Four-Vessel Occlusion Model in Rats

Ping Deng and Zao C. Xu

Abstract Four-vessel occlusion model in rat induces transient forebrain ischemia that resembles cardiac arrest in clinical situations. In this model, two vertebral arteries are coagulated and two common carotid arteries are reversibly occluded. This model produces reliable outcome with selective, delayed cell death. For example, CA1 pyramidal neurons in the hippocampus die 2–3 days after ischemia whereas CA3 neurons survive the same insult. The complications of this model include mortality and seizure. The methods of quantitative analysis of ischemic outcome are introduced. The advantages and limitations of this model are discussed.

Keywords Wistar rat · Transient global ischemia · Hippocampus · Striatum · Ischemic depolarization

Model Selection

Four-vessel occlusion (4-VO) rat model has been widely employed to investigate the mechanisms underlying brain damage after transient global ischemia, which mimics the pathological conditions of cardiac arrest. 4-VO is produced by permanent coagulation of two vertebral arteries and temporary ligation of two common carotid arteries. This model induces transient forebrain ischemia because the blood supply to the hindbrain remains relatively intact [1]. Such insult causes severe, reversible forebrain ischemia with consistent neuronal damage in a species that is large enough to allow physiological monitoring and maintenance with relative low costs. The original model involves a 2-day procedure inducing an ischemic insult in awaked animals. Several modified 4-VO models have been developed in the following decades. For example, to perform the 2-day procedure in anesthetized animals [2]; to perform

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all procedures on the same day in anesthetized rats with maintaining brain temperature and recording of ischemic depolarization (ID) [3]; to coagulate vertebral arteries from ventral approach [4] etc.

Materials

Animal

The choice of animal for 4-VO model is male adult Wistar rats. The strain is an important factor affecting the success rate. 4-VO induces severe ischemia in ~80% of Wistar rats [1] whereas only 50–60% of Sprague-Dawley rats exhibit successful ischemia [5]. It is worthwhile to point out that even Wistar rats from different suppliers may exhibit different outcomes. It is therefore necessary to purchase rats with high success rate from the same supplier. The age of the animal is another factor influences the outcome. Neuronal sensitivity to ischemia significantly decreases in neonatal rats due to the developmental nature. The success rate of complete ischemia is significantly reduced in aged rats due to the increase of blood vessel collaterals to the brain. The rats with 200–300 mg in body weight have higher success rate and more consistent outcomes for 4-VO model.

Anesthesia

Anesthetics used for 4-VO models include ether, barbiturates, and isoflurane, etc. Each anesthetic has its side effects, such as lowering the body temperature and metabolism, blocking certain glutamate receptors or ion channels, which might complicate the ischemic pathophysiology and therefore the data interpretation. Isoflurane is recommended because of its relative fewer side effects and rapid recovery.

Equipment

Anesthesia

Isoflurane mixer (Matrx, VIP 3000)[1]
Nasal masks (Fig. 1)

[1] Equipment in parenthesis is the one used in our lab.

