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Pre-chiasmatic, single injection of autologous blood to induce experimental subarachnoid hemorrhage in a rat model

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Corresponding Author:	Jesper Peter Bömers, M.D. Rigshospitalet Neurokirurgisk Klinik Copenhagen, DENMARK
Corresponding Author's Institution:	Rigshospitalet Neurokirurgisk Klinik
Corresponding Author E-Mail:	jesper.peter.boemers@regionh.dk
Order of Authors:	Jesper Peter Bömers, M.D.
	Sara Ellinor Johansson
	Lars Edvinsson
	Tiit Illimar Mathiesen
	Kristian Agmund Haanes
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TITLE:

Pre-Chiasmatic, Single Injection of Autologous Blood to Induce Experimental Subarachnoid Hemorrhage in a Rat Model

AUTHORS AND AFFILIATIONS:

Jesper Peter Bömers[1,2], Sara Ellinor Johansson[2], Lars Edvinsson[2,4], Tiit Illimar Mathiesen[1,3,5], Kristian Agmund Haanes[2]

[1]Department of Neurosurgery, Rigshospitalet, Copenhagen, Denmark
[2]Department of Clinical Experimental Research, Glostrup Research Institute, Rigshospitalet, Glostrup, Denmark
[3]Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark
[4]Department of Clinical Sciences, Division of Experimental Vascular Research, Lund University, Lund, Sweden
[5]Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden

Email addresses of co-authors:

Jesper Peter Bömers	(jesper.peter.boemers@regionh.dk)
Sara Ellinor Johansson	(sara.ellinor.johansson@regionh.dk)
Lars Edvinsson	(lars.edvinsson@med.lu.se)
Tiit Illimar Mathiesen	(tiit.illimar.mathiesen@regionh.dk)
Kristian Agmund Haanes	(kristian.agmund.haanes@regionh.dk)

Corresponding authors:

Jesper Peter Bömers	(jesper.peter.boemers@regionh.dk)

SUMMARY:

Subarachnoid hemorrhage continues to carry a high burden of mortality and morbidity in man. To facilitate further research into the condition and its pathophysiology, a pre-chiasmatic, single injection model is presented.

ABSTRACT:

Despite advances in treatment over the last decades, subarachnoid hemorrhage (SAH) continues to carry a high burden of morbidity and mortality, largely afflicting a fairly young population. Several animal models of SAH have been developed to investigate the pathophysiological mechanisms behind SAH and to test pharmacological interventions. The pre-chiasmatic, single injection model in the rat presented in this article is an experimental model of SAH with a predetermined blood volume. Briefly, the animal is anesthetized, intubated, and kept under mechanical ventilation. Temperature is regulated with a heating pad. A catheter is placed in the tail artery, enabling continuous blood pressure measurement as well as blood sampling. The atlantooccipital membrane is incised and a catheter for pressure recording is placed in the cisterna magna to enable intracerebral pressure measurement. This catheter can also be used for intrathecal therapeutic interventions. The rat is placed in a stereotaxic frame, a burr hole is drilled anteriorly to the bregma, and a catheter is inserted through the burr hole and placed just

anterior to the optic chiasm. Autologous blood (0.3 mL) is withdrawn from the tail catheter and manually injected. This results in a rise of intracerebral pressure and a decrease of cerebral blood flow. The animal is kept sedated for 30 min and given subcutaneous saline and analgesics. The animal is extubated and returned to its cage. The pre-chiasmatic model has a high reproducibility rate and limited variation between animals due to the pre-determined blood volume. It mimics SAH in humans making it a relevant model for SAH research.

INTRODUCTION:

Non-traumatic subarachnoid hemorrhage (SAH) is a form of stroke, representing around 5% of all cases. The most common cause of non-traumatic SAH is the sudden rupture of an aneurysm (aSAH), which accounts for 85% of SAHs. Other causes include the rupture of an arterio-venous malformation, coagulopathies, and rupture of veins in perimesencephalic hemorrhage[1]. The incidence rate is 9 per 100,000 person-years with mortality around one in three and another third requiring the support of daily living following SAH[2,3].

Following initial stabilization and diagnosis confirmation, treatment depends on the severity of the hemorrhage. The most severely afflicted patients will have an extra-ventricular drain inserted into the ventricles to reduce the intracerebral pressure (ICP) and be admitted to the neurointensive care unit, where they are monitored closely. Patients will undergo an angiography to identify the (probable) aneurysm and afterward have the aneurysm coiled or clipped to prevent rebleeding[4]. Despite numerous trials of pharmacological therapies, only nimodipine, a calcium-channel antagonist, has shown to improve outcomes[5]. Multiple clinical trials are currently underway. Please see the review by Daou and colleagues for an extensive list[6].

The rupture of an aneurysm has been described as the sudden onset of the worst headache ever experienced or a thunderclap headache. The rupture results in a steep rise in the ICP followed by a reduction in the cerebral blood flow (CBF). This reduction results in global ischemia of the brain, which can result in a loss of consciousness. This more mechanistic pathway, along with the initiated breakdown of the extravasated elements of blood, gives rise to cytokine release and activation of the innate immune system resulting in sterile neuroinflammation. Furthermore, breakdown of the blood-brain barrier, resulting in cerebral edema and disturbance in the ion homeostasis, is often observed. All these changes and more, coined early brain injury (EBI), occur within the first couple of days and results in neuronal loss and apoptosis[7].

Approximately 1/3 of patients afflicted with aSAH will develop delayed cerebral ischemia (DCI) between day 4–14[8]. DCI is defined as either the debut of a focal, neurological impairment or a drop of minimum two points on the Glasgow coma scale lasting for a minimum of 1 h, when other causes, including seizures and re-bleeding is excluded. DCI is associated with an increased risk of death and decreased functional outcome following aSAH[9]. Cerebral vasospasm (CVS), the narrowing of the cerebral arteries, has been known to be associated with DCI for decades and was formerly thought to be the sole reason for DCI. It has since been shown that CVS can occur without the development of DCI and more factors, including microvascular thrombosis and constriction, cortical spreading depression, and an inflammatory response of EBI have since been identified[10–12].

Due to the large influence of EBI and DCI on the course of the disease and the outcome of the patients afflicted, animal models need to mimic these to the largest degree possible, while still being reproducible. Researchers have employed a wide range of different models in a variety of animals from mice to non-human primates to try and simulate aSAH. Sprague-Dawley and Wistar wildtype rats are currently the most commonly used laboratory animals, and the most common models are the endovascular perforation model, the cisterna-magna double injection model, and lastly the pre-chiasmatic single injection model, which will be described in this article[13].

