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Laser-induced brain injury in the motor cortex of rats.

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TITLE:

Laser-Induced Brain Injury in the Motor Cortex of Rats

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KEYWORDS:

Brain injury, stroke, laser technique, animal model, middle cerebral artery occlusion, MCAO, motor cortex

SUMMARY:

The protocol presented here shows a technique to create a rodent model of brain injury. The method described here uses laser irradiation and targets motor cortex.

ABSTRACT:

A common technique for inducing stroke in experimental rodent models involves the transient (often denoted as MCAO-t) or permanent (designated as MCAO-p) occlusion of the middle cerebral artery (MCA) using a catheter. This generally accepted technique, however, has some limitations, thereby limiting its extensive use. Stroke induction by this method is often characterized by high variability in the localization and size of the ischemic area, periodical occurrences of hemorrhage, and high death rates. Also, the successful completion of any of the transient or permanent procedures requires expertise and often lasts for about 30 minutes. In this protocol, a laser irradiation technique is presented that can serve as an alternative method for inducing and studying brain injury in rodent models.

When compared to rats in the control and MCAO groups, the brain injury by laser induction showed reduced variability in body temperature, infarct volume, brain edema, intracranial hemorrhage, and mortality. Furthermore, the use of a laser-induced injury caused damage to the brain tissues only in the motor cortex unlike in the MCAO experiments where destruction of both the motor cortex and striatal tissues is observed.

Findings from this investigation suggest that laser irradiation could serve as an alternative and effective technique for inducing brain injury in the motor cortex. The method also shortens the time for completing the procedure and does not require expert handlers.

INTRODUCTION:

Globally, stroke is the second leading cause of death and the third leading cause of disability[1]. Stroke also leads to severe disability, often requiring extra care from medical staff and relatives. There is, therefore, a need to understand the complications associated with the disorder and improve the potential for more positive outcomes.

The use of animal models is the initial step to understanding diseases. To ensure the best research outcomes, a typical model would include a simple technique, affordability, high reproducibility, and minimal variability. The determinants in ischemic stroke models include brain edema volume, infarct size, the extent of the blood-brain barrier (BBB) breakdown, and functional impairment generally evaluated via neurological severity score[2].

The most widely used stroke induction technique in rodent models occludes the middle cerebral artery (MCA) transiently or permanently[3]. This technique produces a stroke model similar to the ones in humans: it has a penumbra surrounding the stroked area, is highly reproducible, and regulates ischemia duration and reperfusion[4]. Nevertheless, the MCAO method has some complications. The technique is prone to intracranial hemorrhage and injury to the ipsilateral retina with a dysfunction of the visual cortex and common hyperthermia that often lead to additional outcomes[5-7]. Other limitations include high variations in induced stroke (arising from the probable extension of the ischemia to unintended regions, like the external carotid artery region), insufficient occlusion of the MCA, and premature reperfusion. Also, rats of different strains and sizes exhibit various infarct volumes[8]. In addition to all the disadvantages mentioned, MCAO model cannot induce small isolated strokes in deep brain areas, because it is limited technically in terms of its requirement of minimum vessel size for catheterization. This makes the

need for an alternative model all the more critical. Another method, photothrombosis, provides a possible alternative to MCAO procedures but does not improve on the efficiency[9]. This technique targets stroke with light and offers some improvements on the previous models. However, photothrombosis requires an invasive craniotomy that is associated with secondary compications[9].

In the light of outlined shortcomings, the protocol presented here provides a capable alternative laser technique for inducing brain injury in rodents. The mechanism of action of the laser technique is based on the laser's photothermal effects imparted on living tissues, which leads to the absorption of light beams by body tissues and their conversion into heat. The advantages of using a laser technique are its safety and ease of manipulation. A laser's ability to produce heat to stop bleeding makes it very important in medicine, while its ability to amplify different beams at a given meet point ensures that lasers avoid destroying healthy tissues that stand in their way to their target point[10]. The laser beam used in this protocol can pass through a low liquid medium, such as bone, without emitting its energy and/or causing any destruction. Once it reaches a high liquid medium, such as brain tissues, it uses up its energy to destroy the target tissues. The technique, therefore, can induce brain injury only in the appropriate area of the brain.

The technique presented here showed a tremendous amount of ability to regulate its levels of irradiation, producing the chosen variations of brain injury intended from the start. Unlike the original MCAO that impacts both the cortex and striatum, the laser technique was able to regulate the impact of brain injury, inducing injury only on the intended motor cortex. Herein, the laser-induced brain injury protocol and a summary of representative results for the procedure performed on the cerebral cortex of rats are provided.

