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TITLE:

Measuring Post-Stroke Cerebral Edema, Infarct Zone and Blood-Brain Barrier Breakdown in a Single Set of Rodent Brain Samples

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KEYWORDS:

Blood brain barrier (BBB) disruption; brain edema; infarct zone; ischemic stroke; middle cerebral artery occlusion (MCAO); rat model

SUMMARY:

This protocol describes a novel technique of measuring the three most important parameters of ischemic brain injury on the same set of rodent brain samples. Using only one brain sample is highly advantageous in terms of ethical and economic costs.

ABSTRACT:
One of the most common causes of morbidity and mortality worldwide is ischemic stroke. Historically, an animal model used to stimulate ischemic stroke involves middle cerebral artery occlusion (MCAO). Infarct zone, brain edema and blood-brain barrier (BBB) breakdown are measured as parameters that reflect the extent of brain injury after MCAO. A significant limitation to this method is that these measurements are normally obtained in different rat brain samples, leading to ethical and financial burdens due to the large number of rats that need to be euthanized for an appropriate sample size. Here we present a method to accurately assess brain injury following MCAO by measuring infarct zone, brain edema and BBB permeability in the same set of rat brains. This novel technique provides a more efficient way to evaluate the pathophysiology of stroke.

INTRODUCTION:
One of the most common causes of morbidity and mortality worldwide is stroke. Globally, ischemic stroke represents 68% of all stroke cases, while in the United States ischemic stroke accounts for 87% of stroke cases[1,2]. It is estimated that the economic burden of stroke reaches $34 billion in the United States[2] and €45 billion in the European Union[3]. Animal models of stroke are necessary to study its pathophysiology, develop new methods for evaluation, and propose new therapeutic options[4].

Ischemic stroke occurs with occlusion of a major cerebral artery, usually the middle cerebral artery or one of its branches[5]. Thus, models of ischemic stroke have historically involved middle cerebral artery occlusion (MCAO)[6-12]. Following MCAO, neurological injury is most commonly assessed by measuring infarct zone (IZ) using a 2,3,5-triphenyltetrazolium chloride (TTC) staining method[13], brain edema (BE) using drying or calculating hemispheric volumes[14-16], and blood brain barrier (BBB) permeability by a spectrometry technique using Evans blue staining[17-19].

The traditional MCAO method uses separate sets of brains for each of the three brain measurements. For a large sample size, this results in a significant number of euthanized animals, with added ethical and financial considerations. An alternative method to alleviate these costs would involve measurements of all three parameters in a single set of post-MCAO rodent brains.

Previous attempts have been made to measure combinations of parameters in the same brain sample. Simultaneous immunofluorescent staining methods[20] as well as other molecular and biochemical analyses[21] have been described after TTC staining in the same brain sample. We have previously calculated brain hemisphere volumes to assess brain edema and performed TTC staining to calculate infarct zone in the same brain set[15].

In the present protocol, we present a modified MCAO technique that measures ischemic brain injury through determining IZ, BE, and BBB permeability in the same set of rodent brains. IZ is measured by TTC staining, BE is determined by calculating hemispheric volume, and BBB permeability is obtained by spectrometry methods[19]. In this protocol, we used a modified MCAO model, based on direct insertion and fixation of the monofilament catheter into the internal carotid artery (ICA) and further blocking of blood flow to the middle cerebral artery (MCA)[22]. This

modified method shows a decreased rate of mortality and morbidity compared to the traditional MCAO method[16,22].

This new approach provides a financially-sound and ethical model for measuring neurological injury after MCAO. This assessment of the main parameters of ischemic brain injury will help to comprehensively investigate its pathophysiology.

PROTOCOL:

The following procedures were conducted according to the recommendations of the Declaration of Helsinki and Tokyo and the Guidelines for 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. Preparing rats for the experimental procedure

1.1. Select adult male Sprague-Dawley rats without overt pathology, each weighing between 300 and 350 g.

1.2. Maintain all rats at room temperature at 22 °C, with 12 hours of light and dark cycles before experiment.

1.3. Ensure that food and water are available ad libitum.

1.4. Perform all procedures between 6:00 a.m. and 2:00 p.m.

2. Preparing rats for surgery

2.1. Anesthetize the rats for 30 min with isoflurane (4% for induction and 2% for maintenance) and 24% oxygen (1.5 L/min).

