Whitney U test.

NOTE: All data should be expressed as mean ± SE. Differences between two groups are determined with a two-tailed Student's t-test or Mann Whitney U test. $P<0.05$ is defined as

statistical significance.

REPRESENTATIVE RESULTS:

A total of 25 animals were used, 3 for control, 6 for 30 µL, 6 for 60 µL, and 10 for 100 µL blood injections. One rat that received a 100 µL injection of autologous blood (1/10) died within 24 hours after surgery.

Behavioral tests were conducted on Day 1, Day 3, Day 7 and Day 14 after surgery. The scores for the control group and blood-injection groups on different timepoints after surgery are presented in **Table 2**. The pontine hemorrhage caused neurological deficits like diminished corneal reflex and circling (**Figure 3B**,**C**). Injection of 100 µL blood also induced the myotonia (**Figure 3A**). The results of the balance beam test, limb placement test, and the modified Voetsch neuroscore revealed that the neurological function was decreased as the volume of pontine hemorrhage increased.

MRI scanning was performed 24 hours after surgery (**Figure 4**). In the blood-injection groups, on T2 sequence, hemorrhage was detected as a hypointense rim with an iso- to slightly hyperintense core in the basilar part of the pons. There was no hemorrhage detected by MRI in other brain areas (**Figure 4**). The volume of hemorrhage was increased as the injection volume of autologous blood increased.

Then rats were sacrificed at 24 hours and Day 14 after surgery, separately, and 2 mm thick sections were made (**Figure 4**). Hemorrhage was detected surrounding the injection site and distributing in the base of pons. There was slight edema around the hemorrhage in the 100 µL blood-injection group.

Some of the rats were sacrificed 3 days after surgery and paraffin-sectioned to do HE staining. Results showed that in the blood-injection groups, inflammatory cells enriched in the peri-hemorrhage zone (**Figure 5C** and **F**). The hemoglobin remains contained within intact red blood cells (**Figure 5D** and **G**).

FIGURE LEGENDS:

Figure 1: Schematic diagrams of pontine hemorrhage model. (A) Autologous blood collection from tail vein. (B) The schematic diagram of drill location. (C) The schematic diagrams of injection location. (D) Experimental design.

Figure 2: Instruments and procedure of surgery. (A) Anesthesia machine. (B) Surgical instruments. (C) Microdrill. (D) Micro-injection pump. (E) Stereotaxic apparatus. (F) A line marked in the middle of the skull. (G) Yellow arrow points to drill location. (H) Drainage of blood from the tail vein. (I) Transfer the blood into Eppendorf tube. (J) Aspirate the blood into Hamilton syringe. (K) Advance the Hamilton Syringe through the skull hole. (L) Injection process of autologous blood.

Figure 3: Representative results of behavioral tests. (A) Myotonia in a rat injected 100 μL of autologous blood. (B) Diminished corneal reflex on the right side in a rat injected 60 μL of autologous blood. (C) Diminished corneal reflex in the bilateral sides in a rat injected 100 μL of autologous blood. (D) A rat received 60 μL of autologous blood circled to the contralateral side of lesion. (E) Results of balance beam test. (F) Results of the modified Voetsch neuroscore. (G) Results of limb placement test. Line means significant difference between the two groups ($p < 0.05$).

Figure 4: Representative results of MRI scanning and gross anatomy. The MRI scanning (Upper) was performed 24 h after pontine hemorrhage surgery, then the rats were sacrificed and cut into 2 mm brain sections (Bottom).

Figure 5: Representative results of HE staining. Brains were harvested from the rats injected 100 μL of blood 3 d after surgery. (A) The whole brain section. Low fields from (B) the normal pontine area, (C) peri-lesion zone and (D) hemorrhage core. High fields from (E) normal pontine area, (F) peri-lesion zone and (G) hemorrhage core. Scale bar was 100 μm.

Figure S1: Representative results of gross anatomy on Day 14 after surgery.

Table 1: Injection of autologous blood.

Table 2: Results of behavioral tests.

DISCUSSION:

In the present study, we provided a protocol to generate a massive pontine hemorrhage rat model. This model can be used for the research on the pathophysiological mechanism and prognosis of massive pontine hemorrhage.

Throughout the experiment, 25 rats were used, of which only one died. The verification of MRI, gross anatomy, and the HE staining indicated that this method had a very low mortality rate and a high success rate. To establish massive pontine hemorrhage model, two problems must be solved, the injected autologous blood tends to leak into the subarachnoid space and flow back to the fourth ventricle along the needle tract. The existing double-injection moderate (60 μL autologous blood in total) pontine hemorrhage model resolved the first problem, barely any blood flowed into the subarachnoid space. However, there was still a small amount of blood backflow. In the present study, several strategies were applied to optimize the existing double-injection method to make it possible to inject a larger amount of 100 μL autologous blood without backflow. First, two different injection spots instead of one were employed. Second, heparin was used to flush the syringe with minimal residual to reduce the dosage, in order to only protect the blood from coagulating during the injection process, but not enough to promote leakage and backflow after the injection. Third, the injection time was long and injection speed was slow, 1 μL/min. Moreover, only a small amount of autologous blood was injected the first time, while the second injection was performed 20 minutes later. Afterwards, the needle was withdrawn only after 10 minutes,

and this procedure was conducted extremely slowly. Using this method, there was barely any blood flowing into the subarachnoid space or fourth ventricle in the rats injected with 30 μL or 60 μL of autologous blood, but there was still a small amount of backflow in the rats injected with 100 μL. Extending the time before removing the needle could solve this problem.

