Video Article

Induction of Ischemic Stroke and Ischemia-reperfusion in Mice Using the Middle Artery Occlusion Technique and Visualization of Infarct Area

Luc Bertrand[1,2], Levi Dygert[2], Michal Toborek[1,3]

[1]Department of Biochemistry and Molecular Biology, Miller School of Medicine, University of Miami

[2]Miller School of Medicine, University of Miami

[3]Jerzy Kukuczka Academy of Physical Education

Correspondence to: Michal Toborek at mtoborek@med.miami.edu

URL: https://www.jove.com/video/54805
DOI: doi:10.3791/54805

Keywords: Medicine, Issue 120, stroke, ischemia, MCAO, mouse, microsurgery, tissue imaging

Date Published: 2/2/2017

Citation: Bertrand, L., Dygert, L., Toborek, M. Induction of Ischemic Stroke and Ischemia-reperfusion in Mice Using the Middle Artery Occlusion Technique and Visualization of Infarct Area. *J. Vis. Exp.* (120), e54805, doi:10.3791/54805 (2017).

Abstract

Cerebrovascular disease is highly prevalent in the global population and encompasses several types of conditions, including stroke. To study the impact of stroke on tissue injury and to evaluate the effectiveness of therapeutic interventions, several experimental models in a variety of species were developed. They include complete global cerebral ischemia, incomplete global ischemia, focal cerebral ischemia, and multifocal cerebral ischemia. The model described in this protocol is based on the middle cerebral artery occlusion (MCAO) and is related to the focal ischemia category. This technique produces consistent focal ischemia in a strictly defined region of the hemisphere and is less invasive than other methods. The procedure described is performed on mice, given the availability of several genetic variants and the high number of tests standardized for mice to aid in the behavioral and neurodeficit evaluation.

Video Link

The video component of this article can be found at https://www.jove.com/video/54805/

Introduction

The study of cardiovascular disease, such as stroke, relies on the use of *in vivo* models. To understand the possible implication of ischemia, drug toxicity, and/or treatment, there is a need to use a suitable, standardized, reliable, and reproducible model of the disease, which enables comparative studies between treatment groups. In this manuscript, we are using mice, given the availability of a large number of transgenic mice and standardized assessment models. Raking scores to assess motor and behavior deficits following experimental ischemic stroke and the following recovery have been developed[1,2].

Several ischemic stroke models are available, such as complete global cerebral ischemia, incomplete global ischemia, multifocal cerebral ischemia and focal cerebral ischemia. The latter group is also the category of stroke most prevalent in patients. The majority of events are initiated by the formation of an embolic or thrombotic occlusion at or near the middle cerebral artery (MCA). Given these parameters, the model presented closely mimics disease etiology of human stroke and makes results obtained highly relevant[3]. Nevertheless, the translation of discoveries from animal models to disease treatment in humans has proven to be challenging. Up to now, only the use of thrombolytic tissue plasminogen activator has been approved for treatment of acute ischemic stroke[4].

Among models of focal cerebral ischemia in mouse, posterior cerebral circulation stroke model and cerebral venous thrombosis model are highly invasive, diminishing their applicability and restricting the range of analyses that can be performed. However, other techniques, such as the embolic model, photothrombosis model, endothelin-1 induced stroke model, and intraluminal suture middle cerebral artery occlusion (MCAO) model, are available for use without such limitations. The MCAO model is a technique described in this protocol. It offers a reliable method of inducing focal cerebral ischemia that can be readily reperfused and performed in a high-throughput manner. There are two approaches to this model, namely, the Zea-Longa and Koizumi methods. They differ slightly in the way the occlusion suture is inserted in the vasculature. In the Zea-Longa technique, the suture is inserted via the external carotid artery[5]. The technique presented here is modified from the Koizumi method in which the occluding suture is inserted via the common carotid artery[6].

