

Video Article

A Middle Cerebral Artery Occlusion Technique for Inducing Poststroke Depression in Rats

Ruslan Kuts^{*1}, Israel Melamed^{*2}, Honore N. Shiyntum³, Dmitri Frank¹, Julia Grinshpun¹, Alexander Zlotnik¹, Evgeni Brotfain¹, Michael Dubilet¹, Dmitry Natanel¹, Matthew Boyko¹

¹Division of Anesthesiology and Critical Care, Soroka Medical Center, Ben-Gurion University of the Negev

²Department of Neurosurgery, Soroka Medical Center, Ben-Gurion University of the Negev

³Department of Biophysics and Biochemistry, Oles Honchar Dnipro National University

*These authors contributed equally

Correspondence to: Matthew Boyko at matthewboykoresearch@gmail.com

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Abstract

Poststroke depression (PSD) is the most recurrent of all psychiatric complications resulting from an ischemic stroke. A greater majority (about 60%) of all ischemic stroke patients suffer from PSD, a disorder considered to be an ischemic stroke-related precursor for increased death and degradation in health. The pathophysiology of PSD is still obscure. To study the mechanism of development and occurrence of PSD further, and to find out a therapy, we attempted to develop a new protocol that requires occluding the middle cerebral artery (MCA) via the internal carotid artery (ICA) in rats. This protocol describes a model of PSD induced in rats through the middle cerebral artery occlusion (MCAO). Also used in the experiment are the Porsolt forced swim test and the sucrose preference test to confirm and evaluate the depressive mood of the rats under investigation. Rather than inserting the catheter through the external carotid artery (ECA), as stipulated for the original procedure, this MCAO technique has the monofilament passing directly through the ICA. This MCAO technique was developed a few years ago and leads to a reduction in mortality and variability. It is generally accepted that the criteria used are preferred in the selection of biological models. The data obtained with this protocol show that this model of MCAO could be a way of inducing PSD in rats and could potentially lead to the understanding of the pathophysiology and the future development of new drugs and other neuroprotective agents.

Introduction

Stroke is fourth on the list of death-perpetrating diseases in the United States^{1,2,3}, while it causes the majority of disabilities in adults in developed countries⁴; this makes stroke a leading contender among the world's most significant health issues. Normalcy in stroke-surviving patients is rare, with about 15%–40% of survival victims suffering permanent disability, 20% requiring institutional care 3 months after stroke onset⁵, and about a third of 6-month survivals needing others to help them live through each day⁶. Stroke reportedly also accounts for the rising national health expenditures⁷. Estimates from the American Heart Association has stroke-related costs in the United States at over \$50 billion in 2010⁸.

Not only does stroke cause individuals' long-term damages, but some survivors tend to suffer emotional and behavioral disorders, such as dementia, fatigue, anxiety, depression, delirium, and aggression^{9,10,11,12,13,14}. The most recurrent psychological sequel after a stroke is poststroke depression (PSD), diagnosed in about 40%–50% of survivals^{15,16,17}. Stroke-induced depression results in increased morbidity and mortality^{18,19,20,21,22}. The pathophysiology of PSD is not known completely, but it is apparently caused by multiple factors and is linked to disability, cognitive impairment, and lesion site²³.

The rat model of focal brain ischemia, created by MCAO, is the most widespread animal model of stroke^{24,25,26,27}. In demonstrating the induction of PSD in rats by occluding the MCA via the ICA, techniques that minimize mortality and variability in the MCAO model are employed²⁸.

The primary objective of this protocol is to outline the steps for inducing PSD in rats by occluding the MCA via the ICA, a modified model of MCAO, which reduces mortality and the outcome of variability²⁸. Specific aims include performing neurological and histological examinations (determining the neurological severity score [NSS], the volume of the infarction zone, and brain edema) to verify the efficacy of MCAO and using behavioral tests to examine the influence of this MCAO procedure on the development of emotional disorders, mainly PSD.

Protocol

The Animal Care Committee of the Ben-Gurion University of the Negev, Israel approved all treatment and testing procedures used in this protocol.

1. Preparation of rats for the experimental procedure

NOTE: Select adult male Sprague-Dawley rats weighing 300-350 g.

1. House the rats, four per cage, in a vivarium at 22 °C and 40% humidity, with a reversed 12 h light/dark cycle (lights off at 8:00 a.m.) and unlimited access to food and water.
2. Randomly assign the rats to two groups, namely an MCAO group ($N = 24$) and a Sham group ($N = 19$).