Behavioral tests were performed on Day 1, Day 3, Day 7 and Day 14 after modeling, including balance beam test, limb placement test, and the modified Voetsch neuroscore. On the first day after surgery, almost all of the rats in the blood-injection groups showed circling behavior (i.e., turning left or right), accompanied by disappearance of unilateral or bilateral corneal reflex. Although autologous blood was injected in the midline of the pontine, it was unevenly distributed in the two sides of the brain. This seemed to be the reason for the different performance in behavioral tests. The activities and reactions of the rats injected 30 μL or 60 μL of autologous blood slowed closer to normal. In the rats injected 100 μL of blood, the sensorimotor functions were significantly weakened and the response was poor. Muscle rigidity appeared in some rats in the resting state. On Day 1, Day 3 and Day 7, there were obvious differences between rats injected 30 μL, 60 μL or 100 μL of autologous blood and the rats in the control group in the modified Voetsch neuroscore. In the balance beam test and limb place test, there was no significant differences between the rats injected 30 μL or 60 μL of blood and the rats in control group at any time points. However, the results of the balance beam test and limb placement test in rats injected 100 μL of autologous blood were significantly different when compared with the control group on Day 1 and Day 3. The possible reason could be that there are fewer evaluation items in the balance beam test and the limb placement test compared to the modified Voetsch neuroscore, which are not sensitive enough to discover subtle neurological deficits. It is inappropriate to use these two methods to evaluate behavior in hemorrhage models with mild symptoms, but they are applicable in the massive pontine hemorrhage model. Overall, the modified Voetsch neuroscore turned out to be more suitable for comprehensively and accurately assessing the neurological functions in different pontine hemorrhage models.

There are several advantages of this method. Based on the previous double-injection method, the second injection location was changed and the dosage of heparin was adjusted to avoid the leakage and backflow in the mild (30 μL) and moderate (60 μL) pontine hemorrhage model. Even in the massive (100 μL) pontine hemorrhage model, the backflow was very limited, and occurred only in a small number of rats. This method can be easily performed with a high success rate and a low death rate. Moreover, the experimental pontine hemorrhage can be observed during a long period, at least 14 days after modeling, which is conducive to investigating the entire disease development and effects of treatments. The major advance of this model was that it mimicked the symptoms of patients with pontine hemorrhage. Clinically, massive pontine hemorrhage results in severe neurological deficits, while previous pontine hemorrhage models only developed relatively small hemorrhagic volume with mild symptoms. The massive pontine hemorrhage in this model distributed in the bilateral pons, which is similar with hemorrhage distribution in pontine

hemorrhage patients. In previous experimental pontine hemorrhage models, the hemorrhage only located in the unilateral pons[9].

However, there are also some limitations of this method. First, pontine hemorrhage in this study was caused by injection of blood, partially heparinized during transition, which might influence the blood coagulation or even homeostasis in the surrounding pons. Second, this model requires special equipment, such as stereotaxic apparatus and injection pump. Third, this model cannot mimic spontaneous hemorrhage.

In conclusion, this study provided a method to create an experimental acute massive pontine hemorrhage model in the rat, which could promote new mechanical and therapeutic research in this field.

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DISCLOSURES:

No conflicts of interest.