The MCAO model has been successfully applied to evaluate different events occurring during ischemic stroke. Following reperfusion, brain edema can be observed along with the breakdown of the blood-brain barrier. Peak neuronal death is usually observed at 24 hr; however, it returns to baseline levels after 7 days[7]. In humans, sex and age are important variables when determining stroke outcome, this is also observed in mice and rats[8,9,10]. Several publications have used the MCAO model to demonstrate treatment efficiency[11,12,13,14].

Protocol

All procedures were approved by the University of Miami Institutional Animal Care and Use Committee (IACUC) in accordance with the National Institutes of Health (NIH) guidelines. The use to sterile equipment and aseptic techniques is required.

1. Preparing the Occlusion Suture

1. Use a suture of 0.21 mm diameter for mice between 20 - 25 g and 0.23 mm for mice between 25 - 35 g of body weight. The choice of the type of sutures for the MCAO procedure depends on animal weight.
2. Using a silver pen, mark the suture at 9 mm starting from the silicone coated tip. This will serve as a guide for insertion length.

2. Preparation for Surgery

1. Anesthetize mouse with isoflurane mixed with oxygen, using a laboratory anesthesia system. Use isoflurane at setting 5 and oxygen flow at 2 on the commercial machine (See **Materials Table**). Transfer the animal to the surgery surface and sustain anesthesia using a nose cone (use isoflurane setting 1.5 - 2.5 and oxygen flow at 2).
    1. Ensure that the mouse respiratory rate is around 1 - 2 respiration per sec without gasping. In addition, ensure that the animal does not exhibit whiskers stimulation reaction and pedal reflex (toe pinch). Monitor respiration rate and effort during the surgery, at least every 5 min.
2. Put a drop of ophthalmic lubricant on each eye using a sterile swab to prevent them from drying during the procedure. Alternatively, apply ophthalmic ointment to the eye from a sterile pharmaceutical tube.
3. Flip the animal on its back and shave the incision area. Afterward, thoroughly disinfect the surgery area using 70% ethanol, followed by Chlorhexidine, and the final swab with 70% ethanol. Place the animal on a warm surgical surface under a stereomicroscope.

3. Dissection of the Common Carotid Artery and Internal/External Branching

1. Using surgical scissors and forceps, perform a shallow midline incision in the neck, from above the breast bone to below the jaw (approximately 3 - 4 cm). Surgical drape should be placed around the surgery area to prevent instruments from coming in contact with non-sterile surfaces. The surgical drape was omitted in the current video to aid filming.
2. Using forceps, carefully separate fatty and connective tissue to expose the trachea. Tissue should easily and naturally separate to both sides.
3. Place a pillow (round object, about 0.5 cm in diameter) on the back of the neck of the mice to extend the neck, further exposing the area for surgery.
4. Open the incision using either a tissue retractor or hooks.
5. On the animal's left side of the trachea, carefully tweeze apart the connective tissue to expose the left common carotid artery (CCA). Be careful not to damage nerves and major veins bundled with the CCA.
6. Continue exposing the CCA, being careful to detach from underlying tissue, and expose the top "Y" branching of the internal cerebral artery (ICA) and external cerebral artery (ECA). To aid in detaching the CCA, insert the curved forceps under the CCA to pierce the connective tissue and then slowly allow them to open.

4. Preparation of the CCA for the MCAO Suture Insertion

1. Insert 3 segments of nylon suture of about 4 cm in length under the CCA. Ensure that the CCA is not twisted as this would drastically complicate the insertion of the suture.
2. At the lowest point possible, close the bottom suture using a permanent knot.
3. Tie the top suture just below the ICA/ECA branching using a removable slip knot.
4. Tie the middle suture using a removable slip knot, but keep it wide open. It is important to leave plenty of space as not to hinder suture insertion.
5. Using microdissection spring scissors, perform an incision in the CCA between the bottom and middle sutures. Perform the 0.2 mm incision close to the bottom suture.

