Video Article

Permanent Cerebral Vessel Occlusion *via* Double Ligature and Transection

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Abstract

Stroke is a leading cause of death, disability, and socioeconomic loss worldwide. The majority of all strokes result from an interruption in blood flow (ischemia) [1]. Middle cerebral artery (MCA) delivers a great majority of blood to the lateral surface of the cortex [2], is the most common site of human stroke [3], and ischemia within its territory can result in extensive dysfunction or death [1,4,5]. Survivors of ischemic stroke often suffer loss or disruption of motor capabilities, sensory deficits, and infarct. In an effort to capture these key characteristics of stroke, and thereby develop effective treatment, a great deal of emphasis is placed upon animal models of ischemia in MCA.

Here we present a method of permanently occluding a cortical surface blood vessel. We will present this method using an example of a relevant vessel occlusion that models the most common type, location, and outcome of human stroke, permanent middle cerebral artery occlusion (pMCAO). In this model, we surgically expose MCA in the adult rat and subsequently occlude via double ligature and transection of the vessel. This pMCAO blocks the proximal cortical branch of MCA, causing ischemia in all of MCA cortical territory, a large portion of the cortex. This method of occlusion can also be used to occlude more distal portions of cortical vessels in order to achieve more focal ischemia targeting a smaller region of cortex. The primary disadvantages of pMCAO are that the surgical procedure is somewhat invasive as a small craniotomy is required to access MCA, though this results in minimal tissue damage. The primary advantages of this model, however, are: the site of occlusion is well defined, the degree of blood flow reduction is consistent, functional and neurological impairment occurs rapidly, infarct size is consistent, and the high rate of survival allows for long-term chronic assessment.

Video Link

The video component of this article can be found at http://www.jove.com/video/50418/

Introduction

In order to induce ischemic conditions that effectively mimic human ischemic stroke, several animal stroke models are widely employed, with varying volumes of infarct resulting. In the photothrombotic model, the brain is irradiated through the intact skull using laser illumination after intravenous injection of a photosensitive substance (such as rose-bengal), resulting in photochemical coagulation, blockage of the irradiated vessels, and ischemia within the surrounding tissue [6,7]. Photothrombosis can result in very small, isolated regions of infarct and is typically used as a means of modeling "mini-strokes", or "micro-strokes".

The more widely adopted technique for inducing ischemic stroke, particularly in middle cerebral artery (MCA), is the intraluminal monofilament model [8], in which a filament is surgically introduced into the external carotid artery and advanced until the tip occludes the base of MCA. A primary challenge of intraluminal filament occlusion is the high mortality rate (70% when MCA is occluded for 3 hr, a relevant time point for stroke research) [9]. Other issues with the method included possible subarachnoid hemorrhage, incomplete occlusion, and variable infarct volume [10,11]. This model results in an extensive degree of infarct both in the cortex and subcortically [12], and models a massive human stroke.

Although both micro and massive stroke models are important, human strokes are typically somewhere in between. In large clinical studies, stroke infarct ranges in size from 28-80 cm^3, which translates to 4.5-14% of the ipsi-ishemic hemisphere [9]. In comparison, our rat pMCAO infarct size ranges from approximately 9-35 mm^3, which constitutes 3 to 12% of the ipsi-ishemic hemisphere. Our pMCAO model, therefore, closely resembles human ischemic stroke infarct volumes by percentage of brain volume.

In addition to modeling the structural damage of stroke, pMCAO results in functional and behavioral deficits similar to the human condition. At minimum, an effective model of stroke results in movement deficits contralateral to stroke damage [13-15], loss or disruption of motor and sensory function [16,17], loss or disruption of evoked neuronal activity [16,18], reductions in cerebral blood flow [19,20], and infarct [21,22]. Accordingly, our pMCAO models a serious occlusion of MCA resulting in physical disability, loss of function within the sensory cortex (and neighboring cortices), disruption of neuronal activity, a severe reduction in MCA blood flow, and infarct- hallmark attributes of ischemic stroke [23-25], therefore serving as an effective model of human stroke.