PROTOCOL:

The following procedure was conducted according to the Guidelines of the Use of Experimental Animals of the European Community. The experiments were also approved by the Animal Care Committee at the Ben-Gurion University of the Negev.

1. Animal selection and preparation

1.1. Select 65 male Sprague-Dawley rats weighing 300 to 350 g with no overt pathology for this procedure. The smaller size poses technical difficulties for the MCAO procedure.

1.2. Assign 3 rats per cage and let them adapt for least 3 days.

2. MCAO procedure

2.1. Select 25 rats for MCAO allowing for 10—20% mortality associated with the procedure[11].

2.2. Perform MCAO using a standard technique, as previously described in detail[12].

3. Laser-induced brain injury experimental procedure

3.1. Assign 20 rats to a group marked as laser group and 20 rats to another control group (sham-operated).

3.2. Subject the laser group rats to laser irradiation at $50\ J\ \times\ 10\ points$ in the following manner:

3.2.1. Anesthetize rat with a mixture of 2% isoflurane in oxygen allowing for the spontaneous ventilation. Check for sufficient anesthetic depth by pinching the tail with forceps to see the absence of the withdrawal reflex.

3.2.2. Maintain the core body temperature of the rat at 37 ˚C throughout the experimental procedure using a rectal temperature regulated heating pad.

3.2.3. Remove local hair with a shaver and disinfect with 70% ethyl alcohol.

NOTE: The size of the surgical incision should be approximately 3 cm. Remove hair by at least 2 cm around the incision area.

3.2.4. Place the rat on a stereotaxic head holder in a prone position and make a 3 cm incision to reflect the scalp laterally and to expose the area between Bregma and Lambda.

3.2.5. Maintain anesthesia through the nose cone.

3.2.6. Use Neodymium-YAG (Nd-YAG) laser (peak wavelength 1064 nm) to administer $50J\ \times\ 10\ points$, with 1 s pulse duration, to the exposed area of the skull above the right hemisphere.

3.2.7. Ensure that the laser generating part of the apparatus is at a 2 mm distance from the exposed area to produce a laser beam. $50J\ \times 10\ points$ was selected after careful evaluation of different energy/surface combinations. This combination is efficient and does not cause bone destruction of the skull after administration for less than a second[10].

NOTE: 2 mm is the distance between the terminal of the laser beam (from the optical cable it is passed through) and the skull bone. In case a focusing lens is used, the distance should be calculated taking into account the angle of inclination of the lens to focus the beam in the desired area of damage. Ensure proper safety when using a laser device including appropriate training and eye protection.

3.2.8. Remove the rat from the device and close the scalp with 3-0 silk surgical sutures.

3.2.9. Discontinue anesthesia and return the rat to its cage for recovery. Administer 0.1 mL of 0.25% bupivacaine locally to reduce the postoperative pain immediately after surgery.

NOTE: The entire procedure should last less than 5 min if performed correctly.

3.3. Observe the rat for any signs of distress during post-anesthesia recovery. Give 0.01mg/kg intramuscular buprenorphine for postoperative analgesia every 12 h as needed for 48 h.

3.4. Subject control rats to the same conditions without subjecting them to the laser.

4. Neurological severity score (NSS)

4.1. Evaluate the neurological severity score 24 h after the laser-induced brain injury using a 43-point score[13]. Test the animals for neurological deficits, behavior disturbances, beam-balancing task, and reflexes, assigning higher scores for more severe disabilities, as previously detailed[13].

5. Post-injury manipulations

5.1. After NSS evaluation, euthanize the rats by exposing them to 20% oxygen and 80% CO_2 (via inspiration). Ensure that CO_2 is delivered at a predetermined rate in accordance with the Institutional Animal Care and Use Committee guidelines.

5.2. Harvest brains and prepare for further examination as described in an earlier protocol[11].

5.3. Evaluate for subarachnoid hemorrhage (SAH) through visual examination of the whole brain after its isolation from the skull. If necessary, a microscope or magnifying glasses may be used for this purpose.

6. Evaluation of the brain injury

6.1. Determining the brain infarct volume and brain edema by TTC staining

NOTE: 2,3,5-Triphenyltetrazolium chloride (TTC) staining is a convenient procedure for brain infarct detection[11].

6.1.1. Section the harvested brains into 6 coronal slices, each 2 mm thickness.

6.1.2. Incubate the set of slices from each brain for 30 min at 37 °C in 0.05% TTC.

6.1.3. Following staining, scan the slices with an optical scanner with a resolution of $1600 \times 1600\ dpi$.

6.1.4. The unstained areas of the fixed brain slices are defined as infarcted[12].

6.1.5. Using an image processing software (e.g., freeware *Image J*) measure the unstained infarcted area, ipsi- and contralateral hemispheres for each of the 6 coronal slices.