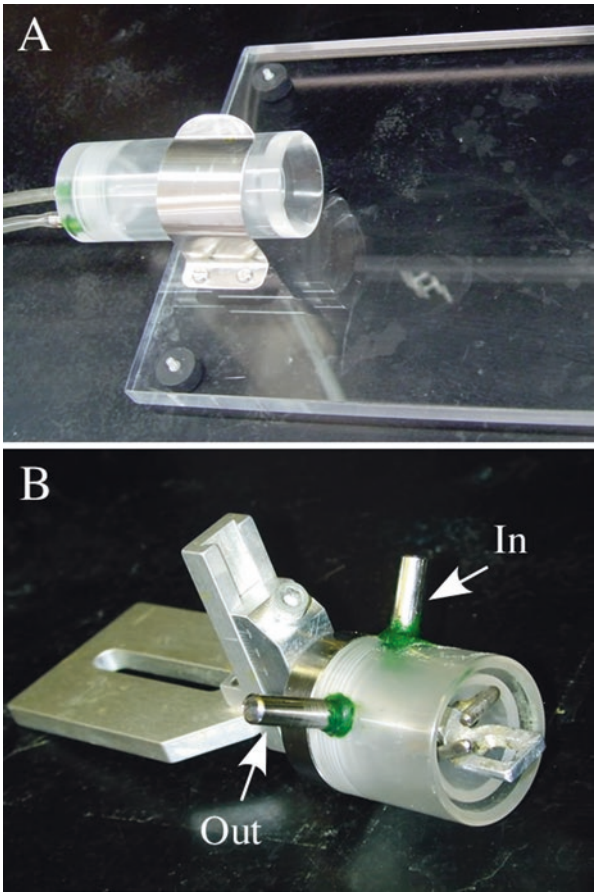

Fig. 1 (**a**) Nasal mask for surgery on ventral neck. (**b**) Nasal mask for stereotaxic apparatus. Both masks have an inlet for anesthesia and an outlet for retrieval of anesthesia by connecting to a vacuum

Surgery

Surgical instruments (scissors, forceps, knives etc.)
Stereotaxic device (KOPF 1430)
Dissecting microscope (Nikon, SMZ-1B)
Driller (Dremel, Multipro, model 395, type 5)
Vessel cauterizer (Cordless soldering iron, ISOTIP, model 7700 with micro-tip)
Buttons (made of Teflon, Fig. 2)
Plastic tube (made of 1 cc syringe, Fig. 2)
Laboratory tubing (SILASTIC, CAT NO. 508-003, OD 1.19 mm 0.025 in., ID 0.64 mm, 0.047 in.)
Hook for occlusion (Ligation aid, model 18062, Fine Science Tools)
Temperature control unit (Temperature controller & alarm, TCAT-1A)
Thermometer (Physitemp, BAT-10)

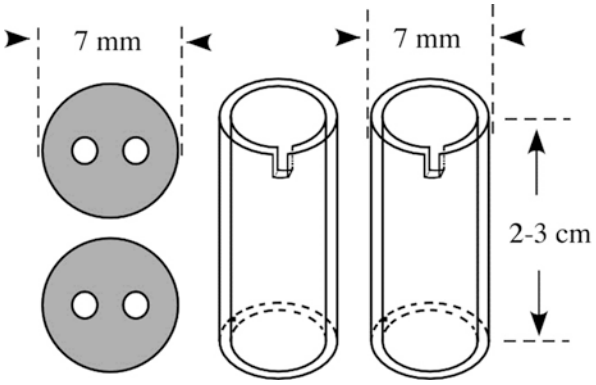

Fig. 2 Drawings of buttons and plastic tube for occlusion

Temperature probe: (Harvard) Rectal: BS4 52-1526; brain: BS4 52-1732 (OD, 0.64 mm)

Recording of Ischemic Depolarization

Amplifier (Neuroprobe, model 1600)
Electrode puller (KOPF model 750)
Electrode (Thin wall glass capillary with filament, OD 2.0 mm, ID 1.5 mm)

Procedures

Original 4-VO Model

Rats are anesthetized with isoflurane (a mixture of 1–2% isoflurane in 33% O_2, 66% N_2), and placed on an operating table. Anesthesia is maintained with a nasal mask.

Placement of Occluding Device Around Carotid Artery

1. Make a ventral midline cervical incision to open the skin.
2. Separate the muscles covering the ventral neck with two small curve forceps to expose the common carotid arteries.
3. Isolate the common carotid arteries free from the vagas nerves and the cervical sympathetic chains.
4. Place the occluding device, a piece of silicone tubing (0.025″ I.D., 0.047″ O.D., 10–15 cm in length), loosely around each common carotid artery and passes through the two holes in a small Teflon button (Fig. 3a).