2.1.1. Test the level of anesthesia in the rats by ensuring they do not have a pedal withdrawal reflex.

2.2. Insert the 24-gauge catheter into the tail vein.

NOTE: Tail warming for vasodilation is not performed.

2.2.1. Place the rats on the table in a supine position. Use medical tape to affix all four of the rats' limbs.

2.3. Place the probe for temperature measurement into the rat rectum before surgery.

2.4. During the procedure, maintain a heating plate to support a 37 °C core body temperature.

2.5. Add ointment in both of the rat's eyes for protection.

2.6. Shave the surgical area and disinfect with three applications of 10% povidone-iodine followed by 70% isopropyl alcohol.

3. Right side middle cerebral artery occlusion

NOTE: MCAO is performed by a modified technique, as previously described[16,22,23], with the use of instruments described by McGarry et al.[24] and Uluç et al.[25].

3.1. Dissect the skin and superficial fascia at the ventral midline of neck with surgical tweezers and scissors with curved blades.

3.2. Identify the muscle triangle, consisting of the ICA, external carotid artery (ECA) and common carotid artery (CCA).

3.3. Carefully separate the right CCA and ICA from the vagus nerve with microforceps for vascular surgery.

3.4. Expose the right CCA and the ICA. Block the blood flow coming from the CCA to the ICA using either micro-clips or special tourniquets for vascular surgery. Make an incision (approximately 1 mm) on the ICA using microscissors for vascular surgery.

3.5. Insert a monofilament catheter (4-0 nylon) directly through the ICA, about 18.5-19 mm from the bifurcation point of the right CCA into the circle of Willis until reaching a mild resistance, to occlude the MCA[26].

3.6. Ligate around the ICA above the bifurcation of CCA.

3.7. For the sham-operated control group, perform an insertion of nylon thread instead of steps 3.5 and 3.6[16,22].

3.8. Administer 5 mL of 0.9% sodium chloride by intraperitoneal injection.

3.9. Close the wound by suture and take the rat to a recovery area.

NOTE: A few minutes after the end of anesthesia, the rat will wake up and move independently around the cage.

3.10. At 23 h after MCAO, inject 2% Evans blue in saline (4 mL/kg)[23,26] into the tail vein for both operated groups via a cannula[27].

NOTE: This is used as a blood-brain permeability tracer. Allow to circulate for 60 minutes.

4. Determination of infarct zone

4.1. Measure IZ at 24 h after MCAO as described previously[9,15,18,19,26].

NOTE: Rats that lost more than 20% of their weight or developed seizures or hemiplegia are excluded from the experiment.

4.2. Euthanize the rat by replacing the inspired gas mixture with 20% oxygen and 80% carbon dioxide until the rat ceases to breathe spontaneously.

4.3. Open the chest with a 5-6 cm lateral incision through the abdominal wall under the rib cage using scissors and surgical forceps.

4.4. Perform a diaphragmatic incision along the entire length of the rib cage with scissors and surgical forceps.

4.5. Carefully displacing the lungs, cut through the rib cage up to the collarbone on the right and left sides[28].

4.6. Perfuse with 200 mL of normal saline through the left ventricle of the heart.

4.7. Puncture or incise the right atrium of the heart with scissors.

4.8. Perform decapitation using a guillotine and collect brain tissue.

4.9. Using iris scissors, cut from the foramen magnum to the distal edge of the posterior skull surface on both sides.

4.10. Separate the olfactory bulbs, nervous connections along the ventral surface and dorsal surface of the skull from the brain.

4.11. Remove the brain from the head.

4.12. Produce 6 brain slices by creating 2 mm thick horizontal sections with a .009" stainless steel, uncoated, single edge razor blade.

4.13. Incubate for 30 min at 37 °C in 0.05% TTC.

4.14. Place the brain tissue on the microscope slides and perform optical scanning of these 6 brain-slices with a resolution of 1600x1600 dpi (see **Supplement 1** for example).