6.1.6. Calculate the infarcted volume as a percentage of the total brain:

$$corrected\ infarct\ size = infarct\ size \times \frac{contralateral\ hemisphere\ size}{ipsilateral\ hemisphere\ size}$$

6.1.7. Calculate brain edema using Kaplan method:

$$extent\ of\ edema = \frac{ipsilateral - contralateral\ hemisphere\ size}{contralateral\ hemisphere\ size}$$

6.3. Determining the extent of blood brain barrier (BBB) breakage

NOTE: Assess BBB breakage 24 h after the laser-induced brain injury as follows:

6.3.1. Administer 2% Evans Blue mixed with 4 mL/kg saline solution intravenously to rats via the cannulated tail vein and allow the solution to circulate for 1 h.

6.3.2. Euthanize rats by exposing them to 20% oxygen and 80% CO_2 (via inspiration) 24 h after the last NSS, as previously described[13].

6.3.3. Harvest the intravascularly localized dye as follows:

6.3.3.1. Open the chests of the rats with surgical pincettes and surgical scissors.

6.3.3.2. Perfuse the animals with cooled 0.9% saline via the left ventricle using 110 mmHg until obtaining a colorless perfusion liquid from the right atrium.

6.3.4. Harvest the brains and slice them rostrocaudally into 2 mm slices.

6.3.5. Separate the left brain slices from the right portions to evaluate injured and non-injured hemispheres separately.

6.3.6. Weigh, homogenize using mortar and pestle, and then incubate the brain tissues in 50% trichloroacetic acid for 24 h.

6.3.7. Centrifuge the homogenized brain slices at 10,000 × *g* for 20 min.

6.3.8. Mix 1 mL of the supernatant from the centrifuged brain with 1.5 mL of 96% ethanol at 1:3 and assess blood-brain barrier breakage using a fluorescence detector at 620 nm excitation wavelength (10 nm bandwidth) and 680 nm emission wavelength (10 nm bandwidth).

NOTE: Both groups of rats undergo the same protocol for determining BBB breakdown.

REPRESENTATIVE RESULTS:

No deaths or SAH were registered in either the control or experimental groups (**Table 1**). The MCAO group had a 20% rate of both mortality and SAH.

The relative body temperature changes in the rats of both groups were also similar, despite a difference in the variability of both groups (**Table 1**).

There was a significantly worse NSS in both the laser (16 ± 1.1) and MCAO (20 ± 1.5) models, compared to the sham-operated control group (1 ± 0.3; **Table 1**; $p<0.01$).

The laser-induced brain injury also caused a significant increase in infarct volume at the target hemisphere, compared to the sham-operated control group (2.4% ± 0.3 vs 0.5% ± 0.1; **Table 2** and **Figure 1A**; $p<0.01$), per the Mann-Whitney U test. However, the infarct volume of the laser model was smaller in comparison to the MCAO technique (2.4% ± 0.3 vs 9.9% ± 2.9).

Brain edema determined 24 h after brain injury are shown in **Figure 1B** and **Table 2**. There was no difference in brain edema between the laser-induced brain injury model and the sham-operated control group (3.4% ± 0.6 vs 0.7% ± 1.2). There was a significant difference in brain edema between the laser model and the MCAO technique (3.4 ± 0.6 vs 7 ± 2.6†). Data are presented as mean ± SEM.

Compared to the sham-operated control group, the laser-induced brain injury and MCAO technique both caused a significant increase in BBB breakage at the non-injured hemisphere (563 ng/g ± 66 and 1176 ng/g ± 168, respectively, vs 141 ng/g ± 14; **Figure 2A** and **Table 2**; $p<0.01$) and target hemisphere (2204 ng/g ± 280 and 2764 ng/g ± 256, respectively, vs 134 ng/g ± 11; **Figure 2B** and **Table 2**; $p<0.01$).

Histological examination of rats' brains are shown in **Figure 3.**

FIGURE AND TABLE LEGENDS:

Table 1: **Assessment of NSS, body temperature, subarachnoid hemorrhage, and mortality.** * = $p < 0.01$

Table 2: Assessment of BBB breakdown, infarct zone, and brain edema. * = $p < 0.01$

Figure 1: Assessment of brain injury in the laser model 24 h after the injury compared to the MCAO model and sham-operated control. (**A**) Assessment of infarct volume. There was an increase in infarct volume in the laser model compared to the sham-operated control (*$p<0.01$). However, the infarct volume in the laser model was smaller compared to the MCAO model (*$p<0.01$). (**B**) Assessment of total brain edema. There was an increase in brain edema in the MCAO model compared to either the laser model or sham-operated control. There was no difference in brain edema between the laser model and sham-operated control. The data are measured as % to the contralateral hemisphere and expressed as mean ± SEM.