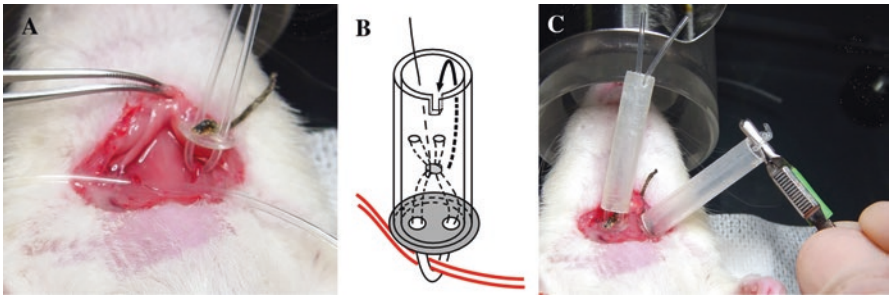

Fig. 3 (**a**, **c**) Photomicrograph of preparation of occluding device. (**b**) Schematic drawing of occluding devices for 4-VO

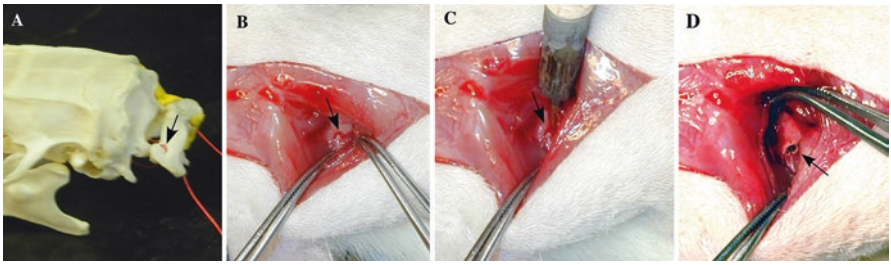

Fig. 4 (**a**) Skeletal specimen showing the alar foramen and vessels (arrow). (**b**) Exposure of alar foramen (arrow). (**c**) Electrocauterization of vertebrate artery. (**d**) Photomicrograph showing the alar foramen after electrocauterization

5. Pass two ends of the silicone tubing through the plastic tube and tie the tubing with a knot to form a loop. The length of the loop should be shorter than the length of the plastic tube that will be used as the supporting apparatus for occlusion. So when the silicone-tubing loop is pulled through the plastic tube, it will press the common carotid artery against the button and stop the blood flow (Fig. 3c).
6. Close the wound with 1–2 suture.

Electrocauterization of Vertebral Artery

1. Place rat on a stereotaxic frame. A dorsal neck incision is made from the occipital bone to the second cervical vertebrae (about 1 cm in length). The paraspinal muscles are separated to expose the alar foramina of the first cervical vertebrae. To improve the exposure of the alar foramina, the cervical spine should be gently extended with a rubber band tied on the rat's tail.
2. Insert the tip of the solder ion into the alar foramen to permanently cauterize the vertebral arteries (Fig. 4). It should be mentioned that the cauterization is crucial for successful ischemia. Heating too long will damage the pons underneath

the cervical vertebrate and increase the mortality, but heating too short will not coagulate the vertebral arteries, resulting incomplete ischemia. There is no perfect method to detect where the vertebral arteries are permanently occluded. In our experience, a small probe with sharp tip, such as syringe needle, is inserted into alar foramina and gently twisted. If bleeding occurs, another cauterization is needed.
3. Close the wounds with surgical clips and return the animal to cage for recovery.

Occlusion of Bilateral Common Carotid Arteries

1. On day 2, the awaked rat (fast over night to provide uniform plasma glucose levels) is gently held by one experimenter and put the animal upside down to expose the ventral neck (rat adapter could be used to secure the head and better expose the operating area).
2. Remove the sutures on ventral neck by another experimenter and pull the silicone tubing loop through the plastic tube and secure the knot to the edge of the tube to completely occlude carotid blood flow (Fig. 5). After occlusion of both common carotid arteries, the rat should become unresponsive and loss its righting reflex within 1 min if the ischemia is completed. The core body temperature should be maintained at 37 °C during occlusion, via a rectal probe coupled to a heating lamp.
3. Release the knot to restore blood flow after occlusion for certain period of time, which depends on the brain region of interest. The animal should recover shortly.