5. Middle Cerebral Artery Occlusion

1. Using forceps, insert the MCAO suture into the CCA incision, guiding it up to the top. Perform this step quickly after step 4.5 to prevent clotting and closing of the CCA opening. In the event that suture insertion is blocked, use the tips of forceps to reopen the incision.
2. Gently tie down the middle suture on the MCAO suture silicone part using a slip knot to restrict blood flow around it, but loose enough to allow it to move freely.
3. Carefully undo the top suture, making sure that the MCAO suture does not slip out.
4. Insert the MCAO suture in the ICA by a couple of millimeters and then reclose the top suture, in the same manner as described in step 5.2.
5. Guide the MCAO suture to the occlusion area. Indication of successful insertion is evident by the low amount of blood backflow from the CCA incision and that the silver 9 mm mark is situated between the CCA incision and the ICA/ECA bifurcation. Further confirmation of successful occlusion can be obtained using monitoring methods such as a laser Doppler blood flow monitoring system[15,16].
   NOTE: A drop in 90% of blood flow to the middle brain area on the occluded area side indicates successful occlusion.

6. After successful occlusion, tie down the middle and top suture tightly. If performing sham insertion, do not reclose sutures but instead immediately skip to step 7.3.

6. Incision Closing and Post-operative Care

1. Tuck in the sutures into the incision area.
2. Remove retractor or tissue hooks and pillow.
3. Clean suture area using sterile saline and a cotton swab.
4. Close the incision using nylon suture/needle and forceps.
5. Administer anti-inflammatory (*e.g.*, Carprofen 10 mg/kg, s.c.) and analgesic (*e.g.*, Buprenorphine 0.1 mg/kg s.c., twice a day) drugs to alleviate post-operative discomfort. Place the mice in a cage placed on a heated pad to prevent hypothermia and give ad labium access to water and softened food.
6. Monitor the recovery of animals (5 - 10 min) and for signs of stroke. They can vary from mild lateral paralysis and circling to severe contraction of the contralateral flank and rolling. Several evaluation points are listed in the discussion. Animals displaying signs of respiratory distress or severe seizures should be euthanized.
7. Depending on ischemia reperfusion protocol, leave the MCAO suture in place from 30 min to 120 min or more. Reducing occlusion time prevents mortality.
8. For permanent occlusion, leave the MCAO suture in place for 24 hr, however significant mortality rate is to be expected. After this time lapse, proceed to step 7.
    1. If using permanent occlusion model, keep mice in cage until end point. At that time, remove suture (steps 7.1 to 7.8). This procedure can also be performed after euthanasia.

7. Reperfusion

1. Anesthetize the animal again (follow instructions in 2.1) and remove wound closing sutures.
2. Using forceps and tissue separators, re-open the incision and expose the CCA.
3. Carefully remove the top suture and gently pull on the MCAO suture until the silicone coated part is situated at the middle knot.
4. Redo the top knot on the suture to prevent blood from flowing pass the suture (it does not have to be a slip knot).
5. Carefully undo the middle knot and pull the suture past the top knot, but keep it inside the CCA.
6. Tightly close the top knot to block artery blood flow.
7. Pull out completely the MCAO suture and close tightly the middle knot.
8. The MCAO suture can be re-used several times. After each use, clean the suture carefully in 70% ethanol to remove contaminants (*e.g.*, blood or tissue) using a sterile gauze. Afterwards, place the suture in a sterilizer pouch and sterilize it.
9. Repeat steps 6.1 - 6.4 and monitor animal recovery.