The pre-chiasmatic, single injection model was originally developed by Prunell and colleagues to counter some of the shortcomings of the other experimental models[14]. The surgery, when mastered, is highly reproducible and minimizes variation between animals. The model mimics SAH in humans on multiple points, including the sudden rise in ICP following the injection of blood, resulting in transient global ischemia due to a fall in the CBF[15,16]. It affects the anterior circulation, which is where most aSAH in humans occur[17]. The mortality ranges from 10%–33% depending on the study and amount of blood injected[14,18]. Delayed cell death and neuroinflammation can be detected on day 2 and 7 thereby providing variables to study the consequences of EBI and DCI[19,20].

The study presents an updated description of the pre-chiasmatic single injection model in the rat along with a description of how to utilize the ICP-probe as a port for intrathecal administration of pharmaceutics.

PROTOCOL:

This procedure is done in accordance with the European Union's Directive 2010/63/EU regarding the protection of animals used for scientific purposes and approved by the Danish Animal Experiments Inspectorate (license no. 2016-15-0201-00940). Surgery is performed using aseptic technique to the widest extent possible, including sterile instruments, catheters, and sutures. The study used male and female Sprague-Dawley rats weighing 230–350 g, group housed in 12-h light/dark cycle, with constant temperature of 22 °C (± 2 °C), and humidity of 55% (± 10%). The animals are provided with standard chow and water ad libitum. The animals are housed in single cages following surgery but can be returned to group caging when the ICP-probe has been removed. The anesthetic in this protocol is isoflurane gas but a 1.5 mL/kg of 3:2 intraperitoneal mixture of ketamine (100 mg/mL) and xylazine (20 mg/mL) is also effectful[21].

1. Preparations

1.1. Modify a 16 G peripheral vein catheter for intubation. To modify, shorten the needle by 1 cm and bend the remaining distal 1 cm by 30° toward the injection valve. Remove the catheter wings (multiple use).

1.2. To make an ICP probe, cut a 20 mm piece of polythene tubing (inner diameter (ID): 0.58 mm, outer diameter (OD): 0.96 mm) and burn one end to make a circular plate, keeping an open

lumen. Circumvent the polythene tubing with 1 mm of silicone tubing (ID: 1.0 mm, OD: 3.0 mm) before connecting 10 mm of silicone tubing (ID: 0.76 mm, OD: 2.4 mm) to the end of the polythene tubing.

1.3. Power on the laptop and open the data acquisition software. Calibrate the blood pressure (BP) and intra cerebral pressure (ICP) transducers, and the Laser-Doppler according to the manufacturer's instructions.

1.4. Prepare the blood gas analyzer apparatus.

CAUTION: Make sure there is enough isoflurane in the vaporizer.

1.5. Turn on the O_2 and N_2O flow. Set the flow of O_2 at 30% and N_2O at 70%.

1.6. Place the heating pad and set the temperature to 37 °C.

2. Anesthesia

2.1. Place the rat in the anesthesia chamber with a flow of 30% of O_2 and 70% of N_2O. Administer 5% of isoflurane gas into the chamber. Adequate anesthesia will take around 4 min. Control the breathing carefully.

2.2. When anesthetized, place the rat in supine position on a heavy plate circumvented by a rubber band. Place the front teeth of the rat below the rubber band.

2.3. Draw the tongue out carefully with curved forceps. Clean the larynx with a cotton tip. Place an external light in the midline of the throat to visualize the vocal cords.

2.4. Intubate during inspiration using the modified 16 G peripheral vein catheter. When correctly inserted, remove the stiletto. Connect the catheter to the ventilator.

NOTE: Correct placement of the tube is confirmed by chest movements in sync with respiration rate. If movements of the abdomen are seen, extubate and reintroduce the rat into the anesthesia bell. Do not repeat the procedure more than three times due to the risk of damaging the airways.

2.5. When intubated, keep the animal on artificial respiration with 30% of O_2 and 70% of N_2O. Maintain the anesthesia at 1.5%–2% of isoflurane. Adjust the isoflurane to keep the blood pressure between 80–100 mmHg.

2.6. Keep the inspiratory volume of the respirator at 3 mL and the frequency at 40–45 inspirations/min. Adjust the inspiratory volume according to the blood gas analysis.

2.7. Make a stitch through the inner soft tissue of the cheek with a 2-0 suture. Tie the suture around the injection tube and the injection valve of the peripheral vein catheter to fasten the catheter.

2.8. Move the rat to the operating field and place it in supine position with the tail facing toward the surgeon.

2.9. Apply the eye gel when needed to counter dry eyes.

3. Tail catheter

3.1. Disinfect the proximal 3–4 cm of the tail with 0.5% of chlorhexidine ethanol.

NOTE: From now on, use the surgical microscope upon the surgeon's discretion.

3.2. Make a 15–20 mm skin incision in the proximal end of the tail on the ventral side. Be careful not to incise the artery.

3.3. Loosen the skin from the underlying connective tissue using a curved forceps.

3.4. Carefully penetrate the fascia exposing the artery.

3.5. Carefully release the tail artery from the underlying tissue using a curved forceps.

3.6. Slip three black silk threads under the vessel. Place one thread as distally as possible and tie a surgical knot tightly around the artery. Hold the loose ends of the thread with a hemostat.

3.7. Tie the two remaining threads loosely around the artery.

3.8. Push the proximal thread as proximally as possible. Apply a hemostat to hold the ends of the proximal thread. Pull the hemostat lightly, but enough to restrict and block the blood flow. Place the hemostat on the abdomen.

3.9. Cut the tip of the catheter at a 45° angle. Cut the sharp point to prevent arterial wall penetration.

3.10. Using a Vannas scissor, make an artery incision 1/3 of the artery's diameter at a 30° angle, 3–5 mm from the distal knot.

3.11. Insert the catheter into the artery using two straight forceps. Use one forceps to hold the catheter and the other to carefully pull the artery over the catheter.

3.12. Insert the catheter up the vessel to the proximal knot and loosen the knot from the hemostat. Visualize the blood flow in the catheter. Fasten the middle thread loosely to the catheter.

3.13. Continue insertion to, and if possible, just beyond, the point where the artery is covered again by fascia.

3.14. Fasten the two proximal threads using surgical knots.

3.15. Control the catheter placement and possible leak by flushing with saline.

NOTE: The blood pressure measurement needs to be pulsatile; if not, the catheter is not properly placed.

3.16. Fasten the catheter at the end of the incision by tying a surgical knot using the distal thread.

3.17. Stitch the skin incision loosely together with two non-resorbable monofilament 4-0 suture. Be careful not to penetrate the catheter.

NOTE: Throughout the surgery be aware of the amplitude of pulsation. If this is low, flush the catheter with saline.

3.18. Loosen the arterial catheter from the pressure transducer to allow blood flow for blood gas sampling. Place a micro capillary tube at the end of the catheter. Let the blood flow into the tube. Re-attach the catheter to the transducer after blood collection and flush the catheter.