Figure 2: The extent of BBB breakdown compared to sham controls. (**A**) Contralateral (non-injured) hemisphere. Both, the laser and MCAO models, led to a significant increase in BBB

breakage at the non-injured hemisphere compared to the sham-operated control group (*p<0.01). (**B**) Ipsilateral (injured) hemisphere. There was a difference in ipsilateral BBB breakdown in the laser and MCAO models compared to the sham-operated control (*p<0.01).

Figure 3: Histological examination of rats' brains from sham, laser and MCAO groups.

DISCUSSION:

It is fair to assume that the laser technique is minimally invasive, given that no deaths or SAH occurred in the laser group. The primary cause of death and SAH is the damage to blood vessels that leads to an elevation of intracranial pressure (ICP), as shown in the original MCAO techniques[10]. The absence of death and SAH in the laser group is likely due to the specific effects of lasers: they do not have direct impact on blood vessels and can induce coagulation in case of leakage. Low infarct volume and brain edema also help minimize the risk of death. The use of lasers should be considered as a suitable technique for inducing brain injury with minimal adverse outcomes, given that the original MCAO techniques for triggering stroke (both transient and permanent) have been shown to produce deaths and SAH[6].

Low body temperature findings in the laser group show that the laser technique does not occlude the hypothalamic artery that regulates body temperature, as the original MCAO does typically[7], supporting the theory that the laser technique is more targeted. Low variability across the board of parameters investigated indicated consistency in the use of lasers to induce brain injury, but such fine results depend very much on the choice of power. Sufficient power provides desired outcomes, while little or surplus calibrations can cause under- or over-performance, which in either case is detrimental. Nevertheless, the ability to aim for the target still makes the technique less risky. Hence, correct handling makes it easier to obtain results with precision using the laser technique, as well as to regulate the method for desirable effects.

The precision and efficacy of the laser technique were evident in its ability to strike only the motor cortex without causing damage to the striatum, suggesting that the laser technique can produce localized injury that is almost impossible to achieve with MCAO[10]. This achievable outcome with the laser technique is due to the ability to regulate the laser beam and its power and makes the laser method a model technique for inducing smaller, peripheral, and deep and defined brain injury that cannot be obtained with MCAO. The simplicity of manipulating a laser machine makes it very desirable. Unlike MCAO techniques that demand arduous training and experts, using lasers is more simple, requiring no experts or expensive training. The use of the laser technique could boost research and help to uncover better outcomes more quickly than the MCAO method alone.

In terms of limitations of the laser technique, the use of laser beams does not produce brain injuries that are perfectly similar to acute vascular occlusive strokes. Specifically, lasers produce immediate tissue scars at the target site that are comparable to a vascular occlusive stroke that is several days old. The technique might, therefore, not be suitable for evaluating drugs that aim to prevent the spread of stroke but should be ideal in assessing isolated motor cortex stroke on

prolonged motor, cognitive, and behavioral impairments. The use of a small number of rats for this research was also a limitation, with only half the number of rats (n = 10) in each group used for brain harvesting and examination of the size of the stroke, the extent of brain edema, BBB breakage, and SAH presence.

The lack of comparisons between our technique and other laser methods may also be deemed a limitation. We deliberated on performing comparative methods but decided not to do so because assessing the damage caused by these other laser methods is difficult. For example, the photothrombosis technique[6] causes weak damage that makes it challenging to evaluate brain swelling and other conditions that may occur. Also, the use of craniotomy in the laser technique for ischemia is problematic because craniotomy is very invasive and can increase BBB's permeability, causing additional brain injury that is not associated with stroke. Assessing such damage for comparison with our method is nearly impossible. The laser model induces stroke with radiation through the skull without craniotomy.

Like many models, the laser model has its benefits and limitations, with the most glaring drawback is its inability to mimic perfectly human stroke as precisely as other models. Nevertheless, the low variability in primary outcomes of most parameters, its precision, affordability, ability to induce smaller brain injuries, and its straightforward application makes it a suitable alternative technique for brain injury in rodents.

ACKNOWLEDGMENT:
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DISCLOSURES:
The authors have nothing to disclose.