2. Preparation of rats for surgery

1. To prepare the rats for the modified MCAO procedure, anesthetize each rat for 30 min with a mixture of isoflurane (4% for induction, 2% for surgery, and 1.3% for maintenance) in 24% oxygen (2 L/min) in an induction chamber and allow it to breathe spontaneously^{29,30}.
NOTE: Consider the anesthesia sufficient for surgery when the tail reflex of the rat ceases to occur.
2. Maintain the core body temperature at 37 °C with a heating plate.
3. Measure the body temperature of all rats through a probe placed in the rat's rectum before beginning the surgical procedure.
4. Keep the body temperature constant (37 °C) for all rats to minimize any hypothermic effect on the neurological outcome and any neurological injury.
5. Apply artificial tears ointment to both of each animal's eyes while they lie on the prep table.
6. Shave the neck of each rat and disinfect the skin with 70% alcohol chlorhexidine (70% alcohol and 0.5% chlorhexidine gluconate).
7. Cover the rats with sterilized surgical drapes.

3. Surgery (the MCAO technique)

NOTE: Perform surgery as described by Boyko et al.²⁸ and use the instruments provided by McGarry et al.²⁹ and Uluç et al.³⁰.

1. Perform a ventral midline incision and dissect the superficial fascia.
2. Carefully perform a sharp and blunt dissection within the three triangle-shaped muscle (the sternohyoid, the digastric, and the sternomastoid muscle) to identify the carotid arteries (the ECA, the ICA, and the common carotid artery [CCA]).
3. Carefully dissect and expose the right CCA and the ICA.
4. Separate the right CCA and ICA from the vagus nerve.
NOTE: Upon identifying the carotid arteries as stipulated in step 3.2, the ICA would be recognized as one-half of the CCA branches alongside the ECA.
5. Insert a catheter (heat-blunted or silicone-coated 4-0 nylon) monofilament directly through the ICA, ~18.5-19 mm from the bifurcation point of the right CCA into the circle of Willis until reaching a mild resistance, to occlude MCA.
NOTE: The heat-blunted filament and the silicone-coated 4-0 nylon play the same role and are the more preferred monofilaments in recent times, given that they provide better occlusion than the plain nylon thread^{28,30}.
6. Tie a 4-0 silk suture around the ICA just above the right CCA (pterygopalatine artery) bifurcation to block the ICA proximal to the filament insertion point permanently and distal to it temporarily.
7. Tie the ICA around the intraluminal thread to fasten the silk suture to prevent bleeding.
8. Subject the sham-operated rats to the same surgical procedure as the MCAO rats but insert a nylon thread instead²⁸.
NOTE: The nylon thread, like the silicon-coated nylon, occludes the ICA, but it is not as effective as the latter.
9. Close the wound on the rat's neck after occluding the MCA, turn off the anesthesia, and place the rat in an incubator under observation until it wakes up.
NOTE: The need for the proximal ligation is to occlude the ICA, and that of the additional distal ligation is to reduce the bleeding around the filament and secure it in place. Also, the rat should wake up a few minutes after its wound has been closed.

4. Postsurgical recovery

1. Administer 5 mL of 0.9% saline solution to each rat intraperitoneally, immediately after surgery, to prevent dehydration.
2. Give diluted dipyrone (0.5 g dissolved in 400 mL of drinking water) to rats showing symptoms of pain during the first 3 days.
3. Sacrifice any rats with seizures (seizures are caused by the increased intracranial pressure from cerebral edema or cerebral hemorrhage²⁸).

5. Neurological severity score³¹

NOTE: This procedure is performed by two observers who do not take part in the surgical proceedings; they test the neurological deficits and grade motor deficits on a cumulative score of 0-4³². The evaluation of this score may be performed at different time intervals; in this investigation, it was performed 50 min, 24 h, 7, 15, and 30 days postsurgery. Find below the steps for evaluating the NSS. Although not a necessity in this situation, this score is required to ascertain stroke in rodents in order to administer treatment.

1. Place the rat on a ceramic floor and allow it to move about freely for 1 min.
2. Gently pull the rat backward by the tail and grade as follows.
 1. Grade the rat with score 0 for no neurological deficit.
 2. Grade the rat with score 1 for forelimb flexion.
 3. Grade the rat with score 2 for contralateral weak forelimb grip.
 4. Grade the rat with score 3 for circling to the paretic side when pulled by the tail.
 5. Grade the rat with score 4 for spontaneous circling³².