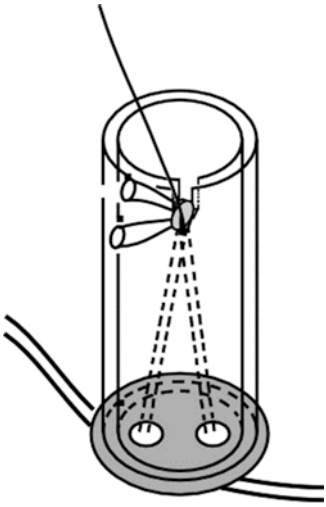

Fig. 5 Schematic drawing of occlusion of common carotid artery

One-Day 4-VO Model (with ID Recording)

Brain temperature is the most important factor influencing the severity of cell death after ischemia. Another potential problem is the collaterals of blood vessels from the skull and neck muscles resulting in incomplete ischemia. This modified 4-VO model resolves these problems by maintain the brain temperature at 37 °C and use ID as an indication of complete ischemia [6].

Surgical Preparation

1. The procedures for placement of occluding devices on common carotid arteries and electrocauterization of vertebral arteries are the same as described above except a surgical suture thread is tied to the knot of the tubing.
2. Drill a burr hole on one side of the skull for placement of temperature probe.
3. Gently insert a tiny temperature probe (0.025″ in diameter) underneath the skull in the extradural space, and the brain temperature is maintained at 37 °C with a heating lamp using a temperature control system (Fig. 6).
4. Drill another hole on the other side of the skull above the brain region of interest. The coordinates for hippocampus are: AP 5.0 mm, ML 2.5 mm; the coordinates for striatum are: AP 9.5 mm, ML 3.0 mm (Interaural).
5. Peal off the dura mater with a sharp tip forceps and keep the brain surface moisture with saline.
6. Put a reference electrode (silver wire) underneath the skin at the opening of the dorsal neck.

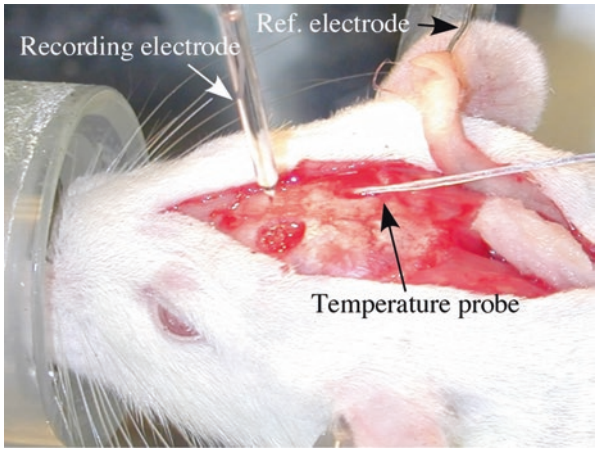

Fig. 6 The operation site of ID recording and temperature monitoring

ID Recording

1. Prepare a glass microelectrode with electrode puller and break the tip of the electrode to yield a tip diameter of 5–10 μm. Fill the electrode with 2 M NaCl.
2. Slowly advance the microelectrode to the region (Hippocampus DV 2.5 mm; Striatum DV 3.0 mm).
3. Connect the recording electrode and reference electrode to the amplifier. Adjust the DC potential to 0 mV.
4. Following baseline recording for 1–2 min, occlude the common carotid arteries bilaterally by pulling the thread and secure the knot to the notch of the plastic tube. A sudden drop of DC potential from 0 mV to ~20 mV should occur 2–3 min after occlusion if a complete ischemia is achieved (Fig. 7).
5. Release the occlusion when ID last for 10 min for studying hippocampus and 20 min for studying striatum. The DC potential should return to 0 mV in 1–2 min.
6. Remove the electrodes and close the wound with surgical clips.

Outcome Evaluation

Several endpoints, including behavioral, physiological and histological features, could be used to assess the brain damage and validate the success of the animal model. Among which, histological changes in ischemia vulnerable regions are the most reliable measurement. Hematoxylin-Eosin (HE) staining of paraffin section is a standard pathological technique and has been widely used to evaluate cell death after transient global ischemia.