8. Tissue Analysis

1. To monitor stroke volume, dissect out the brain.
    1. Euthanize the mouse using a two-step process. First, expose the animal to an overdose of isoflurane until breathing stops. For TTC staining, decapitate the mouse immediately and proceed to brain extraction. For brain sectioning (*e.g.*, for immunostaining analyses), perform perfusion via a cardiac puncture prior to decapitation.
    2. Cut-off the head at the base of the skull and cut the scalp from the neck opening to the top of the skull up to between the eyes. With the skull exposed, cut the bone along both sides starting from the skull base opening up to the eye socket. Make this cut along the wide part of the skull. Be careful not to reach too deep with scissors to prevent damaging the brain.
    3. Afterwards, cut the bone between the two eye sockets.
2. Using forceps, lift up the top of the skull to expose the brain. There should be only a small resistance. If it does not lift easily, skull incisions may be incomplete. Pry out the brain gently using blunt curved forceps. The brain might still be attached by several nerves. Proceed slowly, removing attachments along the way.
3. Place the fresh brain in a 1 mm brain matrix, add a couple of drops of PBS, and slice along the matrix using a razor blade or cryostat blade.
4. Transfer the brain to a 100 mm dish and add staining solution (2% 2,3,5-triphenyltetrazolium chloride [TTC] dissolved in PBS) to cover the bottom of the dish. Using two forceps, separate all brain slices (front facing down) and place them in order at the bottom of the dish.
   NOTE: Staining will turn viable tissue red, with non-viable tissue remaining white. Warming the solution to 37 °C will accelerate staining. Signs of stroke area (white) can be seen as quickly as 90 min post stroke and remain visible for more than 7 days. Imaging should be done along with a ruler to allow calculation of stroke volume.
5. Process dissected brains for sectioning, immuno-staining, protein isolation, or a variety of other procedures[17,18,19].

Representative Results

The insertion route for the occlusion suture is demonstrated in **Figure 1**. The MCAO suture is to be routed to the occlusion area, bifurcating in the ICA. Successful occlusion of the MCA will lead to tissue injury, visible by TTC staining. **Figure 2** presents images of staining from sham treated animal (**Figure 2A**) and from a 60 min MCAO ischemia reperfusion animal (staining at 90 min or 24 hr post-occlusion, **Figure 2B**). To determine stroke volume, first calculate the stroke area for each section, using the ruler included in the imaging procedure, by subtracting the non-infarcted area of the ipsilateral side from the total area of the contralateral side. This calculation can be performed using commercial software or open source software. Afterward, calculate stroke volume for each slice taking into consideration that slides are 1 mm thick and sum the stroke volumes for all slices. This procedure will provide the total stroke volume for each animal, which can then be compared between different animal groups and treatments. On average, a 60 min occlusion followed by 23 hr of reperfusion results in a stroke volume of about 21 ± 3 mm^3 in wild type non-treated C57BL/6J mice of 15 weeks old.

In addition to investigating stroke volume, immunofluorescence can be performed for a multitude of targets, such as microtubule-associated protein-2 (MAP2) for neuronal injury (**Figure 3A**) or glial fibrillary acidic protein (GFAP) for astrogliosis (**Figure 3B**). Such analyses further enable the analysis of stroke progression and recovery as well as other processes involved in stroke tissue damage and repair.