4.15. Add a blue filter with a photo editor (e.g., Adobe Photoshop CS2) using the Channel Mixer function (**Image > Adjustments > Channel Mixer**) and save the image as a JPEG file format.

NOTE: After applying the blue filter, the image will appear greyscale.

4.16. Open the saved image in ImageJ 1.37v[29,30].

NOTE: This computer program uses a threshold function to isolate and calculate the pixels that are either black or white (see **Figure 1**).

4.17. For each of the 6 brain slices of the image, select and save each hemisphere (right injured ipsilateral and left uninjured contralateral) as a separate image file using the "polygon selection" tool from the main menu.

4.18. Set the cut-off for determining IZ by using an auto threshold function from the main menu of the ImageJ software by selecting **Image > Adjust > Threshold**, and measure the number of pixels in each hemisphere of a single brain set.

NOTE: Macros may be used for this step in ImageJ software (see **Supplement 2** for the code). The cut off is a critical parameter for determining which pixels to convert to white and which to convert to black depending on the shade of gray (see **Supplement 3** and **Supplement 4** as examples). ImageJ then compares white and black pixels to determine IZ. Based on the staining protocol and scanner settings, we used a constant cut-off value of 0.220.

4.19. Perform measurement of IZ correcting for tissue swelling using the Ratios of Ipsilateral and Contralateral Cerebral Hemispheres (RICH) method[13,23] (see example in **Supplement 5**).

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

NOTE: Infarct size is assessed as a percentage of the contralateral hemisphere.

5. **Determination of brain edema[31]**

NOTE: Use ImageJ 1.37v for measurement of BE[32,33].

5.1. Measure BE 24 h after MCAO. For calculation of BE, use the data from left and right hemisphere volume (in units).

5.3. Perform optical scanning with a resolution of 1600x1600 dpi (see **Supplement 1** for example).

5.4. Select brain hemispheres and set the cut-off for determining BE with ImageJ 1.37v, as described above in sections 4.17-4.19.

5.5. Express the BE area as a percentage of the standard areas of the unaffected contralateral hemisphere, calculated by the RICH method using following equation (see example in

Supplement 5)[23,34].

$$\text{extent of brain edema} = \frac{\text{volume ipsilateral hemisphere} - \text{volume contralateral hemisphere}}{\text{volume controlateral hemisphere}}$$

NOTE: Extent of BE is assessed as a percentage of the contralateral hemisphere.

6. Determination of BBB disruption

6.1. Measure BBB disruption 24 h after MCAO.

6.2. Divide right and left hemispheres into six slices and put each one into a microcentrifuge tube.

6.3. Homogenize each slice of the brain tissue in trichloroacetic acid, based on the calculation of 1 g of brain tissue in 4 mL of 50% trichloroacetic acid.

6.4. Centrifuge at 10,000 x *g* for 20 min.

6.5. Dilute supernatant liquid 1:3 with 96% ethanol.

6.6. Perform luminescence spectrophotometry by utilizing spectrophotometry software, installing the plate, and performing a sample reading using the following parameters: Fluorescence intensity excitation wavelength of 620 nm (bandwidth 10 nm) and an emission wavelength of 680 nm (bandwidth 10 nm) [23,35] ; Mod top; Number Flesh 25; Manual 100; Shaking 1 sec, 1 mm.

NOTE: Use an excitation wavelength of 620 nm (bandwidth 10 nm) and an emission wavelength of 680 nm (bandwidth 10 nm).[23,35]

REPRESENTATIVE RESULTS:

Infarct zone measurement

An independent-sample t-test indicated that 19 rats that underwent permanent MCAO demonstrated a significant increase in brain infarct volume compared to the 16 sham-operated rats (MCAO=7.49% ± 3.57 vs. Sham = 0.31% ± 1.9, $t(28.49) = 7.56$, $p < 0.01$ (see **Figure 2A**)). The data is expressed as a mean percentage of the contralateral hemisphere ± SD.