3.19. Insert the capillary tube in the blood gas analyzer. Measure the pH, pCO_2, and pO_2 and note them down.

NOTE: Depending on the blood gas and blood pressure values, change the ventilation rate. If the mean arterial pressure (MAP) is too low, try to turn down the flowrate of isoflurane. Test the reflexes to ensure proper depth of anesthesia.

4. ICP probe

4.1. Place the rat in the stereotaxic frame. It is important to position the rat symmetrically.

4.2. Place a cylindrical pillow under the stereotaxic frame to create anterior flexion of the neck.

4.3. Shave the rat's scalp, neck, and the area behind the ears. Remove the superfluous hair.

4.4. Disinfect the area with 0.5% of chlorhexidine ethanol.

4.5. Anesthetize locally with 0.7 mL of 10 mg/5 µg/mL lidocaine with adrenaline, insert the needle at the caudal end of the skull in the midline. Inject into the musculature of the neck with 0.3–0.4 mL. Inject the rest subcutaneously around and anterior to the bregma.

4.6. Make a skin incision from the needle puncture ~8 mm caudally in the midline.

4.7. Dissect all the muscles bluntly in layers to identify the atlantooccipital membrane (marble colored triangle caudally to the skull in the midline).

4.8. Use the Alm retractor to restrain the neck musculature. Place the pronged retractor caudally if needed.

4.9. Check whether the sterile ICP-probe is connected to the ICP transducer. Flush the ICP probe with saline. Ensure no air bubbles are present in the ICP probe.

4.10. Incise the atlantooccipital membrane using a 23 G needle. Make a hole to coax the ICP probe through the membrane.

4.11. Coax the probe through the atlantooccipital membrane gently. Pull the probe lightly and ensure that it shows a pulsating curve ranging between 0–5 mmHg. If not, remove the probe, check the connection to the transducer, and confirm the flow through the lumen.

4.12. Apply two drops of the tissue glue. Move the 1 mm silicone tubing forward to the membrane and apply additional glue to minimize the risk of ICP-probe displacement.

4.13. Remove the retractor(s).

4.14. Make one horizontal mattress suture to the cephalic end of the incision and one simple interrupted suture to the caudal end using a non-resorbable monofilament 4-0 suture.

5. Placement of the needle and the Laser-Doppler probe

5.1. Make an incision in the midline just anterior to the eyes, 15 mm caudally.

5.2. Remove the connective tissue and the muscles with forceps. Use the end of a sterile cotton swab as a rougine making it possible to identify the bregma and the coronal sutures.

5.3. Place the Alm retractor.

5.4. Place a 25 G spinal needle in the stereotaxic frame. Place the needle exactly on the bregma and note the position.

NOTE: Place the midline joint of the stereotaxic frame at 30° toward the animal in the vertical

plane.

5.5. Remove the needle from the bregma, move the frame by 65 mm anteriorly and then replace the needle in the midline to mark the site of drilling.

5.6. Drill until the dura mater is identified below the bone. Gently remove the bone fragments using straight forceps and fill the cavity with bone wax.

5.7. Drill another hole 3–4 mm lateral to the right of the bregma and just anterior to the coronal suture for the Laser-Doppler. It is not necessary to drill all the way through the bone. Be careful not to penetrate the dura mater.

5.8. Look for the vessels where the laser-doppler can measure the blood flow. Place the laser-doppler and check the values. A minimum value of 100 FU is required. Remove the microscope (artificial light).

5.9. If the values are still acceptable, add one drop of glue to fix the probe.

5.10. Recheck to confirm whether the value is above 80 FU. If the value is below 80 FU, remove and reposition the probe to reach a value above 80 FU.

NOTE: The value, FU, is an arbitrary unit showing cerebral blood flow (CBF).

6. Induction of SAH

6.1. Insert the needle gently through the skull in the midline between the hemispheres until resistance of the base of the skull is felt. Retract the needle by 1 mm to ensure correct placement just anteriorly to the optic chiasm.

6.2. Turn the needle 90° clockwise so that the needle tip points to the right to ensure the most homogenous result when injecting the blood. Remove the stiletto (**Figure 3**).

6.3. Equilibrate for 15 min and adjust the level of anesthesia to obtain a mean arterial blood pressure in the range 80–100 mmHg.

6.4. Perform a blood gas analysis. Adjust the level of anesthesia accordingly.

6.5. Withdraw 500 µL of blood from the tail catheter using a 1 mL syringe with a blunt 23 G needle.

6.6. Fill the dead space of spinal needle chamber with blood to avoid injection of air. Remove the 23 G needle from the blood-filled syringe and confirm that the syringe contains 300 µL of blood.

6.7. Connect the syringe to the spinal needle. Grasp firmly and inject the blood manually to surpass MAP.

6.8. Observe a steep rise in ICP and a steep fall in CBF on the laptop.

NOTE: CBF should be 50% or lower compared to the baseline score for at least 5 min for the surgery to be successful, see **Figure 4**.

7. Recovery and awakening

7.1. Administer 0.1 mL/100 g of animal weight of 5.0 mg/mL of carprofen and 1 mL/100 g of animal weight of isotonic saline subcutaneously. Make sure the liquids are at least at room temperature before administering.

7.2. Subsequently keep the rat under anesthesia for 30 min following the SAH.

7.3. Remove the needle, the laser doppler probe, and then fill the cavities with bone wax. Close the incision using two horizontal mattress sutures with non-resorbable monofilament 4-0 suture.

7.4. To use the ICP probe for injections into the cisterna magna, remove the silicone tubing and insert a pinpoint adapter to the polythene tubing.

7.5. If no intervention is planned, cut the simple, interrupted suture. Shorten the ICP probe as much as possible using a scissor and then glue the end to prevent leak of cerebrospinal fluid (CSF). Close the incision with a non-absorbable monofilament 4-0 suture.

7.6. Remove the rat from the stereotaxic frame and place in a supine position. Remove the loose sutures from the tail incision.

7.7. Place a single suture proximal and deep to the arterial catheter. Remove the catheter and tie the suture to prevent bleeding. Suture the tail-incision with a non-absorbable monofilament 4-0 suture.

7.8. Turn off the isoflurane.

7.9. Clean the rat and its fur as much as possible.

7.10. When the pedal withdrawal reflex is regained and the rat has spontaneous respiration when decoupled from the ventilator, extubate it.

7.11. Place the rat in a single cage with food and water ad libitum. Place one half of the cage under a heating plate and place the rat in this area of the cage.

7.12. Perform intrathecal administration by adapting the pinport injector to a precision syringe and administer the treatment through the pinport adapter. This intervention is feasible in animals that are awake. See **Figure 5**.

