

Video Article

Endothelin-1 Induced Middle Cerebral Artery Occlusion Model for Ischemic Stroke with Laser Doppler Flowmetry Guidance in Rat

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URL: <https://www.jove.com/video/50014>

DOI: [doi:10.3791/50014](https://doi.org/10.3791/50014)

Keywords: Medicine, Issue 72, Neuroscience, Neurobiology, Biomedical Engineering, Surgery, Anatomy, Physiology, Nervous System Diseases, Ischemic stroke, Endothelin-1, Cerebrovascular, brain, artery, stroke, occlusion, laser, doppley, flowmetry, rat, animal model

Date Published: 2/16/2013

Citation: Ansari, S., Azari, H., Caldwell, K.J., Regenhardt, R.W., Hedna, V.S., Waters, M.F., Hoh, B.L., Mecca, A.P. Endothelin-1 Induced Middle Cerebral Artery Occlusion Model for Ischemic Stroke with Laser Doppler Flowmetry Guidance in Rat. *J. Vis. Exp.* (72), e50014, doi:10.3791/50014 (2013).

Abstract

Stroke is the number one cause of disability and third leading cause of death in the world, costing an estimated \$70 billion in the United States in 2009^{1,2}. Several models of cerebral ischemia have been developed to mimic the human condition of stroke. It has been suggested that up to 80% of all strokes result from ischemic damage in the middle cerebral artery (MCA) area³. In the early 1990s, endothelin-1 (ET-1)⁴ was used to induce ischemia by applying it directly adjacent to the surface of the MCA after craniotomy. Later, this model was modified⁵ by using a stereotaxic injection of ET-1 adjacent to the MCA to produce focal cerebral ischemia. The main advantages of this model include the ability to perform the procedure quickly, the ability to control artery constriction by altering the dose of ET-1 delivered, no need to manipulate the extracranial vessels supplying blood to the brain as well as gradual reperfusion rates that more closely mimics the reperfusion in humans⁵⁻⁷. On the other hand, the ET-1 model has disadvantages that include the need for a craniotomy, as well as higher variability in stroke volume⁸. This variability can be reduced with the use of laser Doppler flowmetry (LDF) to verify cerebral ischemia during ET-1 infusion. Factors that affect stroke variability include precision of infusion and the batch of the ET-1 used⁶. Another important consideration is that although reperfusion is a common occurrence in human stroke, the duration of occlusion for ET-1 induced MCAO may not closely mimic that of human stroke where many patients have partial reperfusion over a period of hours to days following occlusion^{9,10}. This protocol will describe in detail the ET-1 induced MCAO model for ischemic stroke in rats. It will also draw attention to special considerations and potential drawbacks throughout the procedure.

Video Link

The video component of this article can be found at <https://www.jove.com/video/50014/>

Protocol

This protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida and is in compliance with the "Guide for the Care and Use of Laboratory Animals" (eighth edition, National Academy of Sciences, 2011).

Materials

1. Animals: Eight-week-old, male, Sprague Dawley rats (Charles River Farms, Wilmington, MA, USA) weighing 250-300 g at the time of surgery.
2. Anesthesia
 - a. Inhalation anesthesia system (VetEquip Inc., Pleasanton, CA, USA)
 - b. Isoflurane anesthetic (Baxter Pharmaceuticals, Deerfield, IL, USA)
3. Stereotaxic system (David Kopf Instruments, Tujunga, CA, USA)
 - a. Small animal stereotaxic system
 - b. Non-rupture ear bars for rats
 - c. Gas anesthesia head holder for rats
4. Temperature regulation
 - a. BAT-12 microprobe thermometer (World Precision Instruments, Inc., Sarasota, FL, USA)
 - b. T/PUMP, TP600 Thermal blanket (Gaymar Industries, Inc., Orchard Park, NY, USA)
5. Surgical instruments

