Video Article

Lateral Chronic Cranial Window Preparation Enables *In Vivo* Observation Following Distal Middle Cerebral Artery Occlusion in Mice

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Abstract

Focal cerebral ischemia (*i.e.*, ischemic stroke) may cause major brain injury, leading to a severe loss of neuronal function and consequently to a host of motor and cognitive disabilities. Its high prevalence poses a serious health burden, as stroke is among the principal causes of long-term disability and death worldwide¹. Recovery of neuronal function is, in most cases, only partial. So far, treatment options are very limited, in particular due to the narrow time window for thrombolysis^{2,3}. Determining methods to accelerate recovery from stroke remains a prime medical goal; however, this has been hampered by insufficient mechanistic insights into the recovery process. Experimental stroke researchers frequently employ rodent models of focal cerebral ischemia. Beyond the acute phase, stroke research is increasingly focused on the sub-acute and chronic phase following cerebral ischemia. Most stroke researchers apply permanent or transient occlusion of the MCA in mice or rats. In patients, occlusions of the MCA are among the most frequent causes of ischemic stroke⁴. Besides proximal occlusion of the MCA using the filament model, surgical occlusion of the distal MCA is probably the most frequently used model in experimental stroke research⁵. Occlusion of a distal (to the branching of the lenticulo-striate arteries) MCA branch typically spares the striatum and primarily affects the neocortex. Vessel occlusion can be permanent or transient. High reproducibility of lesion volume and very low mortality rates with respect to the long-term outcome are the main advantages of this model. Here, we demonstrate how to perform a chronic cranial window (CW) preparation lateral to the sagittal sinus, and afterwards how to surgically induce a distal stroke underneath the window using a craniotomy approach. This approach can be applied for sequential imaging of acute and chronic changes following ischemia via epi-illuminating, confocal laser scanning, and two-photon intravital microscopy.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54701/>

Introduction

Stroke is among the principal causes of long-term disability and death worldwide¹, coming second after coronary heart disease. In addition, stroke is the primary cause of long-term disability, underscoring its tremendous socioeconomic impact⁶⁻⁸. Beyond acute treatment, investigating new approaches and mechanisms to accelerate and enhance recovery after stroke remains a prime medical goal⁷.

In the last few decades, data from experimental stroke research has contributed substantially to understanding the complex pathophysiological cascades triggered by ischemia^{9,10}. Excitotoxicity, apoptosis, peri-infarct depolarization, and inflammation have been identified as the most relevant mediators of cell death following focal cerebral ischemia. Moreover, using animal models of cerebral ischemia, important concepts, diagnostic modalities, and therapeutic approaches have been developed and validated (*e.g.*, "penumbra" and thrombolysis)¹¹.

The availability of experimental stroke models, combined with non-invasive imaging modalities (*e.g.*, magnetic resonance imaging (MRI), computed tomography, or laser speckle contrast analysis), enables the researcher to investigate hyperacute and chronic pathophysiological changes induced by the ischemic insult in a longitudinal manner¹². Along with studying the spatiotemporal profile of the evolving lesion, changes resembling neuronal plasticity can be investigated and correlated to functional outcomes and histological findings. Within the last few years, further methodological advances have been made using the combination of cerebral ischemia models and *in vivo* microscopy via cranial windows¹³. These new techniques allow investigators to analyze the neurovascular unit at the cellular and molecular level, with great analytic power in the acute, subacute, and chronic phases following focal cerebral ischemia¹⁴. Moreover, *in vivo* microscopy imaging of microcirculatory dynamics has revealed novel aspects of cerebral microvasculature function and angioarchitecture, with significant pathophysiological relevance¹⁵⁻¹⁷.

In this protocol, we present how to perform a chronic CW preparation lateral to the sagittal sinus and how to surgically induce a distal stroke underneath the window. This mouse model can be applied to sequential imaging of acute, subacute, and chronic changes following focal cerebral ischemia via epi-illuminating, confocal laser scanning, and two-photon intravital microscopy.

Protocol

ETHICS STATEMENT: Experiments involving animal subjects were performed in accordance with the guidelines and regulations set forth by Landesamt fuer Gesundheit und Soziales, Berlin, Germany (G0298/13) and the ARRIVE criteria, as applicable. For this study, 10- to 12-week-old male C57Bl/6J mice were used.