8. ICP-probe removal (if not removed during surgery)

NOTE: Use a surgical microscope upon the surgeon's discretion.

8.1. Place the rat in the anesthesia chamber as described earlier.

8.2. When anesthetized, place the rat in supine position in the operation field with heating pad.

8.3. Place the nose in the anesthesia mask. Set the levels of O_2 to 30%, N_2O to 70%, and isoflurane to 2%.

8.4. Continuously apply the eye gel to counter dry eyes.

8.5. Cut the caudal simple interrupted suture. Open the incision and remove the possible necrotic tissue or blood clots.

8.6. Shorten the ICP probe as much as possible using a scissor and glue the end to prevent the leak of cerebrospinal fluid (CSF). Close the incision with a non-absorbable monofilament 4-0 suture.

8.7. Turn off the isoflurane.

8.8. When the rat starts to move, place it in a single cage with food and water ad libitum. Place one half of the cage over a heating plate and place the rat in this area.

8.9. When returned to habitual state, reintroduce the animals to each other in a joint cage under supervision for the first 15 min.

NOTE: Sham rats do not undergo the steps 6.1–6.7, thereby omitting the introduction of the spinal needle into the cerebrum, minimizing possible spontaneous hemorrhage, and iatrogenic brain damage.

REPRESENTATIVE RESULTS:

Women have an increased risk of aSAH compared to men. Despite this, male rodents are primarily used in experiments due to possible bias from heterogeneity of estrus cycle in females. The representative results presented here are from a recent publication comparing female and male rats, confirming that the model produces similar results in female animals compared to male[21]. The study included 34 female Sprague-Dawley rats (18 SAHs and 16 shams). Shams did not have the spinal needle descended to the optic chiasm or blood injected. All other procedures

were performed on Shams identical to SAHs. All the physiological parameters between groups were comparable. Lastly, a meta-analysis of data from previous experiments on the male rats was done and compared with the results of the present study[21].

The rotating pole test is a test of gross sensorimotor function. The animal is placed on one end of a 150 cm by 45 mm pole, which can rotate up to 10 rpm. The goal is to reach the far end of the pole where a cage is placed. SAH rats did significantly worse on day 1 and 2, compared to sham animals on the rotating pole (**Figure 1**).

Following SAH, both the ET-1 and 5-HT receptor family are upregulated in the cerebral arteries resulting in an increased contraction when stimulated and thereby contributing to CVS[22,23].The basilar artery (BA) and middle cerebral arteries (MCA) were removed following decapitation and used for myograph experiments. Both endothelin 1 (ET-1), an agonist for the ET-1 receptor family and 5-carboxamidotryptamine (5-CT), an agonist for the 5-HT-receptor family produced significantly increased vascular contraction in SAH compared to sham (**Figure 2**). Sensitivity can be observed by the lower concentrations needed to elicit contraction following SAH in both sexes.

Increased water content (edema) following SAH is a measure of reduced functional outcome in humans[24]. Significantly increased cerebral edema was found in SAH compared to sham on day 2. There was also a tendency toward increased edema in the hippocampus, but this was not statistically significant ($p = 0.0508$)[21].

When comparing the above-mentioned data to historical male data, the results are comparable. The metadata shows increased contractility in male SAHs following addition of ET-1 or 5-CT (**Figure 2**). Furthermore, the SAH rats performed significantly worse compared to shams when doing the rotating pole test. The result indicated a decreased sensorimotor function (**Figure 1**).

Figure 5A shows the distribution of the autologous, injected blood following saline perfusion 30 min after induction of the SAH. The figure shows that the blood has been distributed in the subarachnoid space following pre-chiasmatic injection.

Figure 5B and **Figure 5C** shows the distribution of intrathecally injected dyes, followed by whole body saline perfusion for 30 min after the injection. **Figure 5B** shows the distribution of 25 µL of 20 mM Evans Blue (water soluble) and **Figure 5C** shows the distribution of 25 µL of 10 mM Oil Red O (water insoluble). Both dyes were found to be distributed in the subarachnoid space following the injection into the cisterna magna, confirming that this is a feasible model of intrathecal injection of both water soluble and insoluble compounds. Worth noticing is the formation of deposits around the arteries for the water insoluble compound.

FIGURE AND TABLE LEGENDS:

Figure 1: Analysis of sensory-motor cognition in the first 2 days after SAH in male and female rats. Rotating pole test was performed on day 1 and day 2 after SAH. Rats of both genders had significant deficits compared to sham-operated rats of the same gender. Statistical differences in

behavior between groups were tested by 2-way ANOVA on day 0, day 1, and day 2. Female no rotation and 3 rpm: $p < 0.05$. Female 10 rpm and all male data: $p < 0.01$. Values are means ± SEM. Republished with permission from Spray, S. et al.[21].

Figure 2: Analysis of increased sensitivity to ET-1 and 5-CT induced contractions in the basilar artery (BA) and middle cerebral artery (MCA) 2 days after SAH in male and female rats. (**A,B**) 60 mM K^+-evoked (K^+_{max}) contractile responses were used as reference values for normalization of agonist-induced responses. The sensitivity to ET-1 was significantly increased 2 days after SAH compared to sham-operated rats of the same gender in both the BA and MCA. (**C,D**) The sensitivity to 5-CT was significantly increased 2 days after SAH compared to sham-operated rats of the same gender in both the BA and MCA. The concentration-response curves were statistically compared with two-way ANOVA. All data: $p < 0.001$. Values are means ± SEM. Republished with permission from Spray, S. et al.[21].

Figure 3: Overview of the setup before induction of SAH. From the top of the picture, note that the 1) injection needle, 2) laser-Doppler probe, and 3) the ICP probe are all in place.

Figure 4: Sample trace following intrathecal injection. The red graph shows the blood pressure in mmHg. The blue graph shows the ICP in mmHg and the green graph shows the CBF in the arbitrary unit FU. The spike in ICP is the result of blood injection. Notice that this results in a drop in the CBF > 50% of baseline for more than 5 min. The ICP rise furthermore results in a small rise in blood pressure which normalizes within seconds.

Figure 5: Distribution of intrathecally injected blood and colored dyes. (**A**) Distribution of autologous blood 30 min after SAH induction. (**B**) Distribution of 25 µL of 20 mM Evans Blue following intrathecal injection through ICP-catheter. (**C**) Distribution of 25 µL of 10 mM Oil Red O following intrathecal injection through ICP-catheter. All animals were anesthetized with intraperitoneal ketamine/xylazine mixture followed by saline perfusion.