- a. Scalpel handle and #11 blade, iris forceps, Graefe forceps, bulldog clamp retractors, screwdriver, 10 µl syringe with 26 gauge beveled needle (World Precision Instruments, Inc., Sarasota, FL, USA)
 - b. Micromotor drill and stereotaxic holder, Quintessential Stereotaxic Injector (Stoelting, Wood Dale, IL, USA)
 - c. 1.0 mm round drill bur, 1.0 mm inverted cone drill bur (Roboz Surgical Instrument Co., Inc., Gaithersburg, MD, USA)
6. Surgical Supplies
 - a. Mounting screws 0-80 X 3/32 with 2.4 mm shaft length, 21-gauge guide cannula [4mm long below the pedestal] and cannula dummy (Plastics one, Roanoke, VA, USA)
 - b. Jet denture acrylic and liquid (Lang Dental Manufacturing Co., Inc., Wheeling, IL, USA)
 - c. 3.0 nylon suture (Oasis, Mettawa, IL, USA)
 - d. Cotton swabs, Puralube eye ointment (Fisher Scientific, Pittsburg, PA, USA)
 - e. Electric hair clippers (Oster, Providence, RI, USA)
 7. Chemicals
 - a. Endothelin-1 (American Peptide, Sunnyvale, CA, USA)
 - b. Chlorhexidine 2% (Agrilabs, St. Joseph, MO, USA)
 - c. Buprenorphine HCl (Hospira Inc., Lake Forest, IL, USA)
 8. Visualization Equipment
 - a. Surgical microscope (Seiler Instrument and Manufacturing; St. Louis, MO, USA)
 - b. Fiber Optic illuminator (TechniQuip Corp., Livermore, CA, USA)
 9. Laser Doppler flowmetry system (ADInstruments, Inc., Colorado Springs, CO, USA)
 - a. Standard Pencil Probe
 - b. Probe holder
 - c. Blood FlowMeter
 - d. Powerlab 4/30 with LabChart 7
 10. Measurement of infarct volume
 - a. Rat brain matrix (Zivic-Miller Lab., Inc., Allison Park, PA, USA)
 - b. 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich Co., St Louis, MO, USA) diluted to 0.05% in PBS
 - c. Flatbed scanner (Epson Perfection V30, Epson America, Inc., Long Beach, CA, USA)
 - d. Image J software (ImageJ 1.42q software, U.S. National Institutes of Health, Bethesda, MA, USA)

1. Pre-surgical Steps

1. Prior to surgery, the rats are housed under a 12:12 light/dark cycle with free access to water and rodent chow.
2. Anesthesia is induced with 4% isoflurane in 100% O₂ gas mixture in an induction chamber until the rat no longer withdraws to rear paw pinch.
3. Aseptic technique should be maintained during this procedure including use of sterile gloves, sterile surgical instruments, and a sterile surgical drape¹¹.
4. The crown of the head is shaved with electric hair clippers.
5. The rat is placed in the prone position on an absorbent pad lying on a temperature-controlled operating surface (thermal blanket).
6. The head is placed in the stereotactic apparatus starting with placement of the gas anesthetic face mask.
7. Next, the ear bars are inserted and tightened.
8. During the procedure anesthesia is maintained with 2% isoflurane in 100% O₂ gas mixture.
9. Lubricant ophthalmic ointment is applied to both eyes, and the eyelids are closed to prevent eye desiccation during the surgical procedure.
10. A rectal temperature probe is inserted to maintain a constant animal core temperature of 37±0.5 °C.
11. With the head held firmly in the stereotaxic device, the surgical area is cleansed with alternating 2% chlorhexidine and saline three times.