Brain edema measurement

An independent-sample t-test indicated that 19 rats that underwent permanent MCAO demonstrated significant increase in the extent of brain edema after 24 h compared to the 16 sham-operated rats (MCAO=12.31% ± 8.6 vs. Sham = 0.64% ± 10.2, $t(29.37) = 3.61$, $p = 0.01$, $d = 1.23$ (see **Figure 2B**)). The data is expressed as a mean percentage of the contralateral hemisphere ± SD.

Blood brain barrier permeability

An independent-sample t-test indicated that 19 rats that underwent permanent MCAO demonstrated significant increase in the extent of BBB breakdown after 24 h compared to the 16 sham-operated rats (MCAO=2235 ng/g ± 1101 vs. Sham = 94 ng/g ± 36, $t(18.05) = 8.47$ $p < 0.01$, $d = 2.7$ (see **Figure 2C**)). The data are measured in ng/g of brain tissue and presented as mean ± SD.

FIGURE AND TABLE LEGENDS:

Table 1: Protocol timeline. At 23 h after MCAO, the Evans blue solution was injected. One hour later (24 h after MCAO), brain collection was performed, and IZ, BE and BBB permeability were measured in all groups.

Figure 1: Representative brain slices of sham-operated and MCAO rats. (**A-B**) Original scanned image. (**C-D**) Transformation to greyscale. (**E-G**) Threshold function. (**G-H**) Application of a blue filter. (**I-J**) Threshold function after blue filter application. (**K-L**) Using threshold function to assess brain edema.

Figure 2: Histological outcomes of MCAO rats compared to sham-operated rats. (**A**) Infarct zone. The infarct zone volume in 19 rats after MCAO was significantly increased compared to the 16 sham-operated rats 24 h after surgery (*p < 0.01). (**B**) Brain edema. The brain edema volume in 19 rats after MCAO was significantly increased compared to the 16 sham-operated rats 24 h after surgery (*p < 0.01). (**C**) Blood brain barrier permeability. The blood brain barrier permeability in 19 rats after MCAO was significantly increased compared to the 16 sham-operated rats 24 h after surgery (*p < 0.01). Values were expressed as a mean percentage of the contralateral hemisphere ± SD and mean Evans blue extravasation index in ng/g of brain tissue ± SD according to independent-samples t-test. Results were considered statistically significant when p< 0.05, and highly significant when p < 0.01. This figure has been modified from Kuts et al.[23]

Supplement 1: Example scan of brain slices

Supplement 2: Macros that may be used in ImageJ software for the auto threshold function and measuring pixels.

Supplement 3: Example auto threshold

Supplement 4: Example of measured pixels on each hemisphere

Supplement 5: Sample analysis

DISCUSSION:

The principal goal of the present protocol was to demonstrate consistent measurements of three main parameters of ischemic injury: IZ, BE and BBB permeability. Previous studies in this field have demonstrated the possibility of performing one or two of these parameters together in the same sample. Besides the cost reduction that this three-part method offers, it also provides a

more desirable bioethical model that limits the number of animals that must be operated on and subsequently euthanized. As in all histological techniques, the method is limited by the inability to observe ischemic injuries dynamically.

Three computer programs were used in the image analysis: ImageJ 1.37v, Adobe Photoshop CS2 and Microsoft Excel 365. ImageJ was used to measure the extension of infarct zone and brain edema. Adobe Photoshop was used to limit the effect of Evans blue on brain tissue, since a blue color indicates BBB breakdown. Before calculating the infarct zone, it is necessary to remove the blue color from the image, since the color does not allow for accurate measurements of the infarct zone (see **Figures 1B, 1D, 1F**). Excel was used for data processing.

The technique for assessing an infarct zone with ImageJ computer software is based on a comparison of black and white pixels in a healthy hemisphere to pixels in an infarcted hemisphere. In the infarct hemisphere, the infarct zone is not stained with TTC; therefore, it is indicated by a white region in **Figure 1B** that is measured. In order for the program to correctly calculate the infarct zone, it is necessary to convert the pixels to shades of varying intensities of gray colors (see **Figures 1F, 1J**). The blue color, which is caused by Evans blue (see **Figure 1B**), may affect the assessment of the infarct zone (see **Figure 1F**). Therefore, the first step is to remove the blue color using a blue filter and then convert the image to a black and white image (see **Figure 1J**). We used Adobe Photoshop, but other computer programs can also be used for this purpose, such as RawTherapeePortable.