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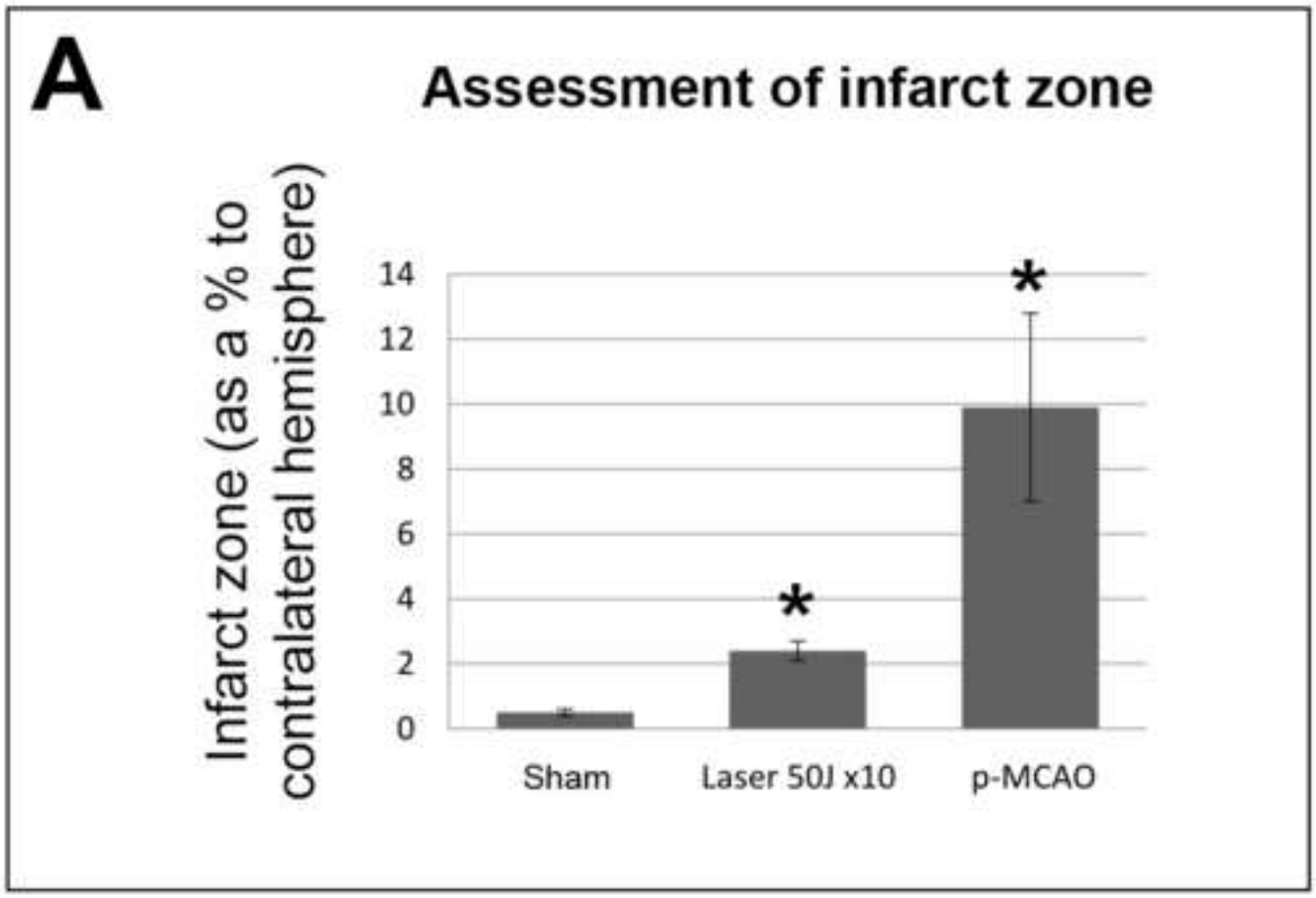
Figure 1