2. Surgical Steps

1. Using a scalpel, a midline incision is made on the skin overlying the calvarium from the most caudal aspects of the eyes (nasion) to between the ears (superior nuchal line).
2. The skin is then retracted laterally with 3 bulldog clamps.
3. Connective tissue is removed from the skull using dry cotton swabs so that multiple structures can be seen clearly. These include bregma, the coronal suture, and the right lateral skull ridge. Cotton swabs are used to remove blood from the surgical field.
4. Using the surgical microscope, bregma is located, and the stereotaxic manipulators are adjusted until the 1.0 mm round drill bur is zeroed at bregma.
5. The drill bur is then moved to 1.6 mm anterior and 5.2 mm lateral to bregma.
6. A bur hole that penetrates the skull is drilled for cannula placement (**Figure 1**). Excess debris and blood are continually cleared using cotton swabs.

At this point, a guide cannula can be inserted (step 7) or a direct ET-1 injection through the bur hole can be performed (proceed directly to step 16).

7. Next, bur holes for 3 mounting screws are drilled through partial thickness of the skull using a 1.0 mm inverted cone drill bur (**Figure 1**). One hole is drilled in each frontal bone about 1-2 mm anterior to the coronal suture and 1-2 mm lateral to the sagittal suture. One hole is drilled in the parietal bone about 2-3 mm posterior to the coronal suture and 2-3 mm lateral to the sagittal suture ipsilateral to the guide cannula bur

- hole. Three 0-80 x 3/32 mounting screws are placed in these bur holes and will provide support for the cement holding in the cannula. The screws should only be advanced 2 or 3 turns so as not to damage the dura matter.
8. The guide cannula is placed in the stereotaxic cannula holder and bregma is located.
 9. The stereotaxic manipulators are adjusted until the guide cannula is zeroed at bregma. The cannula is moved to the bur hole located 1.6 mm anterior and 5.2 mm lateral to bregma.
 10. Finally, the guide cannula is lowered into the bur hole with the final tip position of 4.5 mm ventral to bregma.
 11. Laser Doppler flow probe placement (optional)
 - a. In order to monitor cerebral blood flow using LDF, a probe holder can be placed into position prior to affixing the guide cannula with dental cement.
 - b. The probe holder base is trimmed flush with the pedestal except for small wedge shaped tab.
 - c. The probe holder is then placed posterior to the guide cannula and just medial to the lateral skull ridge with the tab oriented medially (**Figure 1**).
 - d. The probe holder and guide cannula are affixed together using dental cement.
 12. Dental cement is then used to secure the cannula in place. The cement is in contact with all three screws and surrounds the entire base of the cannula.
 13. The cement should be completely dry prior to removal of the cannula holder. This takes about 5 min.
- After these steps, the surgical incision can be closed and the cannula dummy can be screwed into the guide cannula. Alternatively, ET-1 induced MCAO can be performed on the rat after a period of recovery from the cannula implantation surgery. For this method, step 19 should be performed next and steps 14-18 can be performed at a later time. To perform guide cannula implantation and ET-1 injection during the same surgery, step 14 should be performed next.
14. The infusion syringe is loaded with ET-1 (diluted to 80 μ M in PBS) and then mounted in the stereotaxic injector.
 15. The stereotaxic manipulators are adjusted until the needle tip is zeroed at the rim of the guide cannula.
 16. The needle tip is lowered through the guide cannula to a position of 17.2 mm ventral to the rim of the guide cannula. If a guide cannula is not used, the needle tip is zeroed at bregma and lowered through the bur hole to a position 8.7 mm ventral to bregma.
 17. 3 μ l of 80 μ M ET1 is infused at a rate of 1 μ l per min.
 18. The syringe is left in place for 3 min after the infusion is complete and then slowly removed.
 19. The incision is closed with 3.0 nylon suture and the cannula dummy is screwed into the cannula.
 20. A dose of appropriate analgesic (*i.e.* buprenorphine at 0.05-0.1 mg/kg) should be used after the surgery to minimize pain and discomfort during the recovery period.
 21. The rat is removed from the surgical suite and placed in a warm, dry recovery area to prevent hypothermia, with free, easy access to soft food and water.