DISCUSSION:

The pre-chiasmatic single injection model of SAH mimics several important elements of human SAH, including the spike in ICP, reduction of CBF, transient global ischemia, upregulation of neuroinflammatory markers, and CVS[14–16,18–20]. The ICP-probe was also used as a port for intrathecal administration (**Figure 5**). Furthermore, the study shows that the model performs similarly in male and female animals[21]. The model does not include the development of and the subsequent rupture of an aneurysm. A range of models have attempted to produce SAH from a ruptured aneurysm by induction of systemic hypertension either surgically or pharmacologically and by weakening the arterial wall using elastase[25–27]. All attempts have produced aneurysmal SAH in a subset of animals, but these models have an inherent variability including the inability to predict when the aneurysm will rupture. The models are not very suitable for pre-clinical research on SAH[18,28].

Among other murine, SAH models, the endovascular perforation model includes the rupture of a vessel, somewhat mimicking the rupture of an aneurysm, but prone to high variability and

mortality. The model described here is better traceable and more reproducible as the blood volume is pre-determined and injection pressure can be controlled. The double injection model has a higher probability of producing delayed CVS, but primarily affects the posterior circulation and includes an unphysiological second blood injection. In comparison, this model resembles SAH in humans as it is a single injection of the anterior circulation and it produces a reproducible ICP rise[18].

The influence of different anesthesia regimes on experimental SAH is unclear and the experimental data is contradictory. One study reported possible inhibition of cytokines and general neuroinflammation in an endovascular perforation model in mice when using isoflurane inhalations[29]. Another rodent model resulted in reduced respiratory parameters and increased brain edema along with reduced regional CBF when using isofluranes[30]. However, a meta-analysis comparing mortality in mouse models showed no difference in mortality between isoflurane and other types of anesthesia[31]. In agreement, the above protocol has successfully used either isoflurane inhalation or an intraperitoneal ketamine/xylazine mixture with similar results in both groups[21].

To ensure high reproducibility and proper data acquisition, overall emphasis is on the steps regarding placement of the monitoring equipment. Correct placement of the tail catheter facilitates continuous monitoring of blood pressure and the ability to do blood gas analyses. Proper placement of the ICP catheter ensures correct ICP monitoring and the subsequent possibility of intrathecal intervention. Appropriate placement of the Laser-Doppler probe ensures that the reduction of CBF can be monitored, where a reduction of 50% or lower of baseline score for at least 5 min following SAH induction ensures a strong ischemia[32]. By ensuring that all monitoring steps are in order, the researcher can secure correct data collection following the SAH induction.

The protocol describes the pre-chiasmatic single injection model of subarachnoid hemorrhage with updates and modification. The model has been valuable for SAH-research and will probably continue to contribute toward a better understanding of subarachnoid hemorrhage, including early brain injury and delayed cerebral ischemia.

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The authors have no conflicting interests to declare.

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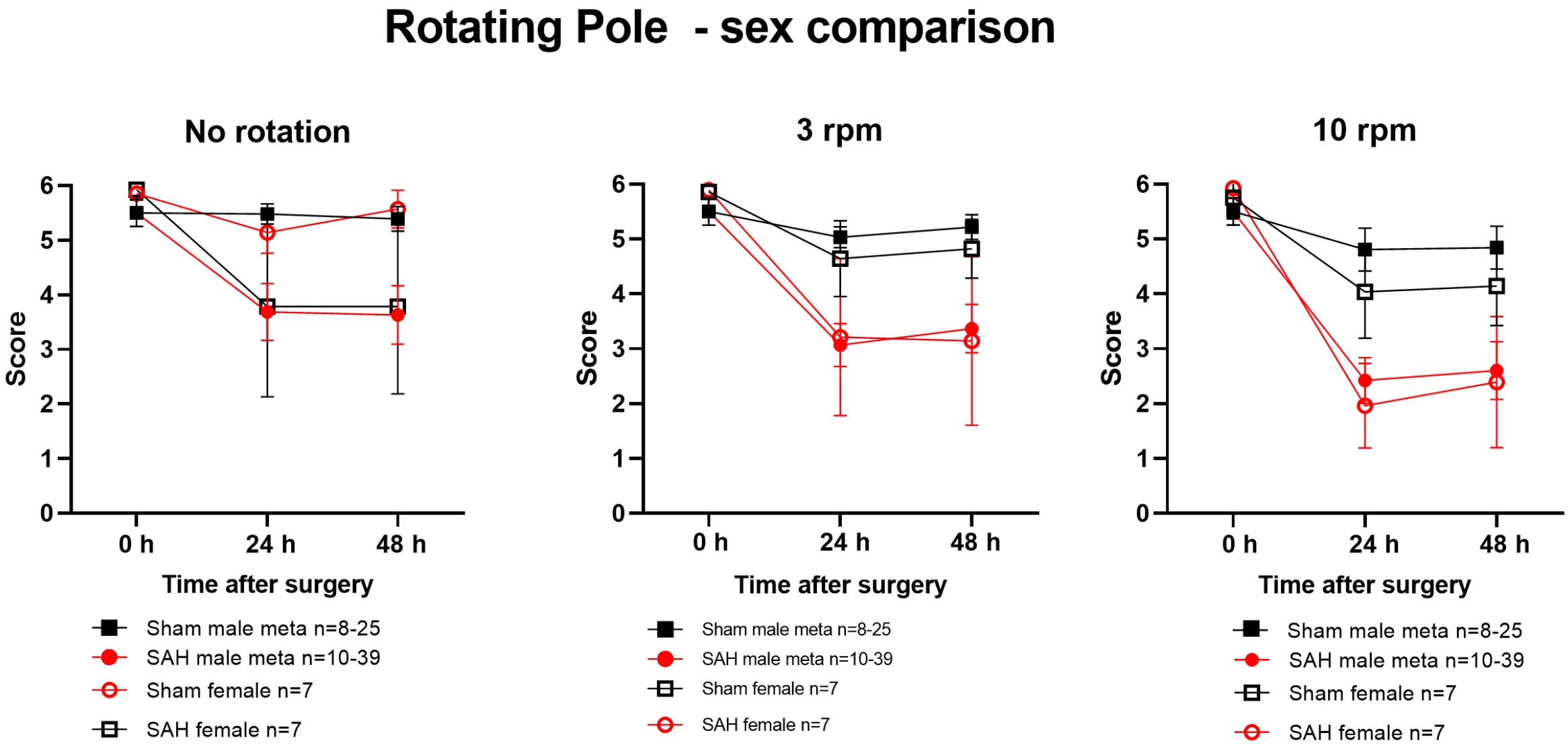