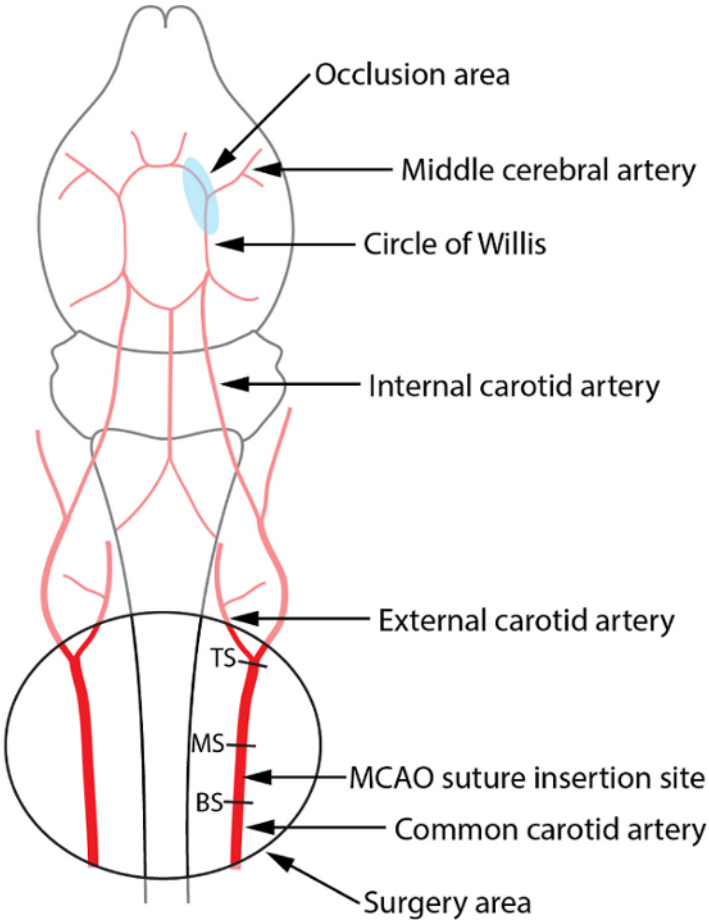

Figure 1: Schematic Representation of Brain Arterial Physiology. Route of the MCAO insertion proceeds from the common carotid artery (CCA) to the occlusion area indicated in blue. The surgery area is located at the bottom of the figure (Oval shape) and suture placement is indicated by black lines (TS: Top suture; MS: Middle suture; BS: Bottom suture). Please click here to view a larger version of this figure.

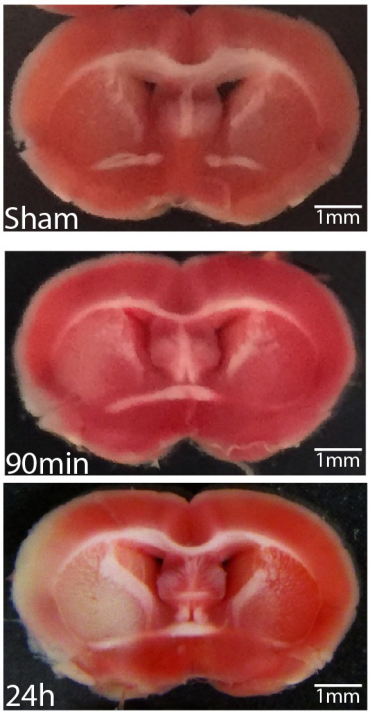

Figure 2: TTC Staining of Brain Sections. Top panel represent typical staining following sham insertion. Middle and bottom panels were obtained after a 60-minute MCAO, followed by 90 min or 24 hr of reperfusion. Scale bar is 1 mm.