Procedurally, pMCAO involves a small craniotomy in which we carefully remove the skull and dura from a 2 x 2 mm "surgical window" over the initial (M1) segment of MCA, just prior to the primary bifurcation of MCA into the anterior and posterior cortical branches (**Figures 1A and 1B**). We pass a half-curve reverse cutting suture needle and thread (6-0 silk) through the pial layer of the meninges, below MCA and above the cortical surface (see Table of specific reagents and equipment for the surgical supplies necessary to carry out pMCAO). We then tie a double ligature, tighten the two knots around MCA, and transect the vessel between the two knots. The double ligature and transection through M1 occurs just distal to the lenticulostriate branching, such that only the cortical branches of MCA are affected- thus only cortical infarct (no subcortical damage) occurs [26,27] (**Figure 2**). Although human stroke often involves subcortical infarct, modeling this in rodents requires increased invasiveness (occluding cerebral vessels prior to cortical branching requires accessing arteries via the carotid artery in the neck and necessitates additional occlusions) in technique and increased variability in infarct size. The model described here cannot be performed more proximally as access to earlier branches of MCA is not possible *via* a simple craniotomy. While it may be surgically possible to induce a subcortical infarct via pMCAO, occlusion would entail an extremely invasive procedure and is therefore not ideal.

Effectiveness of occlusion may be confirmed via laser Doppler, or laser speckle imaging [12,24,25] (**Figure 3**), or histologically post-mortem (**Figure 2**). It should be noted that previous research has shown that sensory stimulation can play a major role in the evolution and outcome of infarct; conferring protection from damage when administered within 2 hr of pMCAO and causing an increase in stroke damage when administered at 3 hr post pMCAO [24,25,28]. We have confirmed that at 5 hr post-pMCAO, stimulation no longer has an effect on outcome (unpublished data). Therefore, sensory stimulation of subjects should be minimized for 5 hr following pMCAO to obtain infarct volumes with minimal variability. Accordingly, our group runs "untreated controls" of this type by keeping rats anesthetized for 5 hr post-pMCAO, in the dark, with minimal sensory stimulation, and expressly no whisker stimulation.

It should be further noted that occasional variation in MCA structure, including excessive branching, multiple primary segments, or the absence of communicating arteries can occur at a frequency of 10 to 30% in male adult Sprague Dawley rats [29,30]. If abnormalities in MCA are observed, it is advisable not to use that particular subject as adding animals with such vascular abnormalities will increase infarct variability.

Additionally, there are several practical aspects of our procedure that make this occlusion method advantageous for stroke investigation. First, sutures may be placed around the artery but not tightened in order to collect a baseline assessment, followed by post-ischemic assessment after ligature and transection. In this manner, surgical preparation necessary for the occlusion is effectively controlled for, within subjects. Because subjects may remain stationary or within a stereotaxic frame throughout occlusion, it is possible to conduct experimental assessment of each subject prior to, during, and after occlusion without moving the subject or disturbing any experimental equipment in use [25,28]. Furthermore, this procedure results in a very low mortality rate, even within aged rodent subjects 21-24 months of age (equivalent to an elderly human) [31], and may therefore be used to evaluate stroke treatments in rats that more closely model the most common age bracket of stroke sufferers [25,28]. Vessel transection also serves several practical purposes. The absence of bleeding after transection confirms that the vessel was completely occluded at both ligature sites. Additionally, transection ensures a permanent disruption of blood flow. Finally, transection ensures that any blood flow detected in the distal portions of the occluded vessel must come from an alternate source.

Finally, although we specifically describe this occlusion technique for MCA in this manuscript and video, the same double ligature transection technique may be applied to any cerebral vessel that can be accessed via craniotomy. Our laboratory, for example, utilized pMCAO in conjunction with several additional permanent occlusions of distal MCA branches in order to block both primary, and collateral blood flow [24] in a manner to similar to techniques designed to selectively induce ischemia within the primary somatosensory cortex [32].

In conclusion, this method for permanent occlusion as applied to MCA closely models three primary facets of human ischemic stroke: the most common location (MCA), type (ischemia), and degree of damage (infarct) associated with the human clinical literature of stroke. Furthermore, this method of occlusion may be applied to single or multiple occlusion sites throughout the brain, and may be conducted in aged subjects with a high rate of survival. Given the dynamic, permanent, and relatively noninvasive nature of this occlusion, this technique represents an additional tool for preclinical researchers evaluating novel approaches for the protection from and treatment of stroke.