1. Lateral Chronic Cranial Window Preparation

1. Perform anesthesia with a subcutaneous injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). Test for adequate sedation with a pain stimulus.
2. Sterilize the surgical instruments and surgical field with 70% ethanol.
3. Remove the scalp hair from the neck to the eyes using a rodent shaver.
4. Fix the head in a stereotactic frame.
5. Use dexpanthenol eye ointment on both eyes to avoid dehydration.
6. Clean the surgical area to remove all hairs and sterilize it with 3 layers of 74.1% ethanol and 10% 2-propanolol.
7. Perform a midline incision from the neck to the eyes using a scalpel.
8. Span the skin flap with 4 tenting sutures.
9. Remove the periosteum carefully on the left hemisphere by scraping it off with a scalpel to the point where the temporal muscle begins.
NOTE: This preparation also serves to create a good adhesion area for the dental glue, which fixes the cover glass.
10. Perform a fronto-parietal craniotomy with a diameter of 4 mm by thinning the skull at the rim of the bone flap using a microdrill. Apply saline solution while drilling to avoid heat injury.
11. Elevate the bone flap with cannulas and remove it using microforceps.
12. Irrigate carefully and extensively with saline solution.
13. Mix the dental glue until it has the proper consistency and is not fluid (*i.e.*, the glue should not produce threads anymore). Place it on the bone around the craniotomy.
14. Place a cover glass with a 6-mm diameter on the prepared glue and fix it with the rest of the dental glue. Be sure that it is watertight. Wait until the glue is dried and hard by testing it with forceps.
NOTE: An additional irrigation accelerates the curing process.
15. Remove the tenting sutures and close the wound with skin sutures.
16. Pull up the skin of the flank of the mouse. Insert a needle subcutaneously and gently substitute 0.5 ml of sterile saline subcutaneously for fluid balance.
17. After surgery, keep the animals in the heated recovery cage for 90 min. Wait until the animals are fully awake before leaving them unattended. Wait until the animals are completely recovered before returning them to a cage with other animals.
18. Repeat the subcutaneous saline volume substitution after about 12 hr, as described in step 1.16.
19. Always apply analgesia via gavage or directly into the oral cavity after surgery (*e.g.*, paracetamol (10 mg/ml) or other non-steroidal anti-inflammatory drugs).
20. Check the condition of the animals every day after surgery, and always provide mashed animal food on the floor in a petri-dish to make eating simple and to avoid critical weight loss after surgery.
NOTE: Intravital microscopy can be performed on the first day after cranial window preparation.
21. Apply isoflurane anesthesia and fix the animal in a head holder. Open the skin suture and clean the window with cotton buds and sterile saline. After 24 hr, the cranial window should be filled with cerebrospinal fluid by that time point, which enables imaging. Perform imaging by using established microscopy protocols¹⁸.

2. Distal MCAo

NOTE: The MCAo procedure should be performed about 5 d after CW preparation. This minimizes interference from the immune reaction caused by the CW preparation with the stroke-induced immune reaction.

Figure 1. Overview of Distal MCAo. **A.** This is a nice overview about the vessels before the op. **B.** The vessels after first bipolar contact. **C.** The vessels after second bipolar contact. **D.** The overview about the vessels, which are completely closed now. **E.** Final overview with lower magnification. [Please click here to view a larger version of this figure.](#)

1. Anaesthetize the mice using an anesthesia mask and an appropriate anesthetic regime, in consultation with veterinary staff (e.g., induction with 1.5 - 2% isoflurane and maintenance with 1.0 - 1.5% isoflurane in 2/3 N₂O and 1/3 O₂ via a vaporizer).
2. Shave and remove the hair and disinfect the skin and surrounding fur with an appropriate agent (e.g., 70% ethyl alcohol), and dry it afterwards.
3. Maintain the body temperature of the mice at 36.5 °C ± 0.5 °C during surgery. A feedback controlled heating pad, heated according to the rectal temperature of the mouse, is highly recommended.
4. Place the animal in the lateral position. Fix the nose in the anesthesia mask and adjust the isoflurane concentration to 1.0 - 1.5%.
5. Apply wet ointment (dexpanthenol) to both eyes.
6. Use the skin incision made for the CW preparation.
7. Gently separate the skin and identify the temporal muscle underneath.
8. Adjust the energy of the high-frequency generator (5 - 7 W) and use the bipolar mode.
9. Use the electrocoagulation forceps and carefully remove the temporal muscle from the skull, creating a muscle flap, without totally removing the muscle.
10. Identify the MCA below the transparent skull in the rostral part of the temporal area, dorsal to the retro-orbital sinus. If the MCA bifurcation cannot be identified, try to identify the vessel most rostral.
11. Thin the skull above the MCA branch with a microdrill while continuously irrigating to avoid heat damage.
12. Lift the bone with cannulas and remove it with microforceps.
13. Decrease the energy of the high-frequency generator to 3 - 5 W.
14. Approach the artery from above and gently touch it with the bipolar forceps on both sides without lifting the vessel.
15. Coagulate the artery proximally and distally to the vessel bifurcation.
16. Wait for 30 sec, and then touch the artery gently to ensure that the blood flow is permanently interrupted. Repeat the electrocoagulation if a recanalization is observed.
17. Fix the temporal muscle with 1 or 2 stitches at the muscle insertion to cover the bone defect, if possible.
18. Suture the wound and place the animal into the heated recovery box. In general, animals recovery quickly after volatile anesthesia.
19. For volume substitution, apply 0.5 ml of sterile saline subcutaneously, as described in step 1.16.
20. After surgery, allow the animals to stay in the heated recovery cage for 90 min. Wait until the animals are conscious before leaving them unattended. Only return them to a cage with other animals when they are fully recovered.