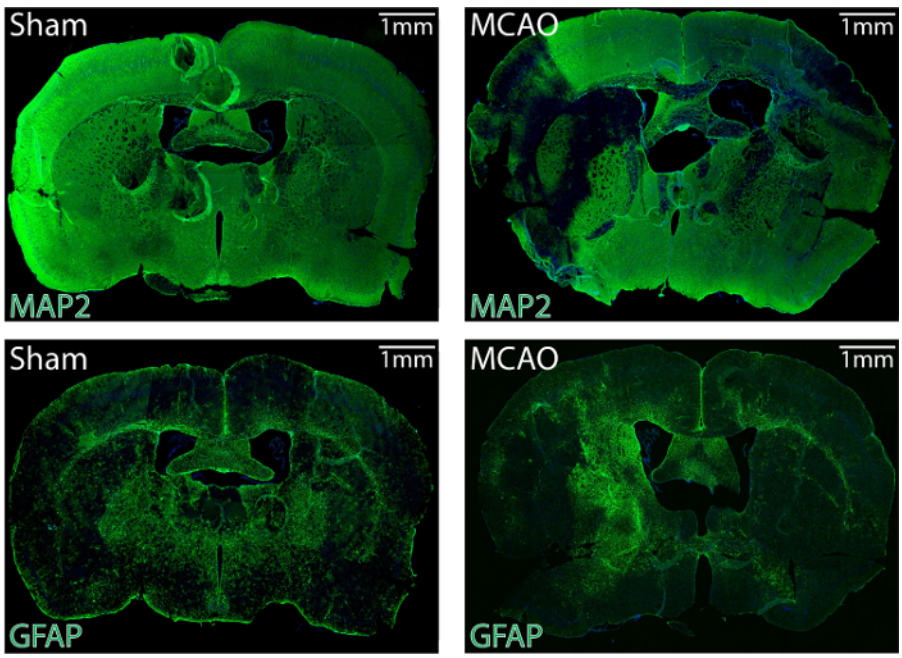

Figure 3: Immunofluorescence Staining of Mouse Brain Following Stroke. Following a 60-minute occlusion, brains were reperfused for 23 hr, harvested, and processed for cryosectioning. Using antibodies to MAP2 (top) and GFAP (bottom), tissue was analyzed for neuronal injury and astroglyosis in either sham (left panels) or 90 min ischemia-reperfusion brains (right panels). Pictures presented are combined from multiple images taken by confocal microscopy using a 10X objective. Scale bar is 1 mm. Please click here to view a larger version of this figure.

Criteria of Evaluation		
General Condition	Hair condition	Self-cleaning behavior (0: None; 2: normal)
(0: Worst; 2: Normal)	Ears	Ears position (Droopy or raised)
		Hearing (Reactive or not)
	Eye condition	Eyelid position
		Response
	Posture	Crawling, leaning, normal
	Spontaneous activity	Unconscious/no movement, low activity, normal activity
Neurodeficit	Body symmetry	When mouse is still (No movement, crawling, circling, partial leaning, normal)
(0: Worst; 4: Normal)	Gait	No movement, rotating, circling, walking to one side, normal
	Climbing	Walking behavior on angled path (Same criteria as Gait)
	Circling behavior	Lifting animal by the tail (no movement, swirling, contracting to one side, both sides but with preference, normal)
	Front limb symmetry	Look at grabbing behavior of front paws (No grabbing at all, only one side grabs, both grab but one paw rigid, both grab but one continually loose first, normal)
	Compulsory circling	Put mouse on flat surface and push on shoulder from the side (No response/ movement, rotating, falling to one side, leaning with resistance to push, normal)
	Whisker response	Touch whiskers one side at the time (No response, whiskers movement only, turn head, turn trunk, normal)
	Epileptic behavior	Behavior after sudden noise or light change (Unconscious, consistent general tonic spasm, transient general tonic spasm, transient focal tonic spasm, normal)

Table 1. Scale for the Evaluation of Mouse Condition and Neurodeficit Following Ischemia Stroke. Adapted from[1,2].

Discussion

The successful utilization of the described MCAO method is highly dependent on an understanding of cerebral blood flow anatomy. Since the correct placement of the suture is hard to discern due to the lack of direct visual clues, repeated practice is important to master the procedure before using it for investigative studies. Stroke volume should be analyzed to ensure consistent results. The addition of a laser Doppler system can help to determine the successful occlusion of blood flow and should be used periodically to ensure that the procedure is done correctly. Routing the MCAO suture to the occlusion area can be facilitated by manipulating the artery. To help in guiding the suture to the MCA, once it has just passed the ECA/ICA branching, press against the ICA slightly above the branching (2-3 mm) with forceps to guide the suture to the left/down and prevent it from going into the pterygopalatine artery. In addition, moving the pillow placed in step 3.3 or pulling the bottom knot up and to the right can help in orienting the artery to ease the insertion. The remaining steps of the protocol involve microsurgery under stereomicroscope and are fairly straightforward. The success rate of the method described, as obtained by our group and reported by others, is of around 80 - 90%. Nevertheless, several factors can influence the survival, including body temperature control and suture selection [20,21,22]. The maintenance of animal body temperature during surgery is important to improve animal survival.

Depending on the experience of the user, this method can be employed in a high throughput manner to study a large cohort of animals. The statistical significance of animal studies depends on the usage of sufficient number of subjects to discern between treatment groups despite intrinsic variation between animal subjects. The protocol presented enables such studies while recreating closely parts of the disease process.