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618

Figure 1

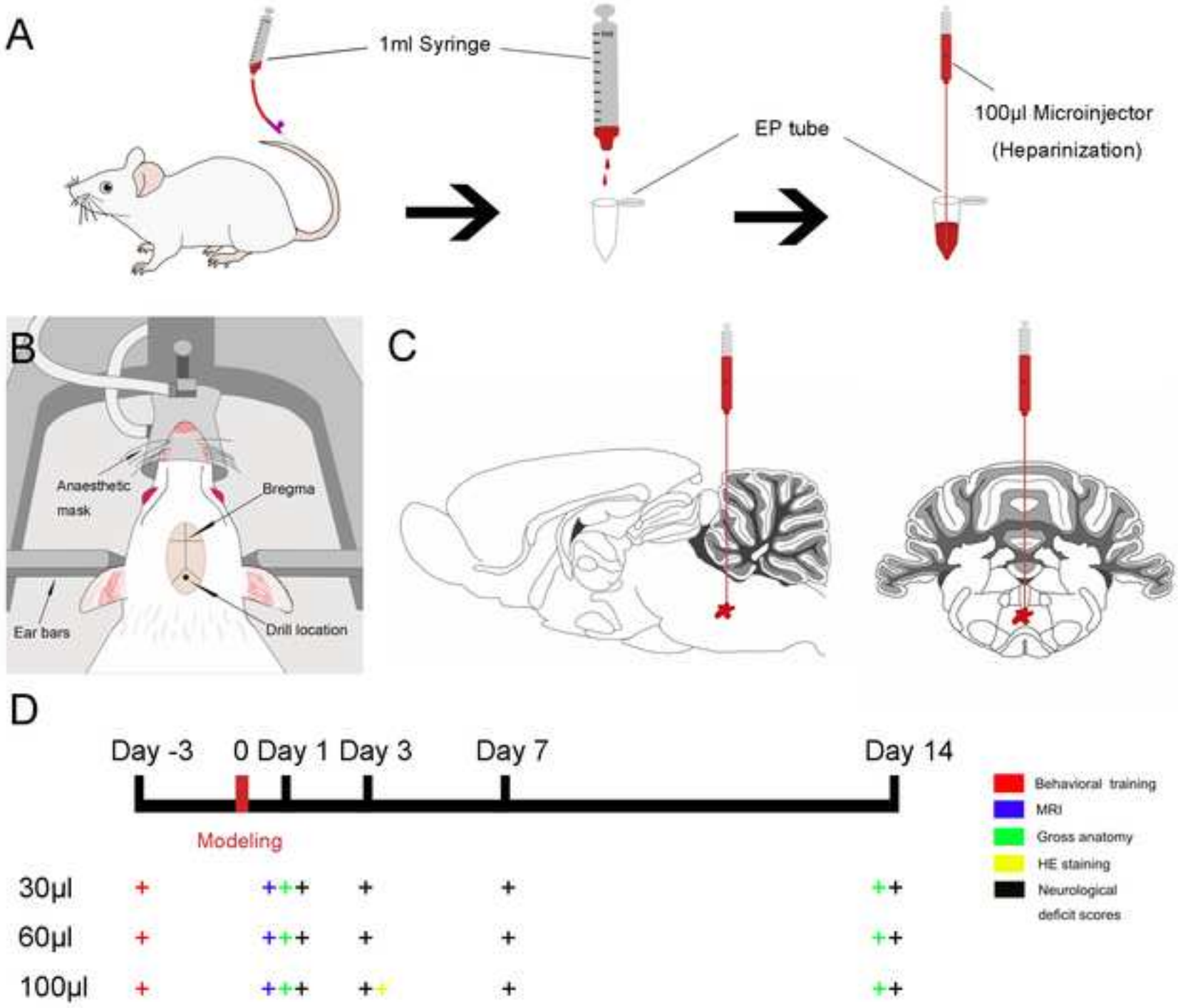

Figure 2

Click here to access/download;Figure;Figure 2.tif

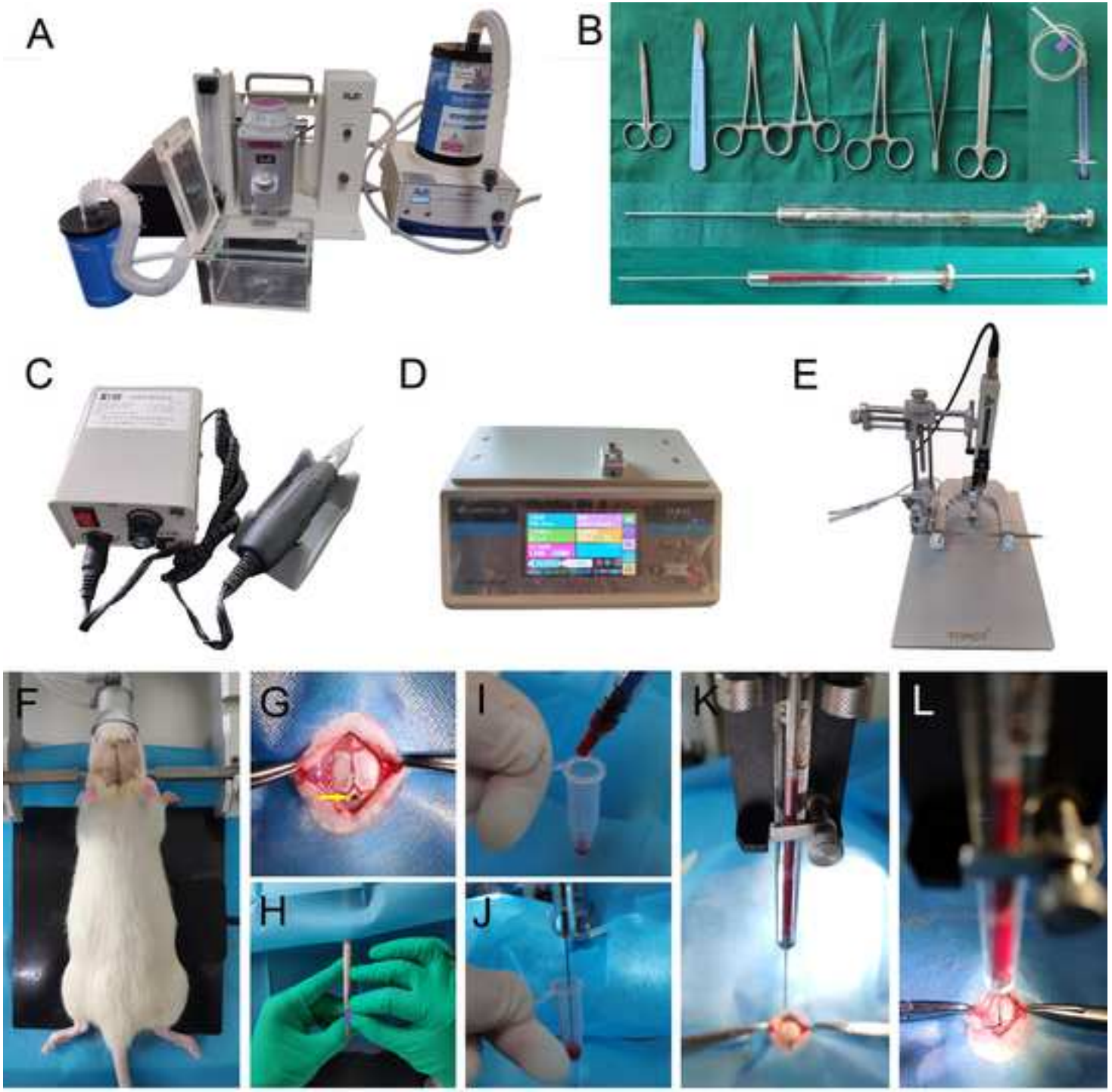

Figure 3

Click here to access/download;Figure;Figure 3.tif

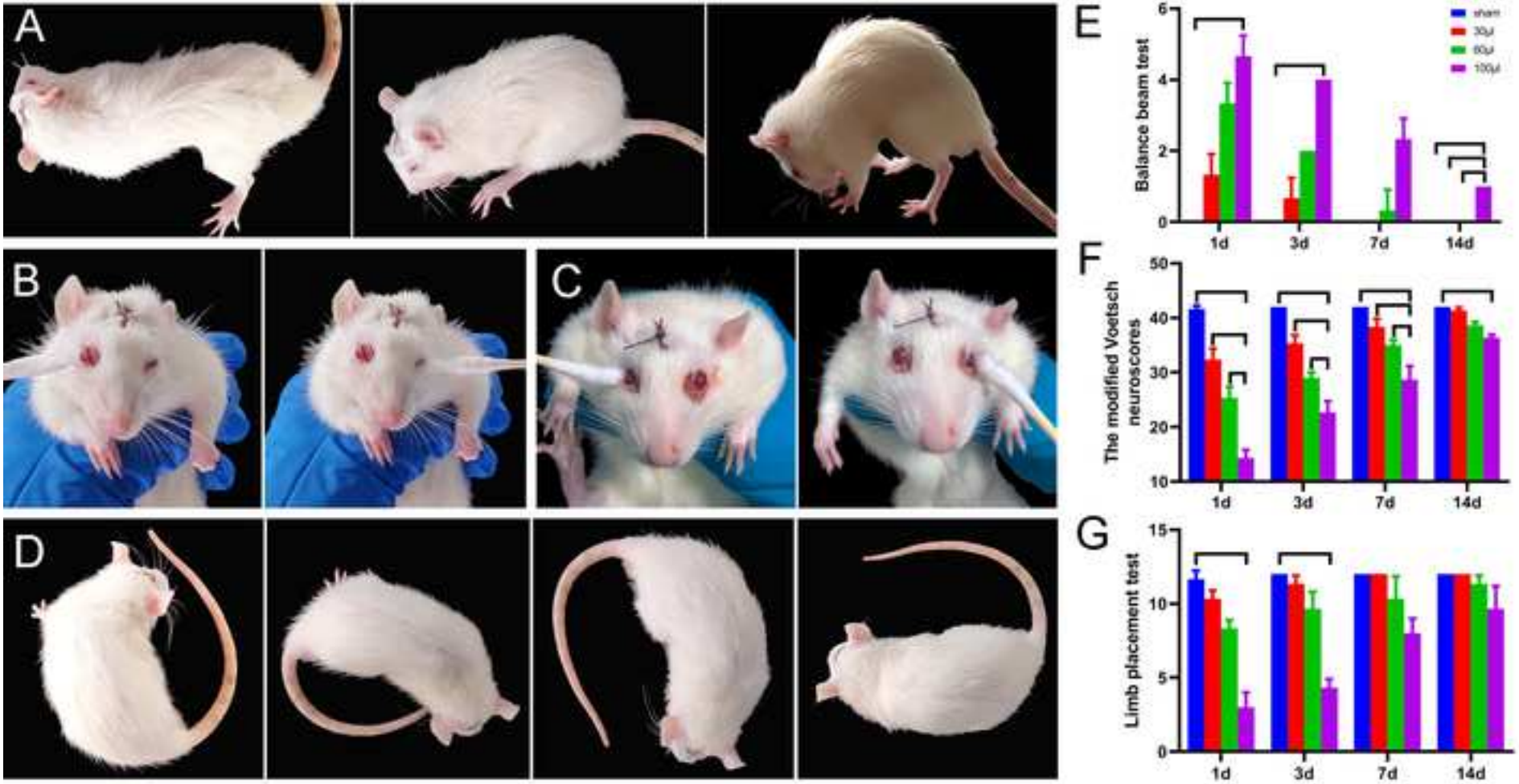