NOTE: If more than one of the reactions are observed, preference is given to the action with a higher score.

6. Determination of the infarct volume (histologic examination)

1. Measurement of the infarct volume

NOTE: Perform this procedure as described previously^{33,34}. Measure brain infarct volume using 2,3,5-triphenyltetrazolium chloride (TTC) staining 24 h after reperfusion.

- Euthanize five rats from each group, 24 h after the last NSS, by exposing them to an isoflurane overdose in an induction chamber.
- Decapitate the rats and quickly isolate their brains using small scissors and forceps.
- Wash the isolated brains in 0.9% saline.
- Examine bleeding points on the brains to exclude the mice that underwent subarachnoid hemorrhage at the circle of Willis.
- Place each brain on a clean glass slide on a -20 °C ice pack and then place them in a -20 °C fridge for 5 min to make the brain easier to slice.
- Take the glass slide with the brain on it out of the -20 °C fridge, put it back on the -20 °C ice pack, and dissect the frontal pole and the cerebellum with blade and forceps.
- Slice the brain sections horizontally into 2 mm thickness with a blade to produce six slices.
- Prepare a 0.05% TTC solution by adding 1.25 g of TTC powder to 500 mL of normal saline solution prior to sacrifice, transfer the solution to a 24-well plate (1 mL per well) covered in foil, and store it at 4 °C.
NOTE: TTC and tissue stained with TTC are light sensitive.
- Using forceps, transfer the brain slices to the 24-well plate containing the TTC solution (one slice per well) and stretch out the slices in the solution.
- Incubate the plate content at 37 °C in a shallow water bath for 30 min.
- Aspirate the TTC solution from the plate with a pipette, wash them with a brainwashing fluid, and incubate at room temperature for 30 min.
- Place the slices in the order in which they were cut on a laboratory glass and examine the segments with a scanner.
- Analyze the infarct size as a percentage of the whole brain slice, using ImageJ analysis software, based on visual identification.

2. Analysis of the infarct volume³⁵

- Quantify the infarct volume by using a standard image analysis software (ImageJ) and analyze the infarct brain volume as a percentage of the whole brain size³³.
- Place the brain slices on glass microscope slides and scan them with an optical scanner at a high resolution (1,600 x 1,600 dpi) for an adequate analysis.
- Crop the images and standardize the scale for all images, using the metric ruler included in the scanned image.
- Measure the area of marked pallor in six consecutive 2 mm coronal sections using a Wand (tracing) tool and freehand selection on the ImageJ 1.37v software³⁶.
- Calculate the indirect infarct volume using the following formula³⁷.

7. Measurement of brain edema³⁸

NOTE: Brain edema was measured 24 h after the last MCAO.

- Assess the magnitude of the edema of the right hemisphere by using the summation of coronally sliced areas to calculate the volumes of the right and left hemispheres in arbitrary units (pixels).
NOTE: Use the ImageJ 1.37v software for this calculation, after optical scanning (resolution: 1,600 x 1,600 dpi). Select the area of interest and use the **Measure** function from the **Analysis** menu. Macros were used in our investigation.
- Express the brain edema area as a percentage of the standard areas in the unaffected contralateral hemisphere.
- Calculate the degree of swelling using the equation developed previously³⁹.

8. Behavioral paradigms

- Perform behavioral tests between days 30 and 33 after surgery in a closed, quiet, and light-controlled room.
- Assign investigators blinded to all experimental procedures to videotape all behavioral tests with a commercially available program.
- Sucrose preference test**^{40,41}
 - Place the rats in individual cages in the same room as where they are housed during the dark cycle.
 - For the next 24 h, place one bottle of 100 mL of 1% (w/v) sucrose solution in each cage with the rat to be tested and allow for the adaptation of the rat.
 - After 24 h, deprive the rats of food and water for 12 h by removing the bottles.
 - Then after 12 h, place two bottles in each cage for 4 h, one containing 100 mL of tap water and another containing 100 mL of sucrose solution (1% [w/v]).
 - Record the amount of sucrose solution and water consumed by rats in milliliters. Calculate the affinity to sucrose preference as follows.

4. Porsolt forced swim test⁴²

NOTE: The Porsolt forced swim test was carried out as described in a previous protocol published by Zeldetz et al.⁴⁰ and Boyko et al.⁴². The principle of this test states that, when rats are forced to swim in a restricted area from whence, they cannot escape, they eventually become immobile, ceasing any attempts to escape the water^{43,44}. This test was carried out in a different room during the dark cycle.