The advantage of the MCAO technique is that, while it involves some surgical procedures, it does not require highly invasive procedures as in the craniotomy model. In addition, it is highly reproducible and the reperfusion is highly controllable, which is not possible using the endothelin-1 or the embolic stroke models. The technique closely mimics the process of a human ischemic stroke and produces characteristic tissue injury seen in humans, which is not the case for the photothrombosis model. As compared to other published techniques, the MCAO procedure does not require the cauterization of the ECA. This is an advantage since it can be combined with our ICA infusion model to deliver therapeutic agents to the affected hemisphere following stroke induction [23,24,25].

In addition to tissue analysis, animal behavior can be monitored to assess longitudinally the stroke severity and recovery. This can help compare recovery between different treatment groups. Several approaches have been developed in order to evaluate behavior. A simple 5 point scale can be used to evaluate neurological deficit post stroke (0: No behavior deficit; 1: No contralateral forepaw extension; 2: Circling on the contralateral side of infarct; 3: Falling on the contralateral side of infarct; 4: Low consciousness level and spontaneous movement) [5]. In addition, in depth analysis of animal condition and behavior can be conducted to assess recovery as described in **Table 1**[1,2].

The surgical procedure presented uses a mechanical method for the induction of stroke, which limits utilization of this technique in studies on stroke susceptibility and/or causative agents. However, this protocol can be remarkably useful in analyzing stroke severity, preventive or mitigating strategies, aggravating factors and possible therapeutic approaches post-stroke. Comparison between treatment groups can help in identifying potential treatment that may minimize stroke damage or accelerate recovery. Indeed, enhancing recovery would be of great importance in helping stroke patients.

Disclosures

Authors have no competing or conflicts of interest.

Acknowledgements

We would like to thank Dr. Lei Chen (Icahn School of Medicine at Mount Sinai, NY) who first established this model in our laboratory. Supported in part by HL126559, DA039576, MH098891, MH63022, MH072567, DA027569, and NSC 2015/17/B/NZ7/02985. Dr. Luc Bertrand is supported in part by a postdoctoral fellowship from the American Heart Association (16POST31170002).