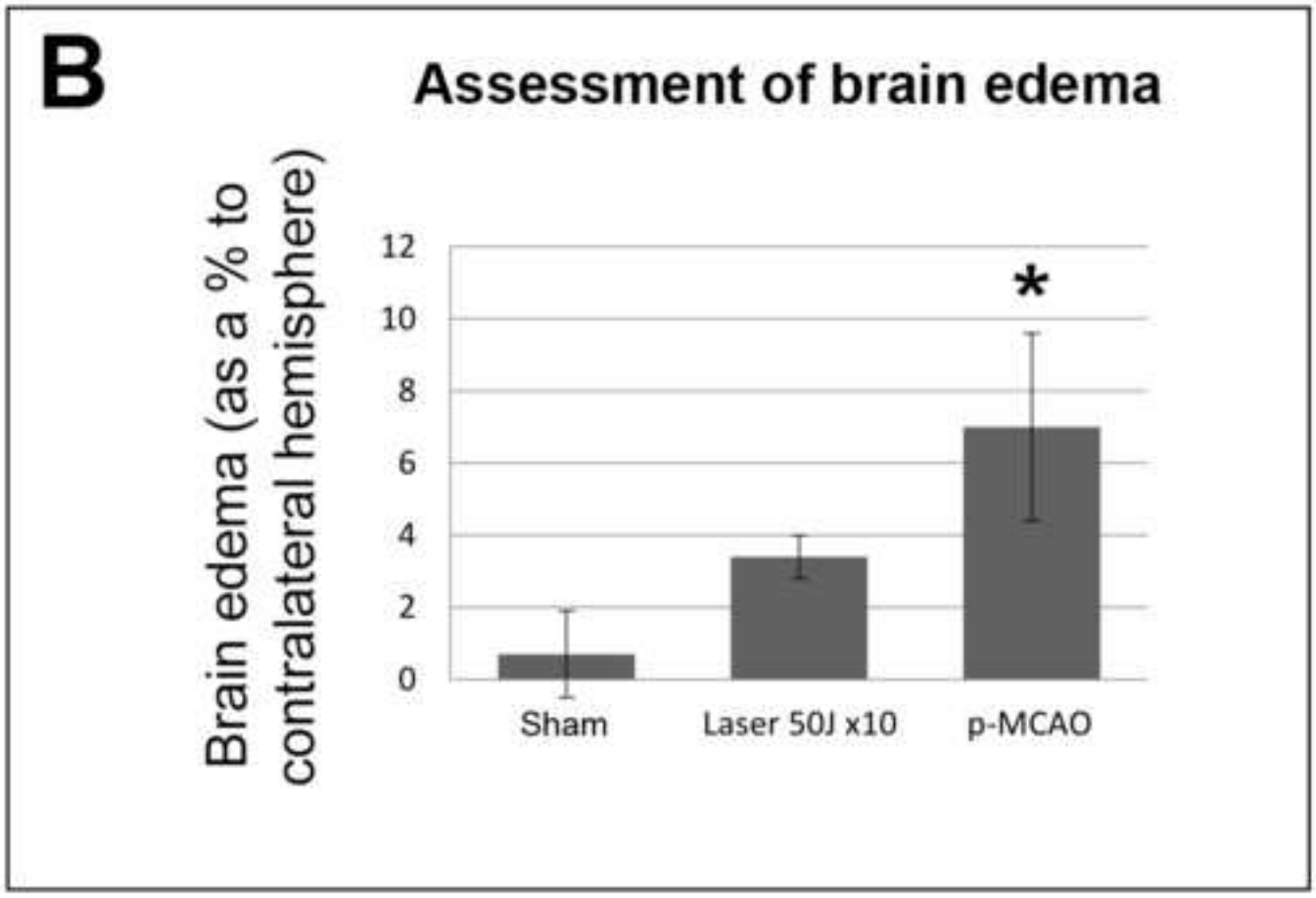


Figure 2

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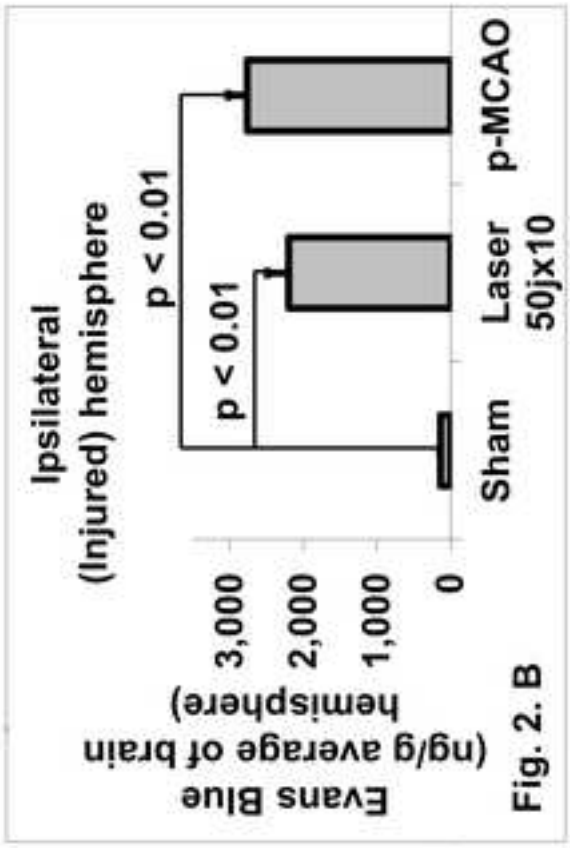