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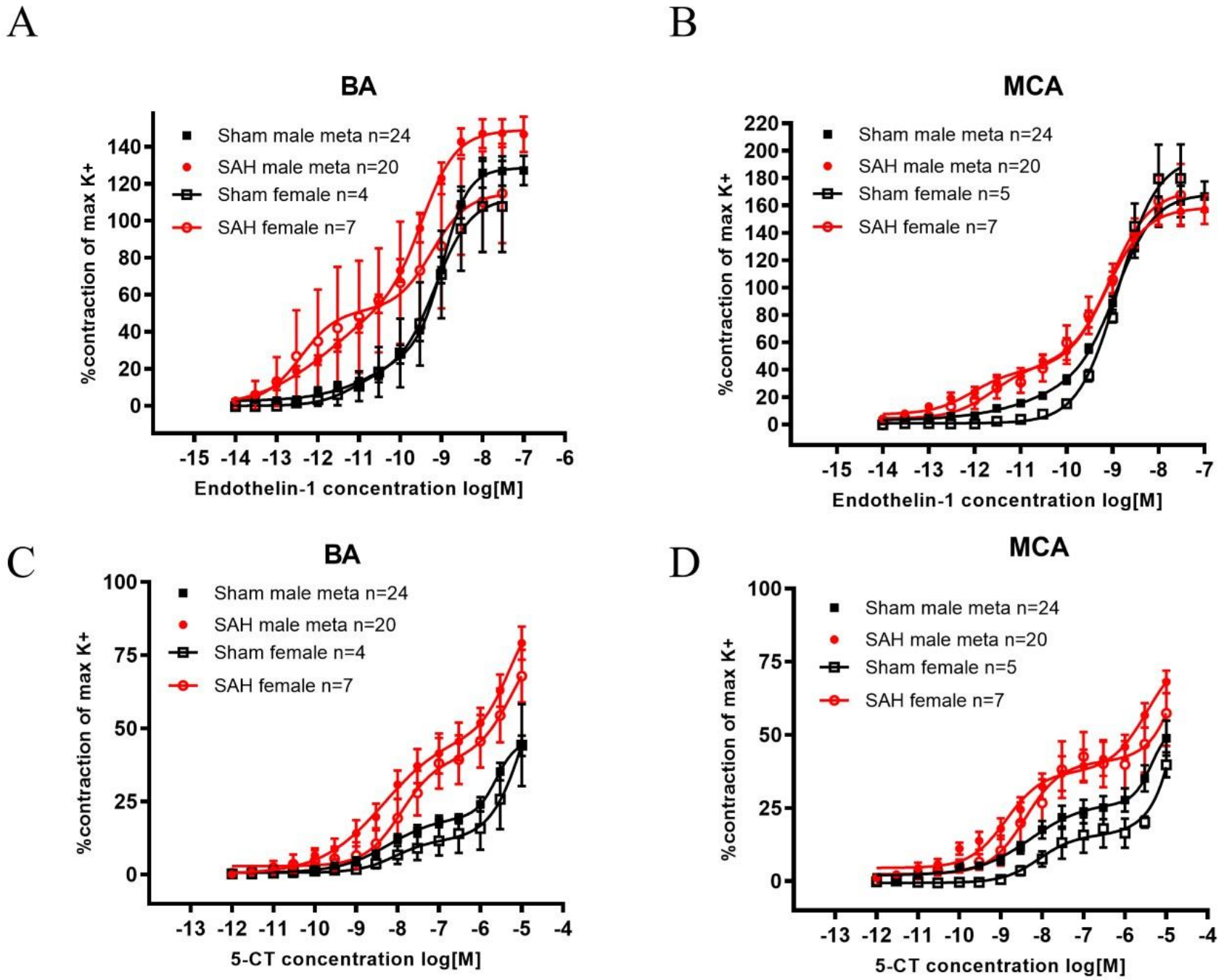

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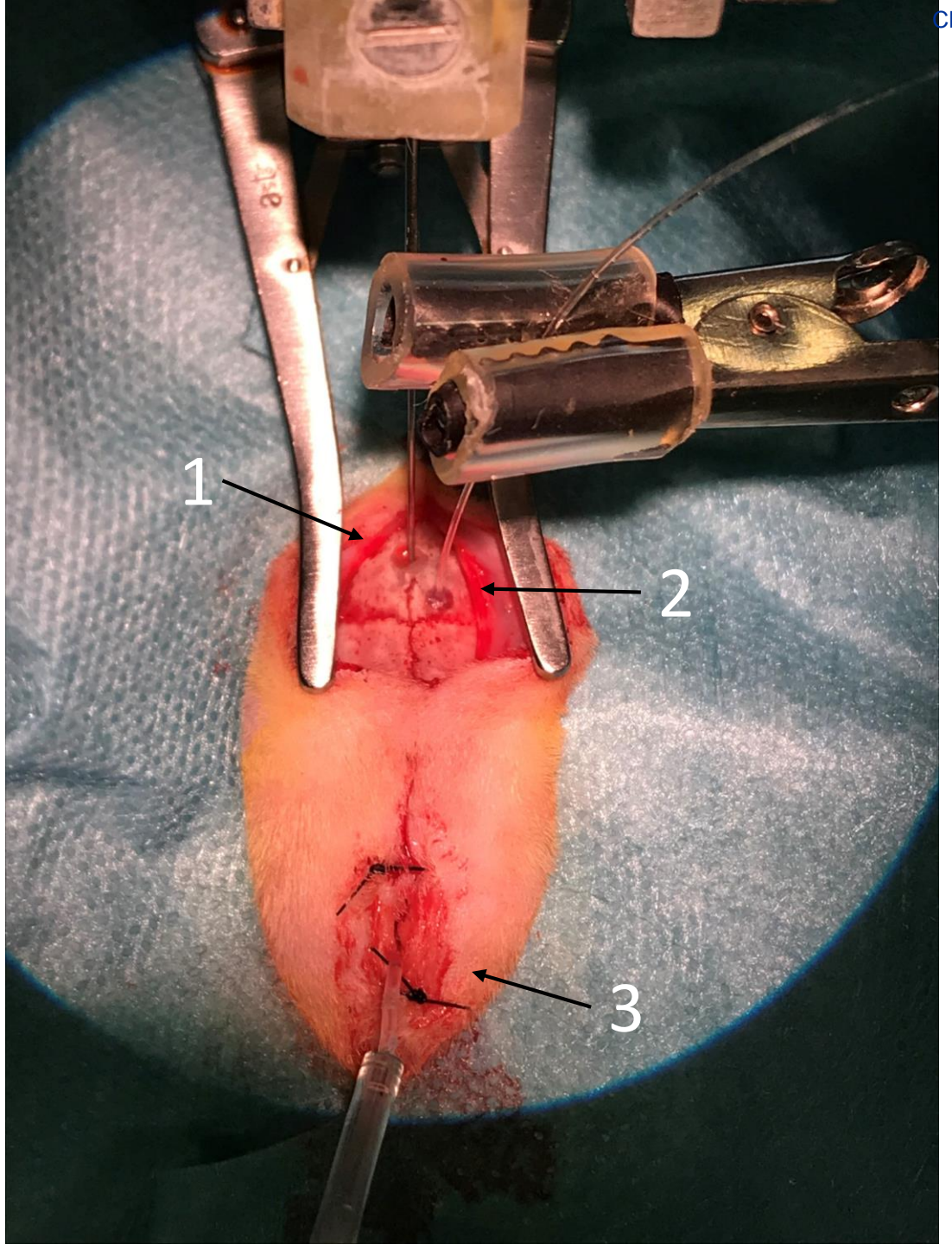