References

1. Cuomo, O. *et al.* Antithrombin reduces ischemic volume, ameliorates neurologic deficits, and prolongs animal survival in both transient and permanent focal ischemia. *Stroke.* **38** (12), 3272-3279 (2007).
2. Wauquier, A., Melis, W., & Janssen, P. A. Long-term neurological assessment of the post-resuscitative effects of flunarizine, verapamil and nimodipine in a new model of global complete ischaemia. *Neuropharmacology.* **28** (8), 837-846 (1989).
3. Liu, F., & McCullough, L. D. Middle cerebral artery occlusion model in rodents: methods and potential pitfalls. *J Biomed Biotechnol.* **2011** 464701 (2011).
4. Chiu, D. *et al.* Intravenous tissue plasminogen activator for acute ischemic stroke: feasibility, safety, and efficacy in the first year of clinical practice. *Stroke.* **29** (1), 18-22 (1998).
5. Longa, E. Z., Weinstein, P. R., Carlson, S., & Cummins, R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke.* **20** (1), 84-91 (1989).
6. Koizumi, J., Yoshida, Y., Nakazawa, T., & Ooneda, G. Experimental studies of ischemic brain edema, I: a new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. *Jpn J Stroke*. **8**, 1-8 (1986).
7. Liu, F., Schafer, D. P., & McCullough, L. D. TTC, fluoro-Jade B and NeuN staining confirm evolving phases of infarction induced by middle cerebral artery occlusion. *J Neurosci Methods.* **179** (1), 1-8 (2009).
8. Liu, F., Yuan, R., Benashski, S. E., & McCullough, L. D. Changes in experimental stroke outcome across the life span. *J Cereb Blood Flow Metab.* **29** (4), 792-802 (2009).
9. Wang, R. Y., Wang, P. S., & Yang, Y. R. Effect of age in rats following middle cerebral artery occlusion. *Gerontology.* **49** (1), 27-32 (2003).
10. Baskerville, T. A., Macrae, I. M., Holmes, W. M., & McCabe, C. The influence of gender on 'tissue at risk' in acute stroke: A diffusion-weighted magnetic resonance imaging study in a rat model of focal cerebral ischaemia. *J Cereb Blood Flow Metab.* **36** (2), 381-386 (2016).
11. Cai, Q. *et al.* Co-transplantation of hippocampal neural stem cells and astrocytes and microvascular endothelial cells improve the memory in ischemic stroke rat. *Int J Clin Exp Med.* **8** (8), 13109-13117 (2015).
12. Cheng, Y. *et al.* Intravenously delivered neural stem cells migrate into ischemic brain, differentiate and improve functional recovery after transient ischemic stroke in adult rats. *Int J Clin Exp Pathol.* **8** (3), 2928-2936 (2015).
13. Nagai, N. *et al.* Intravenous Administration of Cilostazol Nanoparticles Ameliorates Acute Ischemic Stroke in a Cerebral Ischemia/Reperfusion-Induced Injury Model. *Int J Mol Sci.* **16** (12), 29329-29344 (2015).
14. Liu, Y. *et al.* Intravenous PEP-1-GDNF is protective after focal cerebral ischemia in rats. *Neurosci Lett.* **617** 150-155 (2016).
15. Hedna, V. S. *et al.* Validity of Laser Doppler Flowmetry in Predicting Outcome in Murine Intraluminal Middle Cerebral Artery Occlusion Stroke. *J Vasc Interv Neurol.* **8** (3), 74-82 (2015).
16. Tajima, Y. *et al.* Reproducibility of measuring cerebral blood flow by laser-Doppler flowmetry in mice. *Front Biosci (Elite Ed).* **6** 62-68 (2014).
17. Fang, M. *et al.* Scutellarin regulates microglia-mediated TNC1 astrocytic reaction and astrogliosis in cerebral ischemia in the adult rats. *BMC Neurosci.* **16** 84 (2015).
18. Evilsizor, M. N., Ray-Jones, H. F., Lifshitz, J., & Ziebell, J. Primer for immunohistochemistry on cryosectioned rat brain tissue: example staining for microglia and neurons. *J Vis Exp.* (99), e52293 (2015).
19. Kramer, M. *et al.* TTC staining of damaged brain areas after MCA occlusion in the rat does not constrict quantitative gene and protein analyses. *J Neurosci Methods.* **187** (1), 84-89 (2010).
20. Wu, L. *et al.* Keep warm and get success: the role of postischemic temperature in the mouse middle cerebral artery occlusion model. *Brain Res Bull.* **101** 12-17 (2014).
21. Liu, S., Zhen, G., Meloni, B. P., Campbell, K., & Winn, H. R. Rodent Stroke Model Guidelines for Preclinical Stroke Trials (1st Edition). *J Exp Stroke Transl Med.* **2** (2), 2-27 (2009).
22. Tsuchiya, D., Hong, S., Kayama, T., Panter, S. S., & Weinstein, P. R. Effect of suture size and carotid clip application upon blood flow and infarct volume after permanent and temporary middle cerebral artery occlusion in mice. *Brain Res.* **970** (1-2), 131-139 (2003).
23. Wolff, G., Davidson, S. J., Wrobel, J. K., & Toborek, M. Exercise maintains blood-brain barrier integrity during early stages of brain metastasis formation. *Biochem Biophys Res Commun.* **463** (4), 811-817 (2015).
24. Wrobel, J. K., Wolff, G., Xiao, R., Power, R. F., & Toborek, M. Dietary Selenium Supplementation Modulates Growth of Brain Metastatic Tumors and Changes the Expression of Adhesion Molecules in Brain Microvessels. *Biol Trace Elem Res.* (2015).
25. Chen, L., Swartz, K. R., & Toborek, M. Vessel microport technique for applications in cerebrovascular research. *J Neurosci Res.* **87** (7), 1718-1727 (2009).