Fig. 2. B

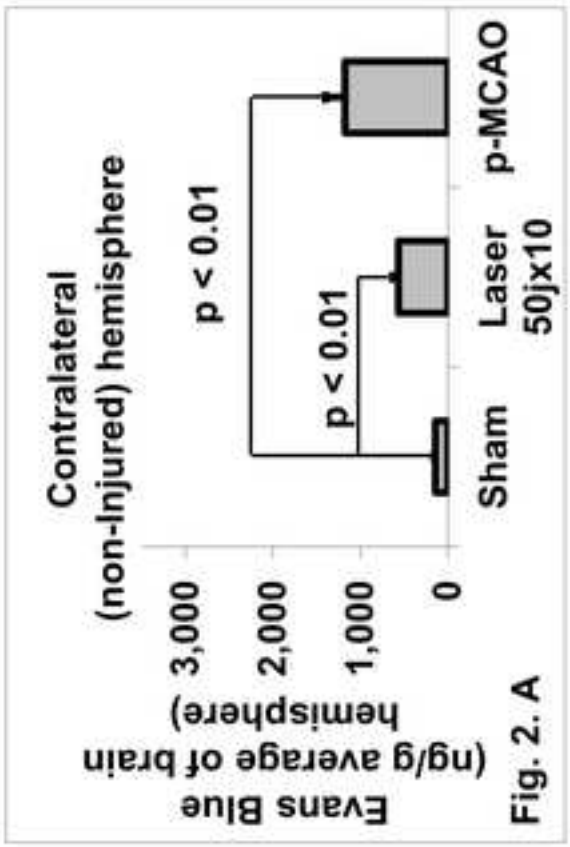

Fig. 2. A

Figure 3

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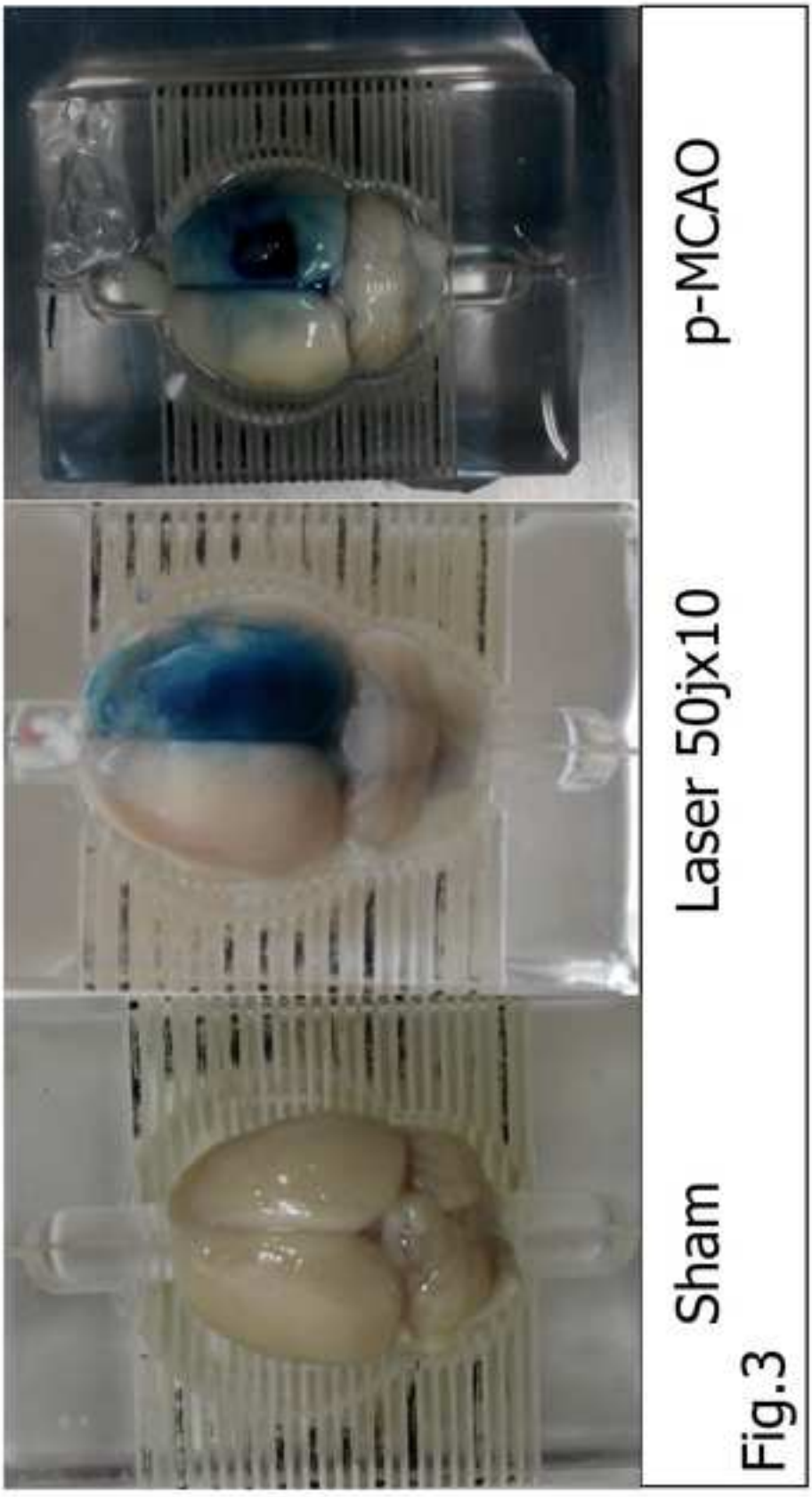