Protocol

1. Getting Started: Required Surgical Instruments

See **Figure 4**

1. Dental drill (Kavo Dental Equipment, Model: UMXL-TM), 2-bit drill, and 3-bit drill
2. Two ~30 gauge hypodermic needles
3. Serrated tweezers, curved tip optional (can be helpful but not essential)
4. Two fine tip tweezers
5. wire cutters
6. Suture thread

7. Micro scissors

2. Creating the Surgical Window

1. Anesthesia: Procedures are in compliance with NIH guidelines and have been approved by UC Irvine Animal Care and Use Committee. Experimental subjects are 295-400 g male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA, USA) and the following anesthesia procedure should be used:
    1. Inject rat intraperitoneally with a sodium pentobarbital bolus (55 mg/kg b.w.) followed by an intramuscular injection of atropine (0.05 mg/kg, b.w.) in the hind leg, and administered 3.0 cc of 5% dextrose in water subcutaneously.
    2. Supplement sodium pentobarbital (27.5 mg/kg, b.w.) injections as necessary. Administer an ophthalmic antibiotic ointment to the eyes to protect the corneas during the following procedures. Administer 5% dextrose (3 ml) and atropine (0.05 mg/kg, b.w.) every six hours decrease respiratory secretions during anesthesia. Measure body temperature via a rectal probe, and maintain body temperature at 37 °C by a self-regulating thermal blanket.

2. Locate the MCA by either:
    a. Thinning a 2 x 2 mm imaging/visualizing window over the somatosensory cortex using a size HP 3 drill bit until the skull is nearly transparent and then thinning to full transparency using a size HP 2 drill bit. MCA's location can then be seen through this window and its proximal trajectory used to approximate the location of the initial segment. MCA will generally run diagonally across this window in a rostral to caudal/ventral to dorsal direction (for example, left to right/bottom to top when viewing left hemisphere from surgeon's point of view). The surgical window can then be created above where the observer estimates the M1 segment (proximal to cortical branching) to be located based on the distal branches visible through the first window. In order to minimize the amount of skull that is removed in order to gain access to MCA, the imaging/visualizing window should be positioned close to, but separate from the surgical window.

    Or

    b. A small surgical window should be positioned approximately 3 mm anterior and 1 mm lateral to the foramen ovale or the mandibular nerve, close to the arch rostrum [30,33,34]. In order to effectively access the stem of the MCA (also known as the M1 segment), the temporalis muscle is temporarily reflected away from the skull surface. (Note: In the case of long-term survival surgeries, our lab's experience has been that by allowing the temporalis muscle to remain attached at its anchor, the muscle will re-anneal to the skull surface, allowing for healthy eating behavior and effective maintenance of body weight.

3. Follow MCA to the rostral, ventral corner of the imaging window (if using this as a reference) in order to estimate where its initial cortical branch lies.
4. Create a new thin-skull region (we refer to this as the surgical window) slightly rostral and ventral to the imaging window (if using this as a reference) where the M1 segment (pre-cortical branching) of MCA should be. IMPORTANT NOTE: Leave approximately a 2 mm gap between the imaging window (if using this as a reference) and the surgical window.
5. Locate the stem of the MCA (also known as the M1 segment) just before cortical branching of the artery as shown in **Figures 1A and 1B**.
6. Using a size HP-3 drill bit, thin the skull above the estimated M1 segment location. When the skull becomes somewhat transparent, switch to the more delicate size HP-2 drill bit and thin the skull until it is completely transparent. Confirm visually as surgical window area becomes thin enough to view vasculature, and assess the location of M1 at this point and complete the window such that there is 2-3 mm on either side of the length of the M1 segment (this allows room for insertion and exit of the suture needle on either side of MCA).

IMPORTANT NOTE: Stop thinning when the thickness of the skull is similar to that of plastic wrap. The vessel will rupture if the drill breaks through the skull and dura. If the skull is not thin enough on the other hand, removing it for the occlusion will be difficult and could result in damage to the cortex or artery.