Figure 4

Click here to access/download;Figure;Figure 4.tif

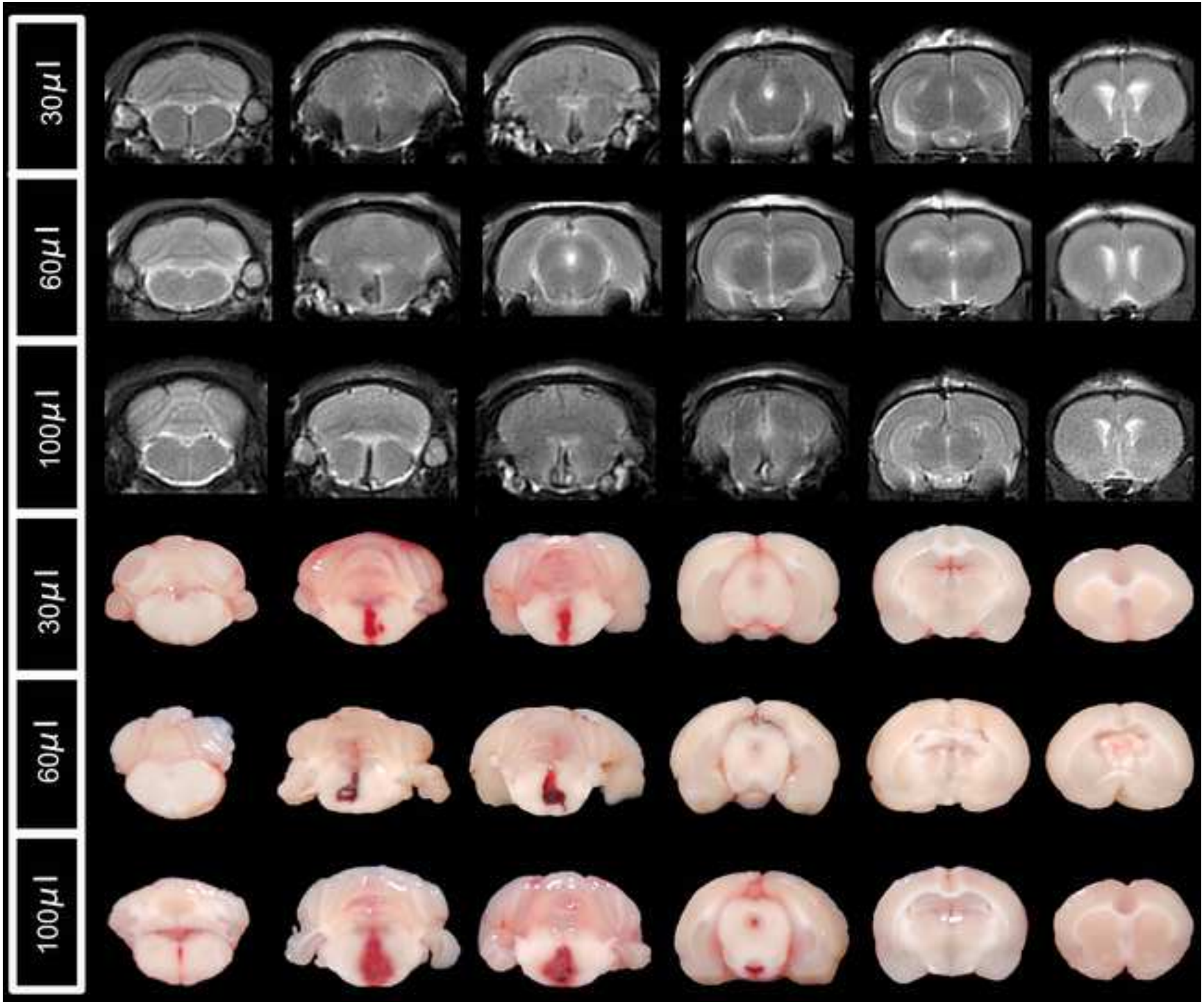

Figure 5

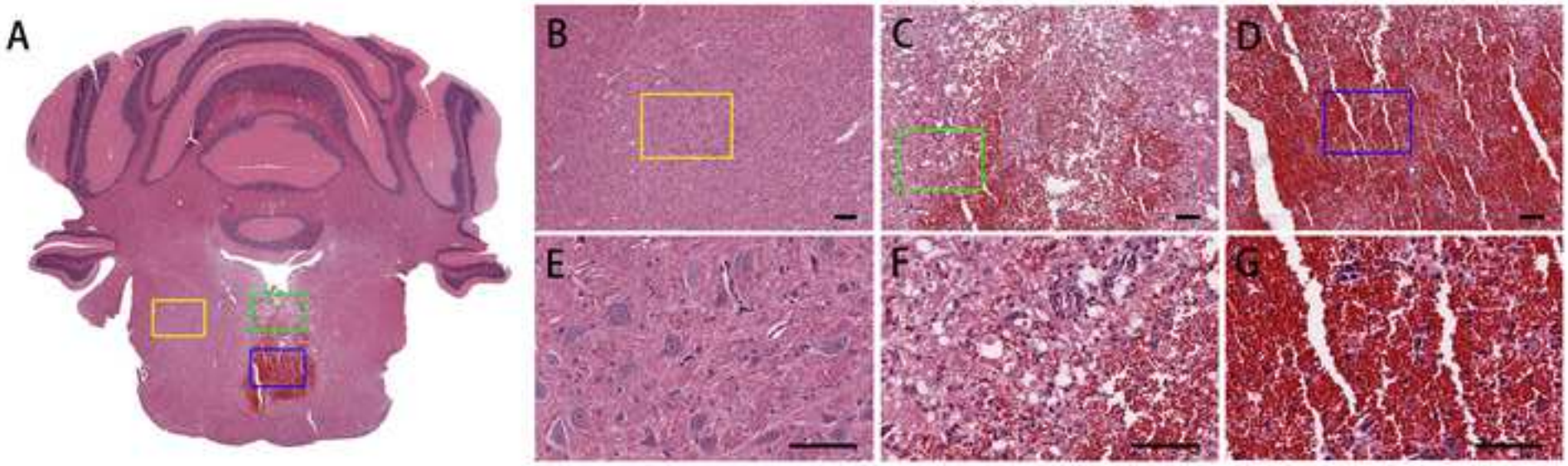

Table 1. Injection of autologous blood

	Mild	Moderate	Massive
Total volume	30 µL	60 µL	100 µL
First injection			
Stereotactic coordinates		AP -9.0 mm; Lat 0; Vert -9.2 mm	
Volume	10 µL	10 µL	10 µL
Speed	1 µL/min	1 µL/min	1 µL/min
Interval time	20 min	20 min	20 min
Second injection			
Stereotactic coordinates	AP -9.0 mm; Lat 0; Vert -9.2 mm		AP -9.0 mm; Lat 0; Vert -9.0 mm
Volume	20 µL	50 µL	90 µL
Speed	1 µL/min	1 µL/min	1 µL/min
Before withdrawal of the needle	10 min	10 min	10 min