Representative Results

1. Post-Op neurological evaluation

After the animal regains consciousness, a wide variety of tests can be used to evaluate neurological deficits including balance, grip strength, paw placing, postural asymmetry and staircase climbing. The sunflower seed task is a gross assessment of motor and sensory function that has significant correlation with infarct volume^{7, 12}. During this task, rats are timed while opening and consuming 5 sunflower seeds. The five seeds are placed in one corner of an empty, dry, plastic cage and the time spent manipulating the seeds is recorded. In addition, the number of shell pieces in the cage after all seeds have been opened and consumed is recorded. Rats with greater neurological deficit will take longer to open and consume the seeds and will break the shells into more pieces. Although not a formal part of this task, it is easy to observe frequent mishandling and dropping of the sunflower seeds by rats after experimental stroke. This includes the use of primarily one forepaw, and ineffective biting of the shell during attempts to open.

2. Staining and quantitative measurement of brain infarct volume

After occlusion and reperfusion of the MCA to establish transient cerebral ischemia, animals are euthanized and their coronally sectioned brains are stained with 2,3,5-triphenyltetrazolium chloride (TTC) to evaluate the infarct volume (**Figure 2**) as discussed in our previous publications^{7, 13}.

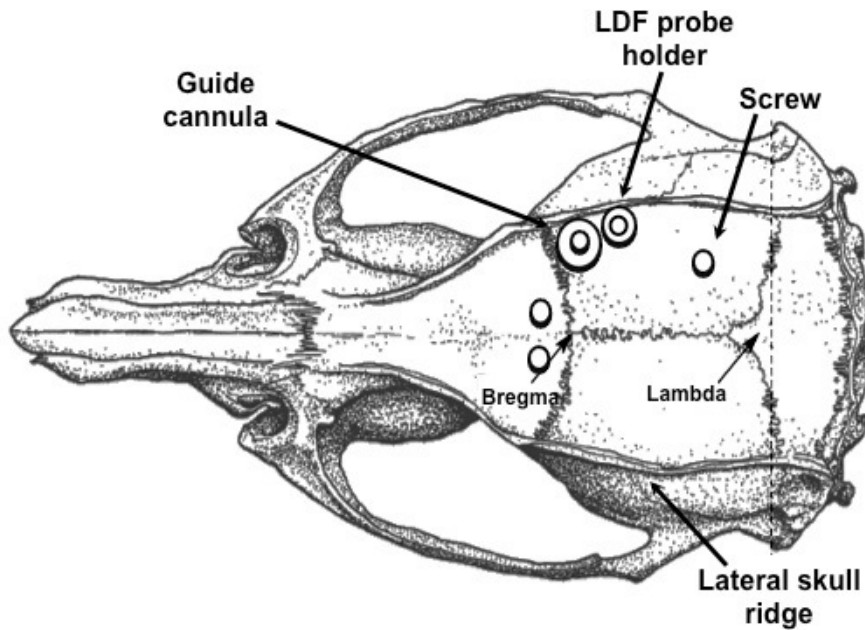


Figure 1. Dorsal view of the rat skull depicting hardware placement. This dorsal view of the rat skull with anterior oriented to the left shows the location of bregma and lambda with respect to the sutures that can be visualized after clearing connective tissue from the skull surface. The lateral skull ridge, guide cannula, LDF probe holder, and anchoring screws are also shown on the diagram with relative relationships to skull land marks and each other.



Figure 2. TTC-stained serial coronal brain sections (2 mm) from rat subjected to ET-1 induced MCAO. A representative brain from a rat after ET-1 induced MCAO is shown. This brain was removed and rinsed in ice cold PBS prior to being sectioned coronally in 2 mm thick sections starting just caudal to the olfactory bulbs. The slices were then incubated in 0.05% TTC diluted in PBS at 37 °C prior to being quenched in PBS and then fixed briefly in formalin. Tissue stained red after exposure to TTC represents viable gray matter. The viable gray matter in the hemisphere ipsilateral to ET-1 injection can be compared to the gray matter of the contralateral hemisphere to calculate the area of the cerebral infarct.