7. Take a 30 gauge (30 G) hypodermic needle and bend the tip of the needle, using serrated tweezers.
8. Use the 30 G needle to puncture the skull carefully in an area not directly above an artery. Use this puncture hole to allow tweezers to grasp skull and carefully remove the thinned area of the surgical window.
9. Take a new 30 G need, bend its tip as in Step 6, and carefully remove the dura.

NOTE: Cutting the dura will cause it to peel back and MCA will become more prominent, as a result of reduced pressure.

3. Occluding the MCA

1. Use wire cutters to trim a half-curve reverse cutting suture needle (round 3/8, 16 mm suture needle) down to about 3-5 mm.
2. Thread the trimmed suture needle as shown in the picture in **Figure 4E. IMPORTANT NOTE:** It is important that the needle is threaded so both ends of the suture thread are of equivalent length. This enables the pulling of both thread ends under M1 at the same time, the needle can then be cut free leaving two lengths of thread to tie the two knots around MCA.
3. Use the serrated the tweezers slip the suture needle under M1. Insert with about 0.5-1 mm distance from MCA, staying as shallow as possible so as to minimize damage to the cortex but avoiding too much strain on MCA as well.
4. When the suture needle comes out the other side such that it is under MCA, use a fine tip tweezer (as shown below) to pull the tip of the suture needle from the opposite side while continuing to feed or pushing the other end of the suture needle with the serrated tip tweezers.
5. Once the suture needle is completely passed under MCA and has been pulled out, continue to pull on the suture needle or thread until the length of the thread is equal on either side of MCA. Pressing down on the thread as it is fed through to minimize strain on MCA can be helpful to prevent rupture as the thread passes under the artery.
6. Cut the thread close to the suture needle.
7. Use both fine point tweezers to untangle the two resulting suture threads so that there are two independent threads strung under MCA that are not touching. Ideally the threads will be about 1 mm apart where they pass under MCA.

8. Use both fine point tweezers to tie two separate knots (two ligatures) with the threads around MCA attempting to maintain that ~1 mm of space between the knots to allow room for transection.

NOTE: If an internal sham control is desired, prepare the occlusion leaving the occlusion knots loose so that they do not constrict MCA at all and collect data prior to tightening the knots and cutting the vessel. Trim the thread to prevent it catching on anything prior to occlusion but leave enough thread to allow tightening of the knots later. This way, any baseline imaging or data collection can be performed with all of the same surgical invasion as the occlusion and the knots tightened at the appropriate time point with little delay.

9. Once the knots have been pulled tight, use the micro scissors to transect M1 in between the two knots.
10. In the case of long-term, survival studies:
    a. Suture incised scalp flap back in place with sterile surgical thread or secure the tissue using sterile wound clips.
    b. Administer antibiotics locally to the wound area (such as bacitracin ointment) and systemically by prophylactic injection of ampicillin (150 mg/kg IM).
    c. While the subject is still anesthetized administer an ophthalmic antibiotic ointment to the eyes.
    d. Administer supplemental atropine (0.05 mg/kg IM) to decrease respiratory secretions during anesthesia.
    e. Inject flunixin meglumine (1.1 mg/kg) subcutaneously at the conclusion of surgery and again the following morning (~12 hr later) for pain control.
    f. Place the animal on a dry, warm, slanted surface such that the animals nose is above its tail on the incline (this facilitates breathing until the animal is awake).
    g. Monitor the animal until it is awake and moving safely on its own.
    h. Once the animal is back in the vivarium, the animal's activity, appearance, vocalization, and feeding and drinking behavior should be monitored daily.

4. Euthanasia

1. At the conclusion of each experiment, rats should be euthanized with sodium pentobarbital (2-3 ml, intraperitoneally).

Representative Results

Successful occlusion of a vessel can be confirmed using laser speckle imaging (LSI) among other blood flow imaging techniques. Blood flow **in the major cortical branches of MCA** should drop to ~25% of baseline or less following occlusion depending on the level of noise in the recording system and sensitivity of the technique. See **Figure 3** for a representative LSI image of a segment of a cortical branch of MCA before and after MCA occlusion. When the described occlusion technique is applied to MCA at the M1 segment, blocking all cortical MCA branches, and sensory stimulation prevented for ~5 hr following occlusion, the result is a cortical infarct of 28.4±2.4 mm^3 (for a representative coronal slice of a 2,3,5-Triphenyl-tetrazolium chloride [TTC] stained brain with the described damage, see **Figure 2**; pale unstained area corresponds to infarct)[25].