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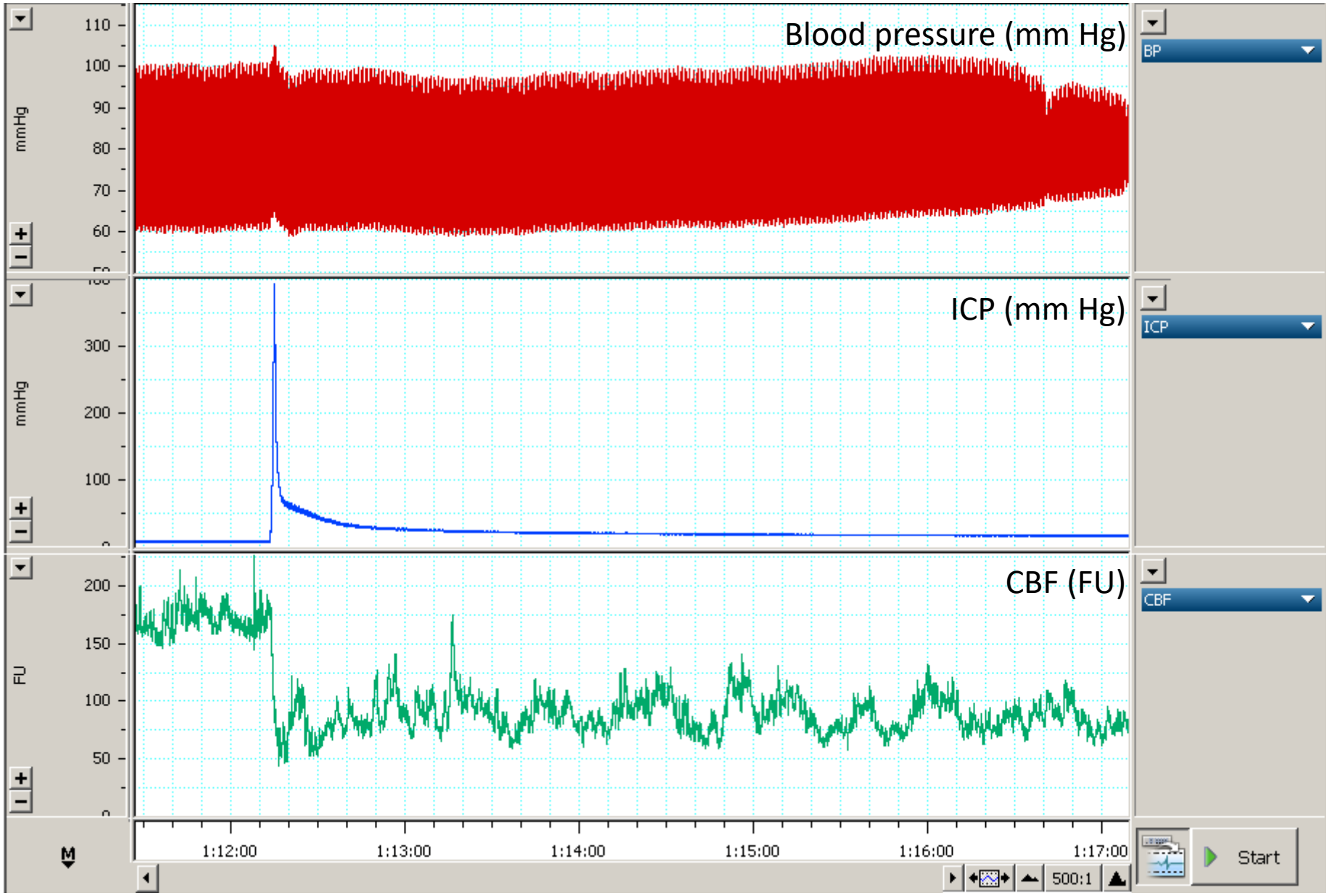

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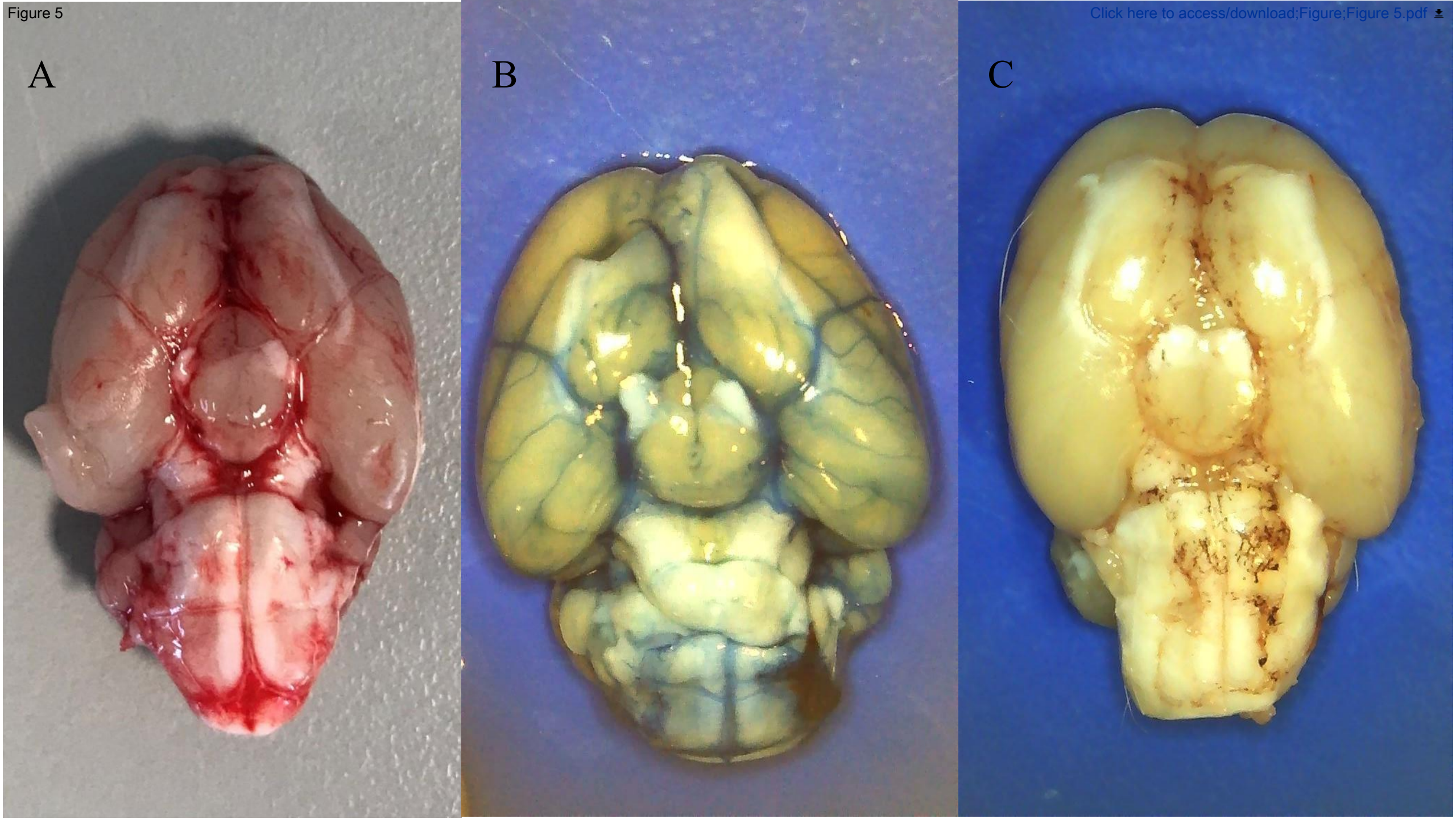