Table 1

	NSS		Temperature, °C		SAH, %
Groups	*mean ± SEM*	*variability, %*	*mean ± SEM*	*variability, %*	
Sham-operated c	1 ± 0.3	97	37.2 ± 0.1	59	0
Laser 50J x10	16 ± 1.1*	30	37.4 ± 0.1	84	0
p-MCAO	20 ± 1.5*	37	38.3 ± 0.1*	129	20*

Mortality, %
0
0
20*

Table 2

	BBB		Infarcted Volume		Brain E
Groups	*mean ± SEM*	*variability, %*	*mean ± SEM*	*variability, %*	*mean ± SEM*
Sham-operated c	134 ± 11	25	0.5 ± 0.1	77	0.7 ± 1.2
Laser 50J x10	2204 ± 280*	40	2.4 ± 0.3*	34	3.4 ± 0.6
p-MCAO	2764 ± 256*	29	9.9 ± 2.9*	92	7 ± 2.6*

Edema *variability, %*
573
58
115

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
2,3,5-Triphenyltetrazolium chloride	SIGMA - ALDRICH	298-96-4	
50% trichloroacetic acid	SIGMA - ALDRICH	76-03-9	
Brain & Tissue Matrices	SIGMA - ALDRICH	15013	
Cannula Venflon 22 G	KD-FIX	1.83604E+11	
Centrifuge Sigma 2-16P	SIGMA - ALDRICH	Sigma 2-16P	
Compact Analytical Balances	SIGMA - ALDRICH	HR-AZ/HR-A	
Digital Weighing Scale	SIGMA - ALDRICH	Rs 4,000	
Dissecting scissors	SIGMA - ALDRICH	Z265969	
Eppendorf pipette	SIGMA - ALDRICH	Z683884	
Eppendorf Tube	SIGMA - ALDRICH	EP0030119460	
Ethanol 96 %	ROMICAL		Flammable Liquid
Evans Blue 2%	SIGMA - ALDRICH	314-13-6	
Fluorescence detector	Tecan, Männedorf Switzerland	model Infinite 200 PRO multimode reader	
Heater with thermometer	Heatingpad-1	Model: HEATINGPAD-1/2	
Infusion Cuff	ABN	IC-500	

Isofluran, USP 100%	Piramamal Critical Care, Inc	NDC 66794-017
Multiset	TEVA MEDICAL	998702
Olympus BX 40 microscope	Olympus	
Optical scanner	Canon	Cano Scan 4200F
Petri dishes	SIGMA - ALDRICH	P5606
Scalpel blades 11	SIGMA - ALDRICH	S2771
Sharplan 3000 Nd:YAG (neodymium-doped	Laser Industries Ltd	
Stereotaxic head holder	KOPF	900LS
Sterile Syringe 2 ml	Braun	4606027V
Syringe-needle 27 G	Braun	305620

Attn: Vineeta Bajaj, Ph.D.

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Title: Laser-induced brain injury in the motor cortex of rats.

Dear Dr. Bajaj,

Please find attached a revised version of the manuscript JoVE60928R2. We have addressed all the specific comments marked in the attached manuscript. We very much hope that this revised manuscript is now suitable for publication in JoVE.

We thank you and the reviewers for your consideration.

Best regards,

Matthew Boyko, PhD

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