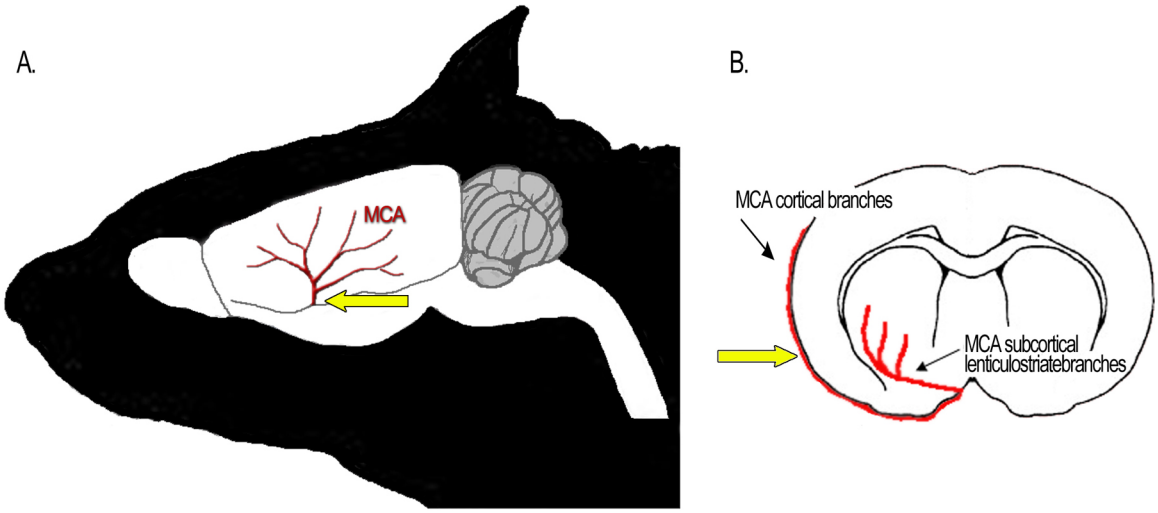

Figure 1. Yellow arrows indicate approximate location of pMCAO at the M1 segment. This occlusion example involves occluding MCA just distal to the lenticulostriate branching, prior to all cortical branching, thus cutting off blood supply to cortical branches only. **(A)** Diagram of MCA on lateral cortical surface. **(B)** Coronal view of approximate MCA cortical and subcortical branch locations. Note that occlusion of MCA proximal to lenticulostriate branching will result in cortical *and* subcortical infarct, though access to this region requires a relatively invasive surgical procedure. Click here to view larger figure.

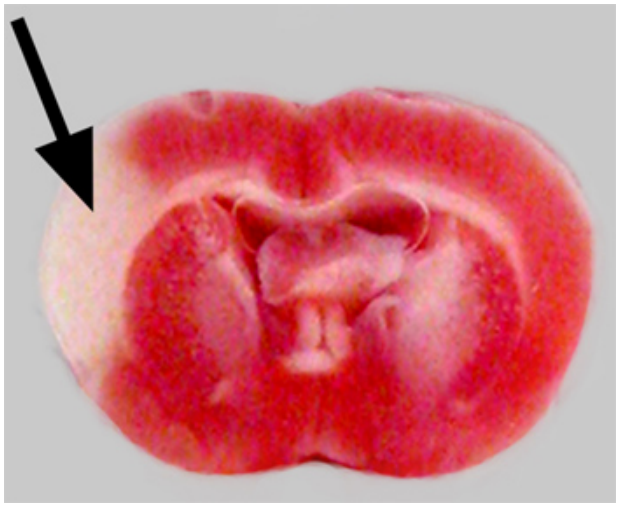

Figure 2. Single representative coronal slice from a rat brain showing infarct resulting from pMCAO (with care taken to minimize protective sensory stimulation for 5 hr following occlusion). 2,3,5-Triphenyl-tetrazolium chloride (TTC) solution stains healthy tissue reddish and leaves areas of cell death or infarct (indicated by arrow) pale. Note that due to the location of occlusion (prior to all MCA cortical branches but distal to subcortical branches) only cortical infarct is observed, and highly myelinated regions of the brain do not take up the TTC solution and will therefore remain white in color, despite being structurally intact.