Table of Materials

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Name of material/ Equipment	Company	Catalog Number
16 G peripheral vein catheter	BD Venflon	393229
Anesthesia bell/ chamber	Unknown	
Blood gas analyzer	Radiometer	ABL80
Blood pressure (BP) monitor	Adinstruments	ML117
Curved forceps, 12 cm x 3	F.S.T	11001-12
Cylindrical pillow, 28 cm x 4 cm	Homemade	
Data acquisition hardware	Adinstruments	ML870 Powerlab
Data acquistion software	Adinstruments	LabChart 6.0
Drill	KMD	1189
Drill controller	Silfradent	300 IN
Flexible light	Schott	KL200
Heating pad	Minco	1135
Hypodermic needle, 20 G	KD Medical	301300
ICP monitor	Adinstruments	ML117
Isoflurane vaporizer	Ohmeda	TEC3
Laptop	Lenovo	T410
Laser doppler monitor	Adinstruments	ML191
Laser doppler probe	Oxford Optronics	MSF100XP
Needle holder, 13 cm	F.S.T	12001-13
Precision syringe, 0.025 mL	Hamilton	547407
Stereotaxic frame	Kopf Instruments	M900
Surgical microscope	Carl Zeiss	F170
Suture needle	Allgaier	1245
Temperaure controller	CWE,INC.	TC-1000
Transducer x 2	Adinstruments	MLT0699
Ventilator	Ugo Basile	7025
Veterinary clipper	Aesculap	GT421
3-pronged Blair retractor, 13.5 cm	Agnthos	17022--13
Blunt Alm retractor	F.S.T	17008-07
Curved forceps, 12 cm x 2	F.S.T	11001-12
Needle holder, 13 cm	F.S.T	12001-13
Straight Dumont forceps, 11 cm	F.S.T	11252-00
Straight Halsted-Mosquito hemostat x 2	F.S.T	13008-12
Straight Iris scissor, 9 cm	F.S.T	14090-09
Straight Vannas scissor, 10.5 cm	F.S.T	15018-10
Absorpable swabs	Kettenbach	31603
Black silk thread, 4-0, 5 x 15 cm	Vömel	14757
Bone wax	Aesculap	1029754
Carbomer eye gel 2 mg/g	Paranova	
Cotton swab	Heinz Herenz	WA-1
Cotton tipped applicator x 4	Selefa	120788
Hypodermic needle, 23 G x2	KD Medical	900284
Hypodermic needle, 23 G x3	KD Medical	900284
ICP probe:	Homemade	
Polythene tubing, 20 mm	Smiths medical	800/100/200

Silicone tubing, 10 mm	Fisher	15202710
Silicone tubing, 2 mm	Fisher	11716513
Micro hematocrit tubes	Brand	7493 11
OP-towel, 45 cm x75 cm	Mölnlycke	800430
PinPort adapter, 22 G	Instech	PNP3F22
PinPort injector	Instech	PNP3M
Polythene tubing, 2 x 20 cm	Smiths medical	800/100/200
Rubberband	Unknown	
Scalpel, 10 blade	Kiato	23110
Spinalneedle, 25 G x 3.5''	Braun	5405905-01
Stopcock system, Discofix x 2	Braun	16494C
Suture, 4-0, monofil, non-resorbable x 3	Ethicon	EH7145H
Syringe, 1 mL	BD Plastipak	1710023
Syringe, luer-lock, 10 mL x 4	BD Plastipak	305959
Tissue adhesive glue	3M	1469SB
0.5% Chlorhexidine spirit	Faaborg Pharma	210918
Carprofen 50 mg/mL	ScanVet	43715
Isoflurane	Baxter	
Isotonic saline	Amgros	16404
Lidocaine-Adrenaline 10 mg/5 µg/mL	Amgros	16318

Comments/Description
Needle shortened, distal 1 cm curved. Wings removed
Connects to Powerlab
For anesthesia
Made from surgical towels
Connects to stereotaxic frame
Connects to Powerlab
Connects to laser doppler monitor
For anesthesia
For anesthesia
Connects to BP and ICP monitor
Connects to stopcock. Remove distal end
Remove distal end. 2 connects to stopcock, 1 to syringe
Made of the following:
Inner diameter (ID): 0.58 mm, Outer diameter (OD): 0.96 mm.

ID: 0.76 mm, OD: 2.4 mm.
ID: 1.0 mm, OD: 3.0 mm.
Connects to syringe. ID: 0.58 mm, OD: 0.96 mm.
Connects to transducer
Connects to transducer
Diluted 1:10

Dear Editor,

Hereby comments regarding the editorial revisions

Comment 1-3: Solved

Comment 4: Solved. Note that the lines 201-213 has been rewritten to accommodate and the two subheadings has been increased to three.

Comment 5-7: Solved.

Comment 8: Solved. Please note the point has been increased to two subheadings.

Comment 9: Solved. Please note the point has been increased to two subheadings

Comment 10-12: Solved.

Comment 13: Solved

Comment 14: Solved. Description added to figure legend and *Vascular constriction* section in representative results.

Comment 15-16: Solved

Best regards,

Jesper

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