Discussion

The ET-1 induced MCAO is an established model of experimental ischemic stroke that is regularly used in multiple rat strains. Many variables, such as rat strain, animal age, body temperature, anesthesia method, and operator expertise can lead to increased variability in infarct volumes when using this model^{5, 14}. However, several investigators have shown that advantages of this model include the relatively non-invasive approach, dose response of cerebral blood flow to ET-1, and ability to avoid anesthesia altogether by infusion of ET-1 in conscious rats^{14, 15}. It is notable that there are many differences in rat strain and age used throughout the literature and it is not surprising that different stereotaxic coordinates are reported depending on these variables. The coordinates used in our protocol are optimized for 8 week old male Sprague Dawley

rats. Any deviation from this strain, sex, or age will likely require intracerebral injection of dye followed by necropsy to identify valid coordinates for this procedure. Additionally, ET-1 induced reduction in cerebral blood flow is dose dependent. The dose used in this method causes an infarction of approximately 30–40% of the gray matter in the hemisphere ipsilateral to the injection with a standard error of the mean of about 4–5% within an experiment^{7, 16}. Direct visualization of proximal portions of the MCA reveals a luminal diameter that goes to 0% of baseline within 3 min and returns to baseline at approximately 30 min⁷. Using LDF, maximum flow was shown to decrease to approximately 10% of baseline flow in 5–7 min with gradual return to 60% of baseline after 1 hr which monitoring was discontinued¹⁶.

Other specific technical details that warrant mention are listed below:

1. Correctly locating and zeroing the stereotaxic equipment at bregma (under microscopic guidance) is crucial to inducing a successful stroke. The stereotaxic coordinates for the ET-1 infusion should be verified for the specific experimental equipment and rat strain prior to experimentation. It is likely that no lesion will occur if the infusion is not within 0.5 mm of the MCA⁶.
2. Take care not to cause damage to the brain when drilling the initial bur hole for the guide cannula. It is recommended that a slow drilling speed be used to start the bur hole and again towards the end so as to minimize the chances of penetrating into the brain.
3. When drilling bur holes for the mounting screws, use the inverted cone drill bur to create a bur hole that only partially penetrates the skull. Only drill deep enough so that the screws are stable.
4. When cementing the cannula in place be sure to keep the cement clear of the skin as well as the stereotaxic arm. The cement can be manipulated prior to drying using a cotton swab.
5. Be sure the cement is completely dry and the cannula is stable prior to removal of the cannula holder. It is critical that the cannula not move prior to ET-1 injection.
6. In our experience, the ET-1 stroke model has an approximately 20% mortality rate due to the surgical procedure. It is important to compare mortality rates between treatment groups and also plan experiments so that sufficient sample sizes are used for neurological and histological endpoints.
7. Guidelines for preclinical trials for acute stroke therapy recommend cerebral blood flow monitoring and physiological monitoring of blood pressure, temperature, glucose, and blood gases to reduce variability during experiments¹⁷. LDF is a method that can be used for real-time monitoring of blood flow in various anatomical regions to verify a predetermined threshold for reduction in flow has been achieved.
8. Be sure to clean the infusion syringe and needle with ethanol and saline between injections, and ethanol prior to storage. This procedure will give consistent withdrawal and injection of ET-1 without air bubbles or blockages during operation.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was supported by grants from the American Heart Association Greater Southeast Affiliate (09GRNT2060421), the American Medical Association, and from the University of Florida Clinical and Translational Science Institute. Adam Mecca is a NIH/NINDS, NRSA predoctoral fellow (F30 NS-060335). Robert Regenhardt received predoctoral fellowship support from the University of Florida Multidisciplinary Training Program in Hypertension (T32 HL-083810).

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