We then established uniform parameters for calculating the infarct zone in all 6 slices of one brain set using ImageJ in order to standardize the measurement procedure. This is necessary because all 6 slices from each set were stained and scanned under the same conditions and require a unified cut-off parameter. The cut off is a critical parameter for determining which pixels to convert to white and which to convert to black depending on the shade of gray (see **Figures 1D, 1H**). We used the Threshold function from the main menu for this purpose. The final step in the image analysis was the calculation of the infarct zone and brain edema.

The infarct zone measurement can be performed by various techniques, including histological staining or radiologic techniques such as a computed tomograph[36], positron emission tomography, and magnetic resonance imaging[23,36]. Previous studies in the lab have demonstrated the assessment of infarct volume using staining with TTC[15,26]. This method is based on a chemical reaction between TTC and mitochondrial dehydrogenases of neurons. The healthy tissues, rich with dehydrogenases, are colored with red with this staining. However, in necrotic cells, this color change does not occur due to damage in the system that participates in the oxidation of organic compounds[37]. In our previous studies, we demonstrated a high correlation between this histological technique and results from brain image scanning of this area[23].

Measurements of cerebral edema can be assessed both in vivo and in vitro. Cerebral edema results from pathological changes in the activities of sodium and calcium ion channels and transporters that lead to an increase in intracellular water[38-41]. Alternatively, swelling can occur by BBB damage that increases extracellular water.[42] In previous studies, cerebral edema was

determined based on wet and dry techniques followed by the calculation of tissue water content[43]. An advantage of the method we present in this protocol is its simplicity and accuracy comparing to other existing techniques[23,44,45].

The most useful method for BBB breakdown detection is luminescence spectroscopy after Evans blue injection. The measurement of BBB permeability is based on the injection of Evans blue, which binds to albumin. In turn, the molecular mass of albumin is 66 kDa and much more significant than the molecular weight of Evans blue 961 Da. Thus, the measurement of the BBB permeability is determined precisely by the molecular mass of albumin which penetrates through the damaged BBB, thereby transferring the Evans blue. In addition to the techniques described above, there are other techniques, in particular those based on a combination of various dextrans and radioactive molecules, which together give more accurate results. Measurement of BBB breakdown by luminescence spectrometry is cheaper and easier to use, compared to more accurate but more expensive techniques. We used this method for the evaluations of BBB disruption together with measurements of infarct zone and brain edema. Injection of Evans blue for assessment of BBB permeability prior to induction of MCAO does not affect the accuracy of measuring these two parameters[23].

The present protocol presents a novel technique for measuring the three most important determinants of ischemic brain injury on the same brain sample. This method can be also applied to models of other brain injuries. This protocol will contribute to the study of the pathophysiology of ischemic injury.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Fig. 1.jpg

A

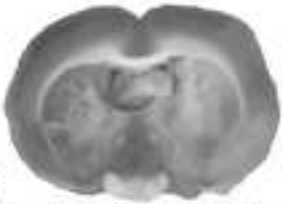
Sham operated rats - original image

B

MCAO rats - original image

C

Sham operated rats - transformation in black and white image



D

MCAO rats - transformation in black and white image

E

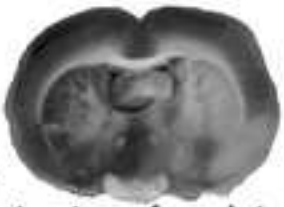
Sham operated rats - use threshold function for black and white image

F

MCAO rats - use threshold function for black and white image

G

Sham operated rats - transform into a black and white image using a blue filter



H

MCAO rats - transform into a black and white image using a blue filter

I

Sham operated rats - use threshold function for black and white image and applying a blue filter

J

MCAO rats - use threshold function for black and white image and applying a blue filter