- Place each rat in a vertical plexiglass cylinder (height: 100 cm; diameter: 40 cm) containing 80 cm of water at 25 °C for 15 min for habituation.

2. Take the rat out and allow it to dry for 15 min in a heated enclosure (32 °C).
3. Return the rat to its home (original) cage.
4. Repeat step 8.4.1 24 h later, this time for the investigation, for 5 min.
5. Videotape the 5 min test and calculate the total duration of immobility during that period.

Representative Results

Histological findings (**Table 1**) revealed a statistically significant infarct volume as a percentage of total brain ($p < 0.0001$) post-MCAO when compared to animals in the sham-control group. Also reported was a statistically significant brain edema when the evaluation from the experimental group ($p < 0.0003$) was put side by side with that of the sham-control group.

The NSS scores obtained, as represented in **Table 2**, show lower neurological performances in the experimental group (MCAO) compared to higher figures for the sham-control group following Mann-Whitney tests: $p < 0.001$ after 50 min, $p < 0.05$ after 24 h, and $p < 0.05$ after 7 days.

Findings from the sucrose preference evaluations revealed that MCAO rats also consumed a significantly less amount of sucrose ($p < 0.0001$, **Figure 2A**) and had a longer immobility duration ($p < 0.0001$, **Figure 2B**) compared to the sham-operated rats.

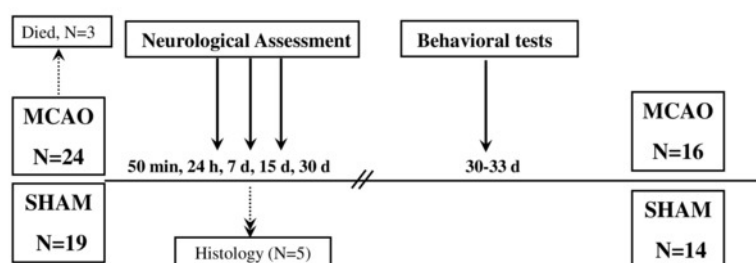


Figure 1: Graphic demonstration of the protocol timeline. The various tests run on rats at different times are shown on the scheme: MCAO = middle cerebral artery occlusion at the beginning of the experiment; NSS = neurological severity score, 50 min, 24 h, and 7 and 30 days post-MCAO; and behavioral tests (sucrose preference and Porsolt forced swim tests) from days 30 to 33 post-MCAO. This figure has been modified from Ifergane et al.⁴⁵. [Please click here to view a larger version of this figure.](#)

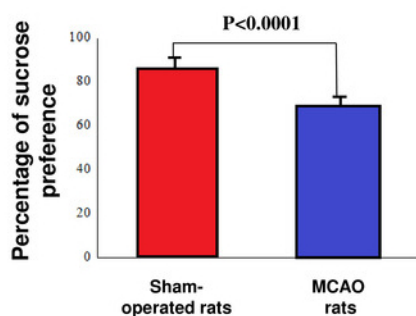


Figure 2: Sucrose preference test performed from days 30 to 33 with post-MCAO ($n = 16$) and sham-control rats ($n = 14$). Percentage (%) of sucrose preference. MCAO rats consumed less sucrose ($p < 0.0001$) than the sham-control rats, with a significant difference in sucrose consumption between the two groups shown in the figure. MCAO = middle cerebral artery occlusion. All data represent the group mean \pm SEM. This figure has been modified from Ifergane et al.⁴⁵. [Please click here to view a larger version of this figure.](#)

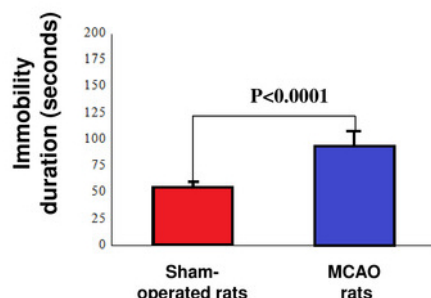


Figure 3: Porsolt forced swim test performed from days 30 to 33 post-MCAO ($n = 16$) and post-sham procedure ($n = 14$). Immobility duration (in seconds). The immobility time in the forced swim test was significantly longer in the MCAO group than in the sham group ($p < 0.0002$). MCAO = middle cerebral artery occlusion. All data represent the group mean \pm SEM. This figure has been modified from Ifergane et al.⁴⁵. [Please click here to view a larger version of this figure.](#)

Histological Findings	Middle cerebral artery occlusion group	Sham-operated group
Total brain (Infarct volume)	8.8% \pm 6.5	0.3% \pm 0.1
Brain Edema	10.2% \pm 4.6	2.6% \pm 1.2
All data represent group mean \pm S.E.M		

Table 1: Histological findings for infarct volume and brain edema. MCAO = middle cerebral artery occlusion ($n = 5$); sham ($n = 5$).