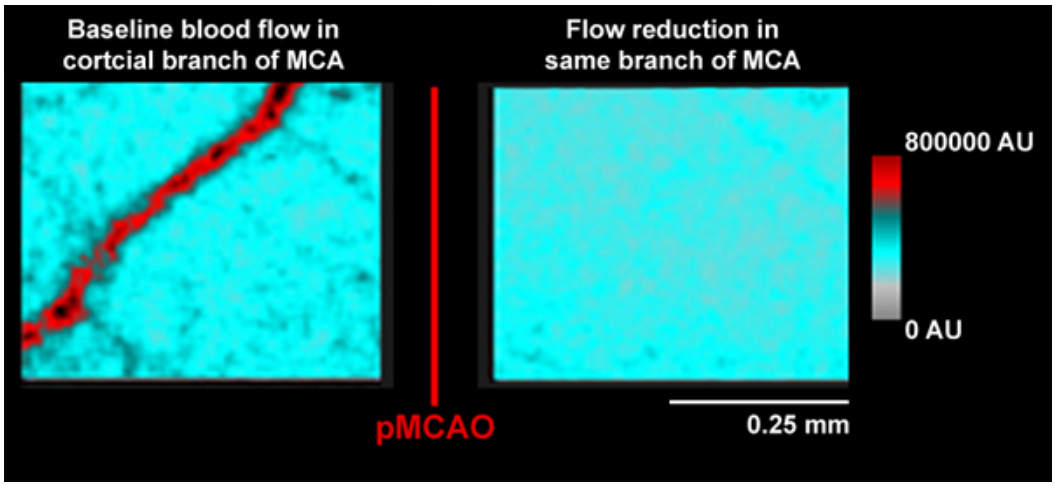

Figure 3. Image depicts flow in a portion of a single cortical branch of MCA before and after pMCAO as imaged using laser speckle imaging (LSI). Warmer colors indicate stronger flow. The described MCA branch is clearly visible traversing the baseline image (left) from the lower left to upper right corners and disappears following pMCAO. Note: occasionally some minimal evidence of flow remains in a given branch, but following pMCAO levels should drop to 20% or less of baseline flow to confirm occlusion success.