AP: anteroposterior position
Lat: lateral
Vert: vertical

Table 2

Rat Number	Day 1	Day 3	Day 7	Day 14
The modified Voetsch neuroscore				
30 μL-1	33	34	38	41
30 μL-2	30	35	37	41
30 μL-3	34	37	40	42
60 μL-4	27	30	36	38
60 μL-5	23	28	34	39
60 μL-6	26	29	35	39
100 μL-7	16	25	31	36
100 μL-8	13	22	29	37
100 μL-9	14	21	26	36
Sham-10	41	42	42	42
Sham-11	42	42	42	42
Sham-12	42	42	42	42
Balance beam test				
30 μL-1	1	0	0	0
30 μL-2	1	1	0	0
30 μL-3	2	1	0	0
60 μL-4	3	2	0	0
60 μL-5	4	2	0	0
60 μL-6	3	2	1	0
100 μL-7	5	4	3	1
100 μL-8	5	4	2	1
100 μL-9	4	4	2	1
Sham-10	0	0	0	0
Sham-11	0	0	0	0
Sham-12	0	0	0	0
Limb placement test				
30 μL-1	11	12	12	12
30 μL-2	10	11	12	12
30 μL-3	10	11	12	12
60 μL-4	9	11	12	12
60 μL-5	8	9	9	11
60 μL-6	8	9	10	11
100 μL-7	4	5	9	11
100 μL-8	3	4	8	10
100 μL-9	2	4	7	8
Sham-10	11	12	12	12
Sham-11	12	12	12	12
Sham-12	12	12	12	12

Table of Materials

Click here to access/download;Table of Materials;62089 materials.xlsx

Name of Material/Equipment	Company
100ml Saline solution	Guangdong yixiang
100μl Microinjector	Shanghai Gaoge
1ml Syringe	Jiangsu Zhiyu
75% Alcohol	Shandong Lierkang
Adhesive tape	Shanghai Jinzhong
Animal anesthesia system	RWD
Balance beam	Jiangsu Saiangsi
Blades	Shanghai Feiying
Bone cement	Shanghai Xinshiji
Brain tank	Shenzhen LEIYEA
Butorphanol tartrate	Jiangsu Hengrui
Electric cranial drill	Nanjing Darwin biotechnology
EP tube	Nantong Surui
Erythromycin eye cream	Yunnan pharmacy
HE dye liquor	Solarbio
Heating pad	Dangerous Jungle
Heparin sodium injection	Chengdu Haitong Pharmacal Company
IndoPhors	Guoyao of China
Isoflurane	RWD
Light dark box	Jiangsu Saiangsi
Micro-injection pump	Baoding Leifu
MRI system	Philips
Needle holder	Shanghai Jinzhong
Penicilin	Guoyao of China
Q-tips	Jiangxi Songhe
Scalp heedle	Jiangxi Hongda
Scalpel	Shanghai Kaiyuan
Shearing scissors	Shanghai Jinzhong
Stereotaxic apparatus	RWD
Surgical towel	Xinxiang Huakangweicai
Suture needle	Shanghai Jinzhong
Suture scissors	Shanghai Jinzhong
Tissue holding forcepts	Shanghai Jinzhong

Catalog Number	Comments/Description
191222201 C1	Preparing heparin diluent
	Injection of autologous blood
20191014	Withdraw autologous blood from the tail vein
	Disinfection of rat tail
	Surgicl instruments
R510-31S-6	Inducing and maintaining anesthesia
	For neurological deficit scores
74-C	For gross anatomy
20180306	Surgicl instruments
	For gross anatomy
	For pain management
20180090018	Making a burr hole on the skull
	Transfer autologous blood
	Eyes protection
G1120	For HE staining
JR01	Keeping warm
190701	Preparing heparin diluent
	Sterilization
20080701	Inducing and maintaining anesthesia
	For neurological deficit scores
TFD03-01-C	Injection of autologous blood
	Confirmation of infarction in vivo
J32020	Surgicl instruments
	Infection Prevention
	Surgicl instruments
20200313	Withdraw autologous blood from the tail vein
170902	Surgicl instruments
Y00040	Surgicl instruments
900-00001-00	for surgical positioning
20070601	Surgicl instruments
	Surgicl instruments
J25041	Surgicl instruments
J31080	Surgicl instruments

Re: JoVE62089

Title: Massive pontine hemorrhage by dual injection of autologous blood

Dear Editors,

Thank you very much for your E-mail of Nov 10th, 2020, with regard to our manuscript (JoVE62089，Title "Massive pontine hemorrhage by dual injection of autologous blood"). We would like to thank the editors and reviewers for the positive and constructive comments. According to your valuable comments, we have revised the manuscript, and listed our responses to the editorial and reviewers' points. Dr. Wu contributed greatly to the revised manuscript and provided the foundation for the publication fee; therefore, we listed Dr. Wu as the co-first author.

We hope these changes will make the manuscript more acceptable for publication.

Thank you very much for your help.

Yours sincerely,

Yongjun Jiang,

The Second Affiliated Hospital of Guangzhou Medical University, 250 Changgang East Road, Guangzhou 510260, China

E-mail: jiangyjnju@gmail.com

Responses to the editorial comments：

1.Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: We are very appreciated with this important suggestion. We have thoroughly proofread manuscript, corrected all the spelling and grammar mistakes, and defined all abbreviations at the first use.

2.Please revise the following lines to avoid overlap with previously published work: 3.3.6 (The apparatus…an entry)

Response: The lines of 3.3.6 (The apparatus…an entry) have been revised and duplicate checked to avoid overlap with previously published work. And since we added more details to the protocol, 3.3.6 has been re-labeled as 3.3.12.