Ischemic Cell Death in the Hippocampus

CA1 pyramidal neurons in the hippocampus are the most vulnerable neurons to ischemia. Neurons in the dorsal hippocampus are more sensitive to ischemia than those in the ventral hippocampus. Therefore only sections from the dorsal hippocampus are used for evaluation (Fig. 8). CA1 neurons start to show morphological signs of cell death, i.e. cell body swollen or shrinkage, and nuclear condensation, 2–3 days after ischemia. The cell death begins from the medial part of CA1 zone, gradually progresses towards the lateral part of CA1 zone and stops at the junction of CA3 zone. If the ischemia is relatively mild, the cell death will stop somewhere in the middle of CA1 zone. In contrast, the CA3 pyramidal neurons, dentate granule cells and interneurons in the CA1 region survive the ischemic insult. Another intriguing phenomenon in hippocampus is the delay cell death after ischemia. Approximately 2 days after 10–15 min global ischemia, CA1 neurons start to die and will reach the maximal cell death in 7 days.

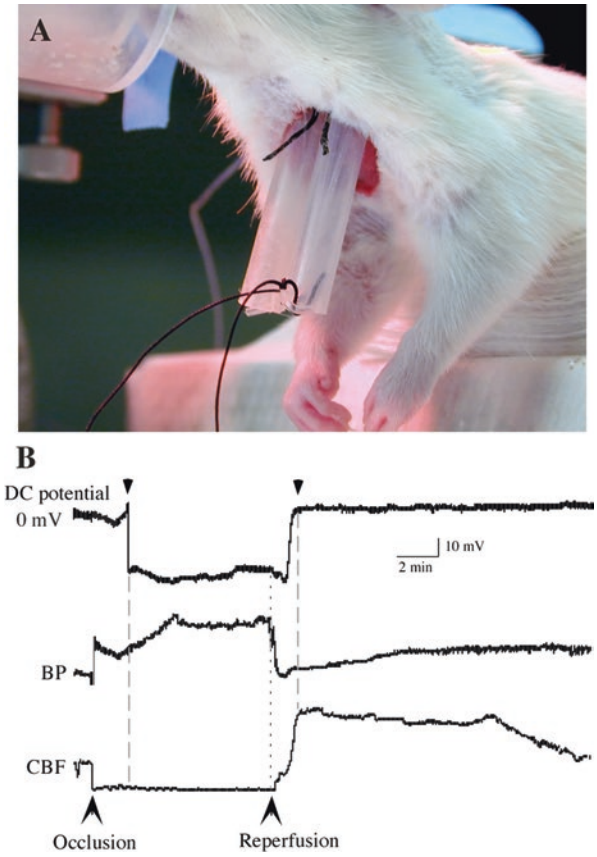

Fig. 7 (**a**) Occlusion of common carotid arteries. (**b**) Traces showing the changes of cerebral blood flow (CBF), arterial blood pressure (BP) and DC potential after ischemia. After occlusion, the blood pressure immediately increases and the DC potential drops to −20 mV (ID). After reperfusion, the blood flow significantly increases (hyperperfusion), the blood pressure and DC potential return to control levels. The ID duration is defined as the distance between the onset of potential drop and the return to 0 mV (arrow heads)

There are many ways to quantify the ischemic cell death. The grading method [7] is the one relatively simple and the results are well correlated to the severity of ischemic insult.

1. Perfuse animal with 4% paraformaldehyde 7 days after ischemia.
2. Embed brain blot containing hippocampus into paraffin.
3. Cut coronal sections of 8 μm thickness of hippocampus and stain with Hematoxylin-Eosin.

   (The details of the above procedure can refer to any histology manual.)