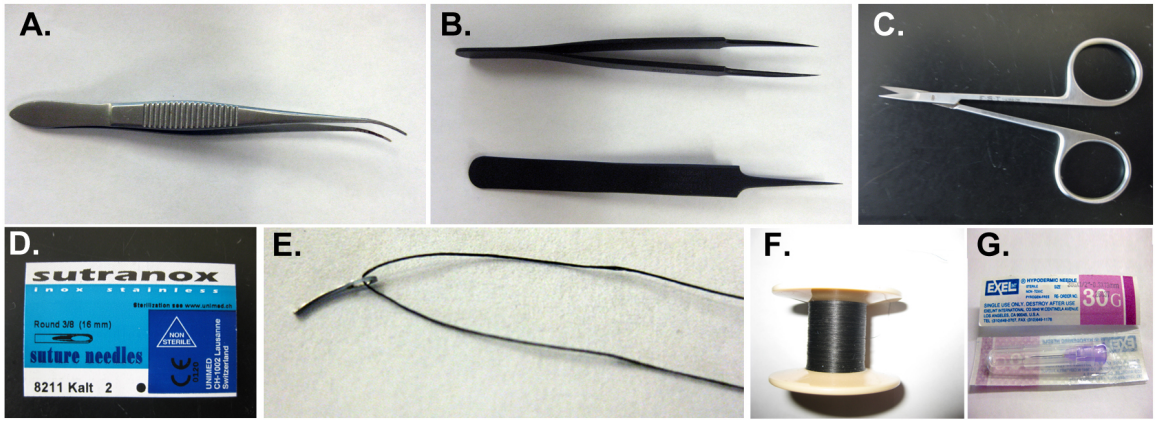

Figure 4. Surgical Tools Required for pMCAO. **(A)** Extra Fine Graefe Forceps - 0.5 mm Tips Slight Curve. **(B)** Ceramic Coated Dumont #5 Forceps. **(C)** Extra Fine Bonn Scissors, straight. **(D)** Round 3/8 (16 mm) Suture Needles. **(E)** NOTE: Suture needles may be shortened via wire cutters according to user preference. After shortening with wire cutters, suture needles should be sterilized. **(F)** 6-0 Braided Silk Suture. **(G)** 30 gauge needle, ½ in length.

Discussion

This protocol was developed in order to induce ischemia within the rodent cortex, and to do so with minimal peripheral impact to experimental subjects. The double occlusion and transection method allows for visual confirmation that the vessel has been permanently occluded, and may be performed without excessive invasion or tissue damage, and with a high survival rate. This occlusion protocol may be applied to any cortical vessel that can be accessed via craniotomy in order to induce ischemia within a specific cortical domain. Furthermore, such occlusions can be performed while an animal is in a stereotaxic apparatus allowing the simultaneous use of various investigational techniques, such as functional imaging or electrophysiological recording. This makes this occlusion technique applicable to a wide range of experimental designs, including within-subject investigation. For example, assessment may be carried out at baseline with sutures in place around the artery (but prior to securing the sutures and transecting), during ischemic onset, and at any post-occlusion time point required.

Successful execution of this occlusion is contingent upon two critical steps. First, proper visualization of the target vessel is critical to inducing ischemia. Occlusion at a location proximal to or distal to the desired location (in our typical case, just proximal to the primary anterior/ posterior cortical bifurcation of MCA) can result in a large degree of infarct volume variability, so care should be taken to confirm the proper site of occlusion and transection. Second, passing the suture needle around the target artery requires careful and precise technique. By necessity, the suture will pass through the most superficial layer of the cortex immediately below the artery. Care should be taken to avoid diving too deep within the cortical surface, because this can result in vessel rupture, hemorrhage, or damage to the brain at the occlusion site. While many types of blood vessel occlusion surgical tools are available, our lab has had the most success using half curve suture needles, truncated according to experimenter preference. Used in conjunction with ultra-fine forceps, this tool allows the user to pass suture thread below an artery and above the cortical surface with only minimal tissue damage.

Upon successful completion of an occlusion, infarct is limited to the cortex alone (**Figure 2**). In the context of using this occlusion method to model MCA stroke, this may have important implication for researchers given that many MCA stroke patients sustain infarct within both the cortex and basal ganglia. However, our laboratory favors this occlusion method as applied to MCA over techniques such as intraluminal suture given the recent findings that impaired mastication, swallowing function, and impaired motor performance occur in 47% of all subjects that undergo intraluminal suture [35]; impaired cerebral perfusion and reduced spontaneous motor activity resulting from reduced food and water uptake also contribute to poorer neurological recovery in rats following intraluminal suture [36-40]. Trueman *et al.* 2011 have also reported abnormal eating, impaired drinking behavior, and sensorimotor disability (as quantified by the adhesive removal task) following this procedure [11]. Critically, we observed the same behavioral deficits in sham intraluminal suture animals [11]. As a result, intraluminal suture may add serious confounding factors to preclinical stroke study- many of which are directly attributable to the surgical procedure and not to cerebral ischemic stroke.

It is impossible to model the variable etiology and pathology of human ischemic stroke - in fact such a high degree of variability would be undesirable in an experimental model. Stroke research in animals should instead focus on producing a result more analogous to human stroke damage and deficits while attempting to model etiology as best possible. We suggest that the minimally invasive nature, occlusion of MCA resulting in ischemia, infarct volume that is comparable to human MCA ischemia, and ability to incorporate multiple investigational techniques alongside pMCAO may make this method an attractive alternative for some preclinical stroke investigators. Additionally, the occlusion method modeled here by pMCAO provides an alternative, minimally invasive, effective means for occluding any surface cortical vessel.

Disclosures

The authors have nothing to disclose at this time.

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