K

Sham operated rats - use threshold function for assessment of brain edema

L

MCAO rats - use threshold function for assessment of brain edema

Figure 2

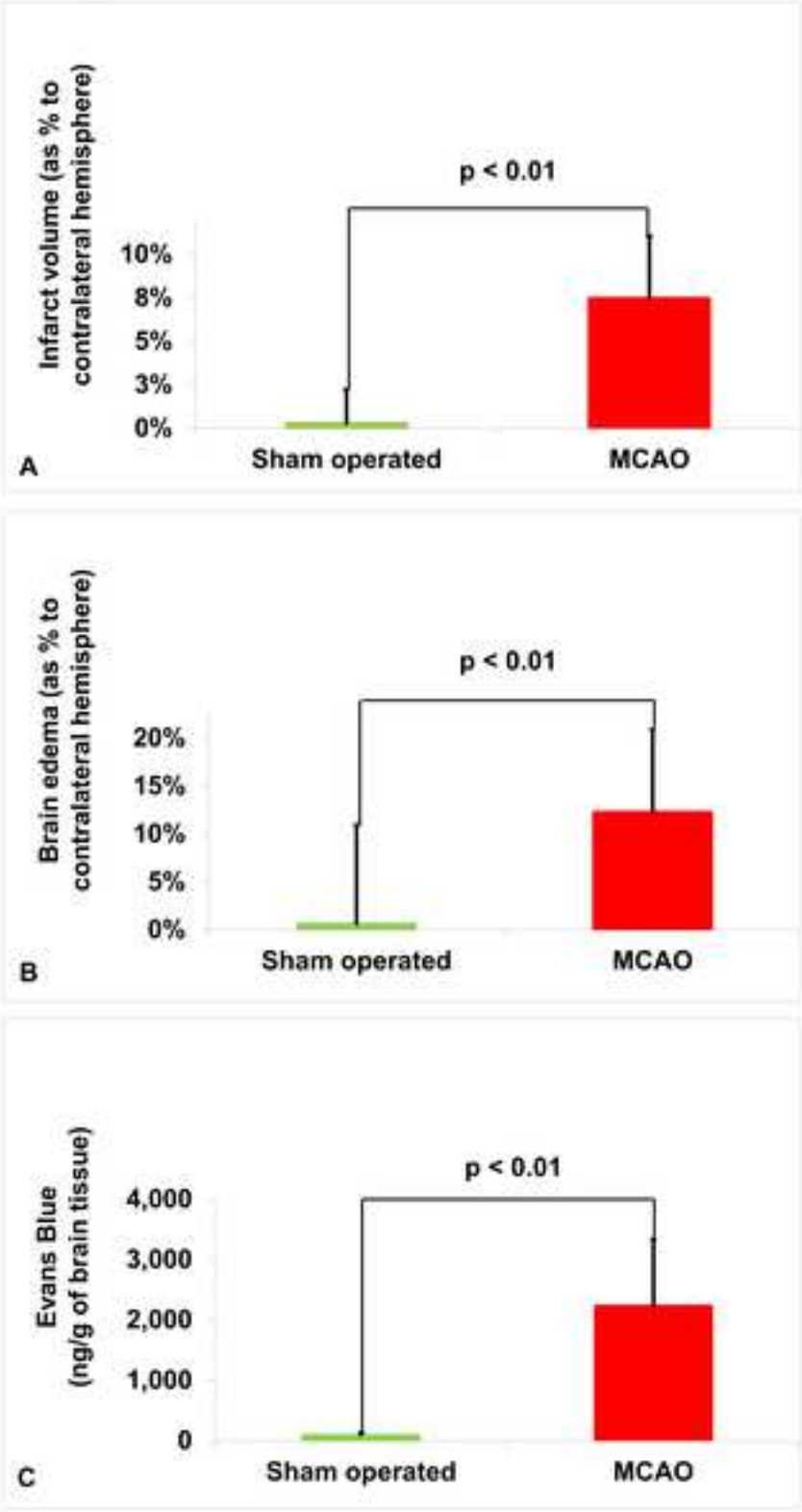


Table 1

Group	Time	Procedures
Sham operated (16 rats) MCAO (19 rats)	0	nd insertion of filament for sham operated group
Sham operated (16 rats) MCAO (19 rats)	23h	Injection of Evans blue
Sham operated (16 rats) MCAO (19 rats)	24h	r measurements of IZ, BE, and BBB disruption