21. Repeat the volume substitution, as explained in step 1.16, after 12 hr.
22. Apply postoperative analgesia *via* drinking water (e.g., paracetamol (10 mg/ml) or other non-steroidal anti-inflammatory drugs).
23. Check the medical condition of the animals every day after surgery. Provide mashed animal food in a petri-dish on the floor to simplify eating and to minimize postoperative weight loss.

3. Sham Treatment

1. Perform all procedures identically to steps 1 and 2, described above-including CW preparation-except do not coagulate the exposed middle cerebral artery.

Representative Results

The timeline and representative results are shown in **Figures 2** and **3**. The cranial window preparation, with a small cranial window lateral to the superior sagittal sinus (**Figure 2 B, C, D**) results in a very low mortality and morbidity rate when performed by an experienced surgeon. All of the 10 animals survived, and all chronic CW could be used for high-quality imaging, even 28 days after surgery. There was no problem with wound infections or other complications.

Due to the short exposure to volatile anesthesia and only minor brain damage, approximately 10 - 15 min after distal MCAo and transfer to the heated recovery cage, all animals were awake, freely moving in the recovery cage, and interacting with littermates. In general, mortality of the distal MCAo model is less than 5% and mainly occurs as a result of vascular injury and subsequent hemorrhage during MCAo surgery. Mortality during the 28-day observation period following distal MCAo occurs only very rarely. Morphologically, the lesion can be assessed using histology or MRI (**Figure 3 A, B**). In addition, MRI measurements offer the opportunity to assess lesion volume and progression in a longitudinal manner. An MRI performed 24 hr after ischemia clearly depicts the ischemic lesion located underneath the chronic CW, while following sham surgery, no lesioned cortical tissue was found (**Figure 3A**). The MRI results clearly show the strict restriction of the lesion to the cortex, thus sparing the striatum, in contrast to the filamentous MCAo model (**Figure 3B**). The lateral chronic CW preparation enables long-term visualization of cortical vasculature and microcirculation by epi-fluorescence microscopy (**Figure 3C**, upper part) and of subcortical areas by two-photon microscopy (**Figure 3C**, lower part). Further, it is possible to image molecular pathways and cell-to-cell interactions with fluorescence-labeled cells or autofluorescence measurements, such as fluorescence lifetime imaging. As shown in **Figure 3D**, the distal MCAo model causes highly reproducible ischemic lesions. Regarding the infarct volume, we expect a standard deviation below 15% in a single set of surgeries. As mentioned above, in contrast to models of proximal MCAo, mortality rates are rather low for the distal model⁵. Using sequential MRI, lesion, volume, and edema progression following focal cerebral ischemia can be assessed. The MRI at 24 hr and 96 hr after permanent distal MCAo showed no significant progression of T2 hyperintensities.

Figure 2: Chronic Cranial Window Preparation. (A) Representative timeline. (B) Marking the area where the craniotomy takes place, lateral to the temporal muscle, medial to the superior sagittal sinus, and dorsal to bregma. (C) Brain surface after a craniotomy, with an intact dura layer and the cover glass in place; the circle shows the location of the ischemic area after distal MCAo. (D) Finished chronic cranial window with fixed cover glass, ready for repetitive imaging for several weeks (b = bregma, tm = temporal muscle, SSS = superior sagittal sinus, ai = area of ischemia). [Please click here to view a larger version of this figure.](#)

Figure 3: Combination of the Lateral Chronic CW and Distal MCAo or Sham Surgery. (A) The MRI performed 24 hr after sham surgery does not show any lesioned cortical tissue. (B) The MRI performed 24 hr after ischemia clearly depicts the ischemic lesion (*) located underneath the chronic CW. (C) Intravital epi-fluorescence imaging (upper part) and two-photon imaging (lower part) of the cortical vasculature. (D) Infarct volume assessed via MRI at 24 hr and 96 hr after ischemia shows a mean lesion volume of 13.16 $\pm$ 2.3 mm³ at 24 hr and 12.2 $\pm$ 1.9 mm³ at 96 hr. Each dot represents an individual animal (n = 10 animals per group, mean $\pm$ SEM). [Please click here to view a larger version of this figure.](#)