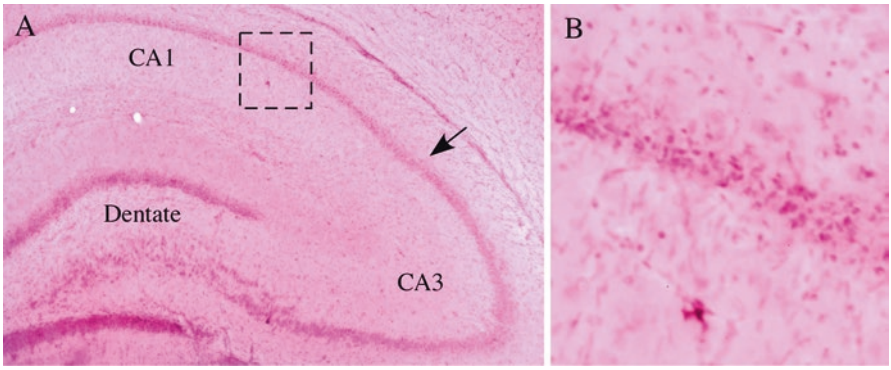

Fig. 8 Photomicrograph of paraffin sections showing the HE staining of the hippocampus 7 days after 10 min forebrain ischemia. (**a**) Most of CA1 pyramidal neurons have degenerated at this time. The arrow indicates the boundary of CA1 and CA3 zone where the cell death stops. (**b**) Higher magnification picture of square area in (**a**) showing the morphological features of cell death in CA1 region after ischemia

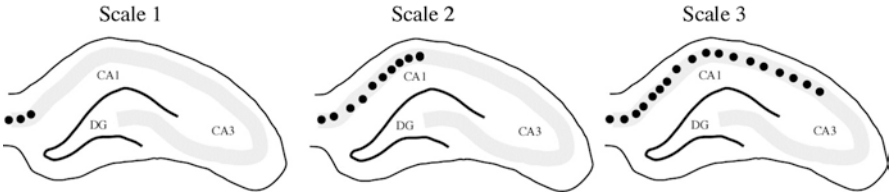

Fig. 9 Schematic drawing showing the grade scale method to quantify the cell death in the hippocampus after transient global ischemia

4. Select sections from the dorsal hippocampus (between AP 4.8–5.8 mm, interaural or AP -3.3~−4.3 mm, Bregma) and grade the damage with the following scale:

   No damage (control) = 0;
   A few neurons damage = 1;
   Many neurons damage = 2;
   Majority of neurons damage = 3.

   According to the natural curves in the coronal plane of the CA1 cell layer, the cell death within the medial portion (subiculum) is defined as scale 1. The cell death reaches the middle part of CA1 zone (the peak of the dorsal curve) is defined as scale 2. The cell death reaches the junction of CA3 zone is defined as scale 3 (Fig. 9). Cell death progresses to between these landmarks could be defined as 1.5 or 2.5 respectively. This method could obtain reliable results across the experimental groups [8].

5. Evaluate at least four sections from each hemisphere. Get average grading from two hemispheres of each animal and use it as the score of that animal.
6. Compare the data between groups using nonparametric Mann-Whitney U-test.

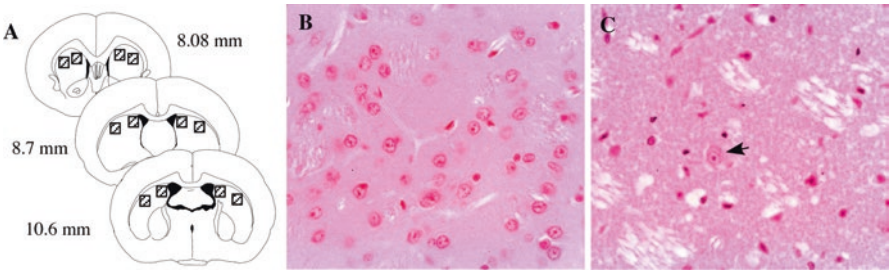

Fig. 10 (**a**) Schematic drawing showing the section plane and selected region (square) for cell counting. (**b**) HE staining of striatal section of a control rat. (**c**) HE staining of section 1 day after 20 min ischemia. Moat of the cells have died but some interneurons (arrow) remain viable

Ischemic Cell Death in the Striatum

In the striatum, small to medium-sized spiny neurons are highly sensitive to transient global ischemia. It requires ~25 min ischemia to induce cell death in the dorsal striatum. The cell death starts about 6–8 h after reperfusion and most of them die in 24 h. The neurons in the dorsolateral striatum are more vulnerable than those in the ventrolateral striatum. Interneurons in the striatum are resistant to ischemia (Fig. 10b, c). Quantification of cell death in the striatum is achieved by counting the number of survived neurons in a given area.

1. Prepare paraffin sections as described above.
2. Select four sections at the anterior, middle and posterial plans (AP 10.6 mm, 8.7 mm and 8.8 mm, interaural). Identify a region in the dorsomedial and a region in the dorsolateral striatum with the same size and approximately the similar location (Fig. 10a).
3. Count the number of survived neurons in the selected region.
4. Compare the data between groups using nonparametric Mann-Whitney U-test.
5. Get average grading from two hemispheres of each animal and use it as the score of that animal.