Name of Material/Equipment	Company	Catalog Number
2 mL Syringe	Braun	4606027V
2% chlorhexidine in 70% alcohol solution	Sigma-Aldrich	500 cc
27 G Needle with Syringe	Braun	305620
3-0 Silk sutures	Henry Schein	1007842
4-0 Nylon suture	4-00	
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
Fluorescence detector	Tecan, Männedorf Switzerland	Model: Infinite 200 PRO multimode reader
Fluorescence detector	Molecular Devices LLC	VWR cat. # 10822 512 SpectraMax Paradigm Multi Mode Microplate Reader Base Instrument
Gauze sponges	Fisher	22-362-178
Heater with thermometer	Heatingpad-1	Model: HEATINGPAD-1/2
Hemostatic microclips	Sigma-Aldrich	
Horizon-XL	Mennen Medical Ltd	
Infusion cuff	ABN	IC-500
Micro forceps	Sigma-Aldrich	

Micro scissors	Sigma-Aldrich	
Multiset	Teva Medical	998702
Olympus BX 40 microscope	Olympus	
Operating forceps	Sigma-Aldrich	
Operating scissors	Sigma-Aldrich	
Optical scanner	Canon	Cano Scan 4200F
Petri dishes	Sigma-Aldrich	P5606
Purina Chow	Purina	5001
Rat cages	Techniplast	2000P
Scalpel blades #11	Sigma-Aldrich	S2771
Software		
Adobe Photoshop CS2 for Windows	Adobe	
ImageJ 1.37v	NIH	
Office 365 ProPlus	Microsoft	-
Windows 10	Microsoft	
Reagents		
2,3,5-Triphenyltetrazolium chloride	Sigma-Aldrich	298-96-4
50% trichloroacetic acid	Sigma-Aldrich	76-03-9
Ethanol 96 %	Romical	
Evans blue 2%	Sigma-Aldrich	314-13-6
Isoflurane, USP 100%	Piramamal Critical Care, Inc	NDC 66794-017

Comments/Description

Provides general antisepsis of the skin in the operatory field

Optional.

Optional.

Resolution 3200 x 6400 dpi

Rodent laboratory chow given to rats, mice and hamster is a life-cycle nutrition that has been used in biomedical research for over 5 d
Conventional housing for rodents. Cages were used for housing rats throughout the experiment

The source code is freely available. The author, Wayne Rasband (wayne@codon.nih.gov), is at the Research Services Branch, National I
Microsoft Office Excel

Flammable liquid

ecades. Provided to rats ad libitum in this experiment.

nstitute of Mental Health, Bethesda, Maryland, USA

Attn: Alisha DSouza, Ph.D.

Senior Review Editor

Journal of Visualized Experiments (JoVE)

JoVE61309R1

Title: Measuring post-stroke cerebral edema, infarct zone and blood-brain barrier breakdown in a single set of rodent brain samples

Dear Dr. DSouza,

Please find attached a revised version of the manuscript JoVE61309R1. Changes are marked in the revised manuscript. We have made a concentrated effort to include more details to the protocol to ensure reproducibility. We have also clarified the reasoning for these steps in our discussion. Given the protocol's reliance on computer software for data processing, certain steps will be made clearer with visualization in the video. 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

Answers to the Reviewers' Comments

[Editorial comments]

1) More details are needed in the protocol to ensure reproducibility of the protocol. Please the comments in the attached manuscript.