Discussion

Stroke is among the principal causes of long-term disability and death worldwide¹. Beyond acute treatment, investigating new approaches and mechanisms to accelerate and enhance recovery after stroke remains a prime medical goal⁷. Experimental stroke researchers frequently employ rodent models of focal cerebral ischemia. In fact, models inducing transient or permanent MCAo mimic one of the most common types of focal cerebral ischemia in patients⁴. Besides proximal occlusion of the MCA, the filament model for surgical occlusion of the distal MCAo is probably the most frequently used model in experimental stroke research^{5,19}. Here, we describe the basic technique of permanent distal MCAo combined with a lateral CW, offering the opportunity for longitudinal intravital microscopy in mice. High reproducibility of lesion volume, as well as very low mortality rates, in particular with respect to studying long-term outcomes, are the main advantages of this murine model. In this cortical stroke model, blood vessels in the stroke area and peri-infarct region can be visualized via the chronic CW. Using a multi-fluorescence epifluorescence videomicroscopic system, blood flow dynamics and the dynamic recruitment of circulating cells can be visualized. Blood vessels are visualized through the use of fluorescently labeled macromolecules, like dextrans or albumin. Cells can be labeled either by fluorescent dyes or by genetic models, such as bone marrow chimeras with GFP-positive animals. Further, to study cell-to-cell interactions and the dynamics of extravascular cells, a two-photon laser scanning confocal microscope can be applied. Imaging up to 250 µm below the cortical surface can be performed. Again, blood vessels are stained using fluorescently labeled macromolecules, and cells are labeled genetically (e.g., by using the transgenic GFP-Nestin mouse).

The cranial window surgery is performed via a craniotomy without opening the dura. One major pitfall is to accidentally injure the dural layer and cortex underneath when opening the skull with the microdrill. Therefore, this technique requires some technical skill in order to avoid damage to the dura and cortex, which induces an immune reaction and influences microscopy results. Alternatively, a potential thinned-skull model is limited by less reliable microscopy quality because of the remaining skull, especially in the long term. Frequently, repetitive skull thinning is necessary, whereas in the CW model, the window quality lasts for several months, until skull regrowth or dural thickening influences imaging quality^{14,20}. A modulation of this model with a thinned-skull preparation would be possible. The dural layer should be left on the brain to avoid any injury or excitation of the cortex. Only if a direct application to the ischemic area is desired in an experimental model can the dura be removed, carefully and without injuring any bridging veins.

In contrast to models combining a CW and inducing targeted vessel occlusion *via* irradiation of circulating photosensitizers, leading to only very small ischemic lesions, the distal MCAo model mimics the majority of human strokes that are located in the cortical MCA territory¹³. To avoid interference with a transient inflammatory reaction due to CW preparation, the window should be prepared several days before distal MCAo surgery.

A number of tests to assess functional as well as behavioral aspects in rodents are available (e.g., gait analysis, rotarod test, pole test, adhesive removal test, staircase test, open field test, and Morris water maze)²¹. In all of these tests, mice subjected to MCAo perform less successfully than control animals with respect to short-term and intermediate-term outcomes. However, regarding the assessment of long-term outcomes, it must be acknowledged that the sensitivity of functional testing is very limited in terms of distal MCAo, as well as mild proximal MCAo^{19,21-23}.

Distal MCAo, performed by well-trained surgeons, can be induced within less than 20 min and can produce highly reproducible ischemic lesions. However, reproducibility requires a thorough control of confounders. Differences in surgical technique may lead to differences in infarct size²⁴. Different mouse strains might show a different stroke outcome due to variances in cerebral vascular anatomy between strains. Furthermore, as body temperature affects neuronal damage and susceptibility to ischemia, with hypothermia leading to smaller lesions and hyperthermia to more severe deficits^{25,26}, temperature control and maintenance are highly relevant in this model, as well as in other ischemia models²⁷. In general, physiological parameters, such as blood pressure and blood gases, are important confounders of outcome and must be controlled for²⁸. In addition, the choice of the anesthetic is highly important, as some agents may exert direct neuroprotective effects or act indirectly via vasoactive properties²⁹. Therefore, exposure to anesthesia should be standardized and kept as short as possible. Finally, housing conditions, like the use of enrichment, may affect stroke outcome as well, and thus should be standardized and described in research reports³⁰. To produce relevant preclinical results for the development of new therapeutic approaches, standardization, quality control, and reporting are of the utmost importance³¹.

Disclosures

The authors have nothing to disclose.

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