Advantages, Complications and Limitations

Advantages

1. Rat is widely used in neuroscience research so the results can be compared to the previous studies.
2. 4-VO model in rat is well established and yields reproducible neurophathological results.

3. The species is large enough to allow relatively easy surgery, physiological monitoring and maintenance.
4. Low costs in purchasing and maintenance.

Complications

Developing seizure is a common complication after cerebral ischemia. The incidence of seizure is positively correlated to the severity and duration of ischemic insult. In Wistar rats, a 20 min ischemia might induce seizure in 20–30% of the animals. The brain damage caused by seizure is different from that caused by ischemia. Therefore, the animals with seizure after ischemia must be identified and excluded from the study. One of the signs of seizure is the spreading of the flooring materials around the cage because these materials are kicked out of the cage due to the seizure activities.

Limitations

Technical Difficulties

In some animals, the alar foramina could deform or disappear, making the electrocauterization impossible. The more difficult task is the successful electrocauterization of the vertebral arteries that are hidden underneath the alar foramina. Unsuccessful electrocauterization is the major cause of incomplete ischemia. Therefore it is essential to make sure that the vertebral arteries are cauterized and the blood flow is completely stopped using the methods described above.

Recording of DC potential change during ischemia might be another difficulty for someone doesn't have experience in electrophysiology. Actually it is not as difficult as it sounds. Recording of ID only involves very basic electrophysiological technique and can be easily learn and master.

Mortality

Approximately 10% of the rats will die either during ischemia or shortly after reperfusion even in experienced hands. The respiratory failure during the first 2–3 min of occlusion is a major complication that contributes to animal death during ischemia. Immediate resuscitation normally rescues the animals. Death after reperfusion may stem from severe ischemia, cerebral vesicular blood clot, combined with surgical trauma in the neck and head. The mortality of stains other than Wistar rats might be even higher.

Variation

Despite the reproducible outcomes, the results of 4-VO might vary among animals. Even in animals with successful ischemia, the severity of cell death between two hemispheres might be different. Many factors, including cerebrovascular collaterals, brain temperature, brain glucose, anesthesia, oxygen and carbon dioxide tension etc., influence the ischemic outcome [9]. It is important to strictly control the variables and optimize the experimental conditions in order to obtain consistent results.

References

1. Pulsinelli WA, Brierley JB. A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke. 1979;10(3):267–72.
2. Schmidt-Kastner R, Paschen W, Ophoff BG, Hossmann KA. A modified four-vessel occlusion model for inducing incomplete forebrain ischemia in rats. Stroke. 1989;20(7):938–46.
3. Xu ZC, Pulsinelli WA. Responses of CA1 pyramidal neurons in rat hippocampus to transient forebrain ischemia: an in vivo intracellular recording study. Neurosci Lett. 1994;171(1–2):187–91.
4. Yamaguchi M, Calvert JW, Kusaka G, Zhang JH. One-stage anterior approach for four-vessel occlusion in rat. Stroke. 2005;36(10):2212–4.
5. Ginsberg MD, Busto R. Rodent models of cerebral ischemia. Stroke. 1989;20(12):1627–42.
6. Xu ZC. Neurophysiological changes of spiny neurons in rat neostriatum after transient forebrain ischemia: an in vivo intracellular recording and staining study. Neuroscience. 1995;67(4):823–36.
7. Pulsinelli WA, Brierley JB, Plum F. Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol. 1982;11(5):491–8.
8. Xu ZC, Gao TM, Ren Y. Neurophysiological changes associated with selective neuronal damage in hippocampus following transient forebrain ischemia. Biol Signals Recept. 1999;8(4–5):294–308.
9. Pulsinelli WA, Jacewicz M. Animal models of brain ischemia. In: Barnett HJM, Stein BM, Mohr JP, Yatsu FM, editors. Stroke: pathophysiology, diagnosis, and management. New York: Churchill Livingstone; 1992. p. 49–64.