Neurological Severity Score	Middle cerebral artery occlusion group	Sham-operated group
50 min post-surgery	2.75 \pm 0.14	0.0 \pm 0.0
24 hours post-surgery	3.2 \pm 0.15	0.0 \pm 0.0
7 days post-surgery	0.91 \pm 0.2	0.0 \pm 0.0
All data represent group mean \pm S.E.M		

Table 2: Neurological severity score (NSS) for MCAO and sham-operated rats. MCAO = middle cerebral artery occlusion ($n = 16$); sham ($n = 14$). This table is taken from Ifergane et al.⁴⁵.

Discussion

One of the ways in which the MCAO technique presented here could be deemed safer than the original MCAO model is illustrated by the fact that the ECA and its branches, including the occipital artery, the terminal lingual, and the maxillary artery, are not compromised when occluding the MCA via the ICA. The original MCAO model's offset of the ECA (and its branches), by distally dissecting and coagulating them⁴⁶, causes impaired mastication, owing to a compromise to the vascular supply to masticating muscles⁴⁷. The destruction of the muscle could eventually cause the release of additional computational fluid dynamics. Contrary to the original MCAO technique in which access to the MCA occurs through the ECA, the technique described here is modified for stroke, with no occurrence of blood flow interruption in the ECA and its tributaries.

The most important arguments for preferring the novel MCAO model to the original MCAO rest in its ability to reduce variability in brain edema, infarct volume, and weight changes, significantly, as well as decrease MCAO-related mortality. Mortality in MCAO procedures is an important factor⁴⁸; a 20% mortality rate is deemed reasonable^{49,50}. The mortality rates (20% for original MCAO, 12.5% for novel MCAO, and 0% for control) in the current investigation were all within range of the acceptable rate, but the novel MCAO technique fared better than the original MCAO.

Rats undergoing the novel MCAO procedure lost less weight right after surgery and gained more weight by the end of the investigation than rats subjected to the original MCAO technique²⁸. More weight loss and less weight gain in rats subjected to the original MCAO could result from ligating the ECA during surgery, which causes distal hypoperfusion in the facial, lingual, and maxillary arteries, as well as ischemia-related damages to the muscles that support mastication. If mastication is impaired, oral intake lessens, which, when coupled with catabolism, could account for weight loss, and in the long run, for a poor neurological outcome, morbidity, and death⁴³. Access to the MCA via the ICA does not require interfering with the ECA and its branches. Thus, no impairments are triggered during the novel MCAO procedure, and no weight, morbidity, or mortality problems are experienced by rats undergoing the procedure.

The tests used to assess underlying depressive factors, such as depressive behavior, anhedonia, immobility, and learning and memory impairment, are standard procedures applied in animal models of depression^{51,52}. A behavioral test, like the Porsolt forced swim test, is commonly affected by unusual motor abilities following MCAO. Here, this test was applied from days 30 to 33 after the surgical procedure to ascertain that the induction of PSD in the modified MCAO rats did not overly affect their motor abilities. According to the results, rats from the experimental group showed a comparable total escape behavior to that of sham-control rats. MCAO rats had significantly more escape failures, a significantly raised duration of immobility, and a reduced preference for sucrose when compared with sham-operated animals. This would suggest that this MCAO technique is a capable alternative to the original MCAO method.

The difference between inclusion and exclusion of rats in the MCAO procedure for a broader experimental evaluation depends very much on the outcome of the operation. With the MCAO-related PSD-inducing model, some unwanted side-effects of MCAO could be curtailed, leaving room for the inclusion of smaller, and possibly fragile rats in the MCAO procedure. The animal model of MCAO presented here provides a scenario for

lessening unintended outcomes after MCAO-induced PSD because it has the potential to reduce variability in weight changes, brain edema, and infarct volume, as well as MCAO-related deaths. This technique could potentially serve as a tool for assessing future PSD therapies and provide preclinical data on the efficacy of therapeutic substances, as well as monitor other factors modifying modalities on the clinical outcome of stroke and PSD.

Disclosures

The authors have nothing to disclose.

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