3.For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Response: We have modified the in-text format of the reference numbers according to your requirements.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: Thank you for your kind reminding, we realized that our protocol should

contain everything that we would like shown in the video. We have added more details to our protocol steps to ensure we answer the "how" question, and contained everything that we would like to show in the video. We believe there are enough details in each step for viewers to understand and easily replicate the protocol.

5. 2.2: what should the outcome of the balance beam test be for the experiment to proceed?

Response: We trained the rats to walk on balance beam 3 times per day, during the 3 days before surgery, to ensure normal rats could pass the balance beam without pause. So the score of the balance beam test before surgery should be 0 for each trained rat.

6. 2.3: To what temperature should the heating pad be preheated?

Response: Preheat the heating pad to 37℃ before anesthesia.

7. 2.27: How much heparin do you take in the syringe?

Response: It is hard to estimate the dosage of heparin we took in the syringe, since we only flushed the syringe with heparin saline: inhale heparin solution (12500u heparin diluted in 100ml saline) to 100ul, and drain it all. We are not sure how much heparin remained in the syringe.

8. 3.2.2: What do you mean by "apply tactile and proprioceptive stimuli to the paw with the table edge"?

Response: We re-wrote the protocol for limb placement test.

Tactile stimulus refers to:

3.2.4. Let the rat grasp the edge of the table. Normal rat would use the chin to prevent the nose and nose hair from touching the table edge.

Proprioceptive stimuli refer to:

3.2.5. Put the rat on the table, push the rat with a gentle lateral pressure behind the rat's shoulder toward the edge of the table, and observe the placement of the forelimbs and hindlimbs. Normal rat would grasp the edge with its forelimbs and hindlimbs.

3.2.6. Place the rat on the table with face toward the edge, and gently push it from the back to the edge. Normal rat would grasp the edge with its forelimbs.

3.2.7. Place the rat on the table with its back to the edge, and push it by the back to the edge of the table. The reaction of hindlimbs is observed. Normal rat would grasp the edge with its hindlimbs.

9. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. Please do not highlight steps describing euthanasia and anesthesia.

Response: We have included a one line space between each protocol step and highlighted protocol text for inclusion in the protocol section of the video, without highlighting the steps of euthanasia and anesthesia.

10. As we are a methods journal, please add to the Discussion the following in detail with citations:

a) Critical steps within the protocol

b) The significance with respect to existing methods

Response: We have added critical steps within the protocol and the significance with respect to existing methods to the Discussion.

a) Critical steps within the protocol: In the present study, several measures were applied to optimize the existing double-injection method to make it possible to inject a larger amount of 100 ul autologous blood without backflow. First, two different injection spots instead of only one injection spot were employed. Second, heparin was used to flush the syringe with minimal residual to reduce the dosage, in order to only protect the blood from coagulating during the injection process, but not enough to promote leakage and backflow after the injection. Third, the injection time was long enough and injection speed was slow enough, 1ul/min in this study. Moreover, only a small amount of autologous blood was injected at the first time, while the second injection was performed 20 min later. Afterwards, before withdrawal of the needle, 10 min was waited for, and this procedure was conducted extremely slowly. By this

method, in the rats injected with 30ul or 60ul of autologous blood in total, there was barely no blood flowing into the subarachnoid space or fourth ventricle; however, there was still a small amount of backflow in the rats injected 100 ul blood. Nevertheless, extending the time before removing the needle could solve this problem.

b) The significance with respect to existing methods: To establish massive pontine hemorrhage model, two problems must be solved, the injected autologous blood tends to leak into the subarachnoid space and flow back to the fourth ventricle along the needle tract. The existing double-injection moderate (60 ul autologous blood in total) pontine hemorrhage model resolved the first problem, barely no blood flowed into the subarachnoid space, but there was still a small amount of blood backflow.

11. 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. Please include volume and issue numbers for all references. Do not abbreviate journal names.

Response: We have modified the format of references according to your requirement to make the manuscript more suitable for publication.

12. Please sort the Materials Table alphabetically by the name of the material.

Response: We have sorted the Materials Table alphabetically by the name of the material.

Responses to the reviewers' comments:

Reviewer 1:

Manuscript Summary:

the language need to be polished and it would be better if some grammar errors could be corrected and the accuracy of expression could be enhanced. Besides, the further discussion on the results of behavioral tests is required so that the accuracy as well as reliability of the protocol is confirmed further.

Response: We are very appreciated with this important suggestion by the reviewer and agree with this. We have revised our manuscript thoroughly, corrected the grammar errors and enhanced the accuracy of expression. Further discussion on the results of behavioral tests has been added to increase the accuracy and reliability of the protocol.

Major Concerns:

The authors used the heparinized blood that can be used to avoid coagulation during the modeling; however, if using heparinized blood, the risk of backflow is greater, especially when injection of 100 ul, the amount of which has not been reported up to now. The authors should clarify what strategies they used to achieved the stable modeling.

Response: We agree with the reviewer's point that the use of heparinized blood would increase the risk of backflow, especially when injected in large amount. Therefore, we applied several strategies to achieved stable modeling.

First, we used as few heparin as possible to avoid coagulation. We flushed the syringe with heparin saline with minimal residual to reduce the dosage, in order to only protect the blood from coagulating during the injection process, but not enough to promote leakage and backflow after the injection: inhale heparin solution (12500u heparin diluted in 100ml saline) to 100ul, and drain it all.

Second, two different injection spots instead of only one injection spot were employed.

Moreover, only a small amount of autologous blood was injected at the first time, while the second injection was performed 20 min later.

Afterwards, before withdrawal of the needle, 10 min was waited for, and this procedure was conducted extremely slowly.

By this method, in the rats injected with 30ul or 60ul of autologous blood in total, there was barely no blood flowing into the subarachnoid space or fourth ventricle, there was still a small amount of backflow in the rats injected 100 ul blood. However, extending the time before removing the needle could solve this problem.

Minor Concerns:

1. introduction part (paragraph 2 , line 4)：what is the meaning of the collagenase-induced collagenase?

Response: We have corrected the mistake to "collagenase".

2.protocol part （2.18）：tense consistency of the word chose should be noted.

(3.2.3): in the expression success and failing, the noun of fail should be failure.

(3.3.7 last line): tense consistency of the word fell should be noted.

(5.11): tense consistency of the word inserted should be noted.

Response: We are very appreciated with these important corrections by the reviewer. Since we thoroughly revised the manuscript and added more details to the protocol, (2.18) has been revised as, 2.17. Mark the central point of the bregma as the origin point via a marker pen.

(3.2.3) has been deleted, and the limb placement test protocol has been re-written.

(5.11) has been revised as, 5.11. Insert a 0.21mm thick stainless-steel blade into the hemorrhage center according to the hole in the surface of the brain.

(6.11): what is the meaning of Scan the sections with?

Response: It was a clerical error, and has been corrected to "Scan the sections".

3.the further discussion on the results of behavioral tests is required

Response: Further discussion on the results of behavioral tests has been added to

increase the accuracy and reliability of the protocol.

Reviewer 2:

Manuscript Summary:

The manuscript describes a method of inducing massive pontine hemorrhage by dual injection of autologous blood in rats. Though the methods described are generally clear, there is a mix-up of tenses which makes the text difficult to comprehend. Some portions of the manuscript are not described with clarity to facilitate comprehension.

Response: We are very appreciated with this important suggestion by the reviewer. And we have thoroughly proofread the manuscript, corrected the grammar mistakes, and described more clearly to facilitate comprehension.

Major Concerns:

1. Plagiarism check gave a rather high value, authors can re-work the article

Response: We agree with this suggestion and have revised the manuscript to avoid overlap with previously published work.

2. There is a mix-up of tenses in the entire manuscript which makes the text difficult to comprehend

Response: We have thoroughly proofread the manuscript to ensure the tense consistency of tense.

3. A native English speaker can help edit the final manuscript

Response: Thank you for this great suggestion. Dr. Li Wu, who has been working in University of California, San Francisco as a visiting scholar for 2 years, edited the final manuscript.

Minor Concerns:

1. The abstract needs to be re-constructed

Response: We are very appreciated with this important suggestion by the reviewer and agree with this. We have re-constructed the abstract.

2. The second paragraph of the introduction section should be re-worded.

Response: We have re-worded the second paragraph of the introduction section，including correcting the spelling, grammar and expression.

3. The methodology could be more specific, for example

i. What behavioral tests were conducted before the induction of hemorrhage?

Response: We revised the protocol and mentioned in the Part 2. Inject the blood in the pons, that we conducted balance beam test before the induction of hemorrhage, as following: During the 3 days before modeling, train the rats to walk on balance beam 3 times per day to ensure normal rats could pass the balance beam without pause.

ii. How long after the surgery were the behavioral tests conducted?

Response: The behavioral tests were performed on Day 1, Day 3, Day 7, and Day 14 after the modeling, including balance beam test, limb placement test, and the modified *Voetsch* neuroscore.

iii. Why was butorphanol administered?

Response: Butorphanol is a powerful synthetic analgesic with anesthetic agonist and antagonistic effects. As a painkiller with good analgesic effect on postoperative incision pain and no obvious side effects such as respiratory depression[1-4], it is often used for postoperative pain management in rats[5]. In previous rat survival surgery study published in this journal, butorphanol was also used as a postoperative analgesic[6-8].

4. The second paragraph of the discussion section should be re-written

Response: We have re-written the second paragraph of the discussion section according to the reviewer’s advice.

Reference

1. Liles, J.H. and P.A. Flecknell, *The effects of buprenorphine, nalbuphine and butorphanol alone or following halothane anaesthesia on food and water consumption and locomotor movement in rats.* Lab Anim, 1992. **26**(3): p. 180-9.
2. Flecknell, P.A., *Anaesthesia of animals for biomedical research.* Br J Anaesth, 1993. **71**(6): p. 885-94.
3. Chen, Q., et al., *Analgesic effect and pharmacological mechanism of fentanyl and butorphanol in a rat model of incisional pain.* J Clin Anesth, 2016. **28**: p. 67-73.
4. Horan, P.J. and I.K. Ho, *Comparative pharmacological and biochemical studies between butorphanol and morphine.* Pharmacol Biochem Behav, 1989. **34**(4): p. 847-54.
5. Richardson, C.A. and P.A. Flecknell, *Anaesthesia and post-operative analgesia following experimental surgery in laboratory rodents: are we making progress?* Altern Lab Anim, 2005. **33**(2): p. 119-27.
6. Papazoglou, A., et al., *Non-restraining EEG Radiotelemetry: Epidural and Deep Intracerebral Stereotaxic EEG Electrode Placement.* J Vis Exp, 2016(112).
7. Roh, M., et al., *Acrylic Resin Molding Based Head Fixation Technique in Rodents.* J Vis Exp, 2016(107): p. e53064.
8. Sanderson, T.H. and J.M. Wider, *2-vessel occlusion/hypotension: a rat model of global brain ischemia.* J Vis Exp, 2013(76).

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