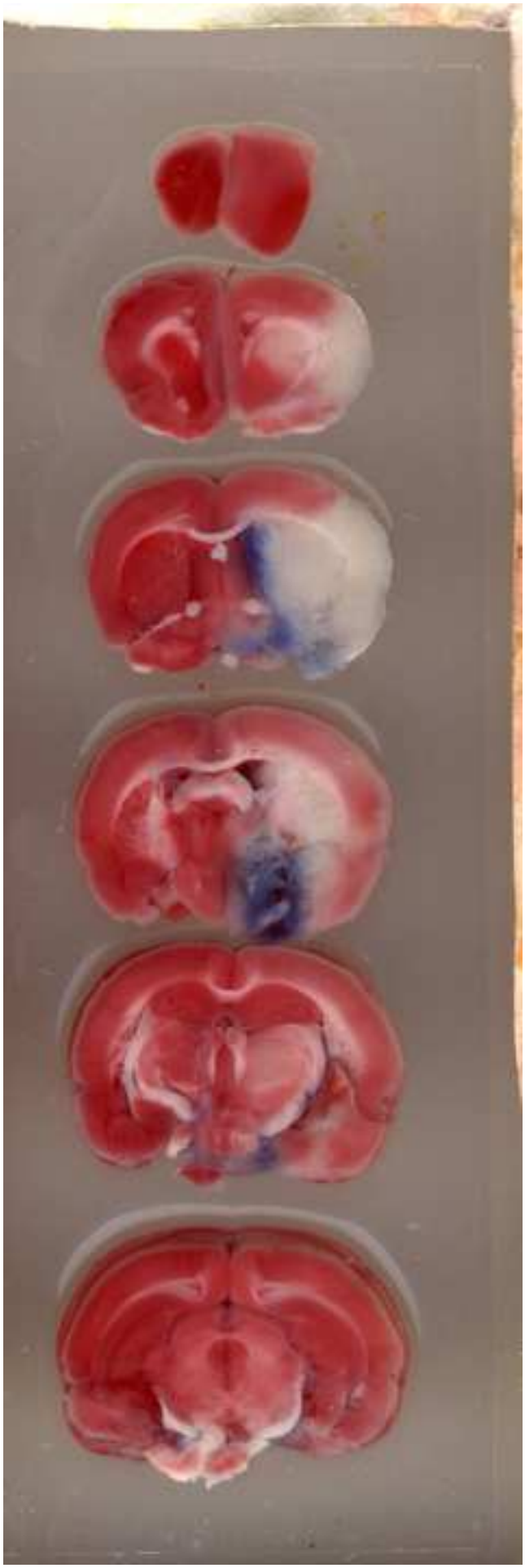
These comments have now been addressed.

2) There are some issues with the figures. Please see the comments in the attached manuscript.

Thank you for your vigilance. We have corrected these issues in the revision.

Supplemental File 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supliment1.jpg



A. Selecting and saving brain slices as separate images in ImageJ

```
run("Copy");
newImage("Untitled", "RGB White", 2000, 2000, 1);
run("Paste");
run("Select None");
saveAs("Jpeg", "c:\\rat 1\\1");
close();
```

B. For the auto threshold function in ImageJ: Below is a macro for ImageJ software that must be run from the main menu using the command plugins> macros> run.

```
run("Measure");
run("Copy");
newImage("Untitled", "RGB White", 2000, 2000, 1);
run("Paste");
run("Select None");
run("8-bit");
setAutoThreshold();
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Threshold...");
```

C. Measuring pixels in ImageJ: Below is a macro for ImageJ software that must be run from the main menu using the command plugins> macros> run.

```
open("C:\\ rat 1\\1.jpg");
run("8-bit");
setThreshold(0, 220);
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
run("8-bit");
                setThreshold(0, 111  );
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
close();
open("C:\\ rat 1\\2.jpg");
run("8-bit");
setThreshold(0, 220);
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
run("8-bit");
                setThreshold(0, 111  );
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
close();
open("C:\\ rat 1\\3.jpg");
run("8-bit");
setThreshold(0, 220);
```

```
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
run("8-bit");
                setThreshold(0, 111  );
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
close();
open("C:\\ rat 1\\4.jpg");
run("8-bit");
setThreshold(0, 220);
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
run("8-bit");
                setThreshold(0, 111  );
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
close();
open("C:\\ rat 1\\5.jpg");
run("8-bit");
setThreshold(0, 220);
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
run("8-bit");
                setThreshold(0, 111  );
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
close();
open("C:\\ rat 1\\6.jpg");
run("8-bit");
setThreshold(0, 220);
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
run("8-bit");
                setThreshold(0, 111  );
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
close();
open("C:\\ rat 1\\7.jpg");
run("8-bit");
setThreshold(0, 220);
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
run("8-bit");
                setThreshold(0, 111  );
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
```

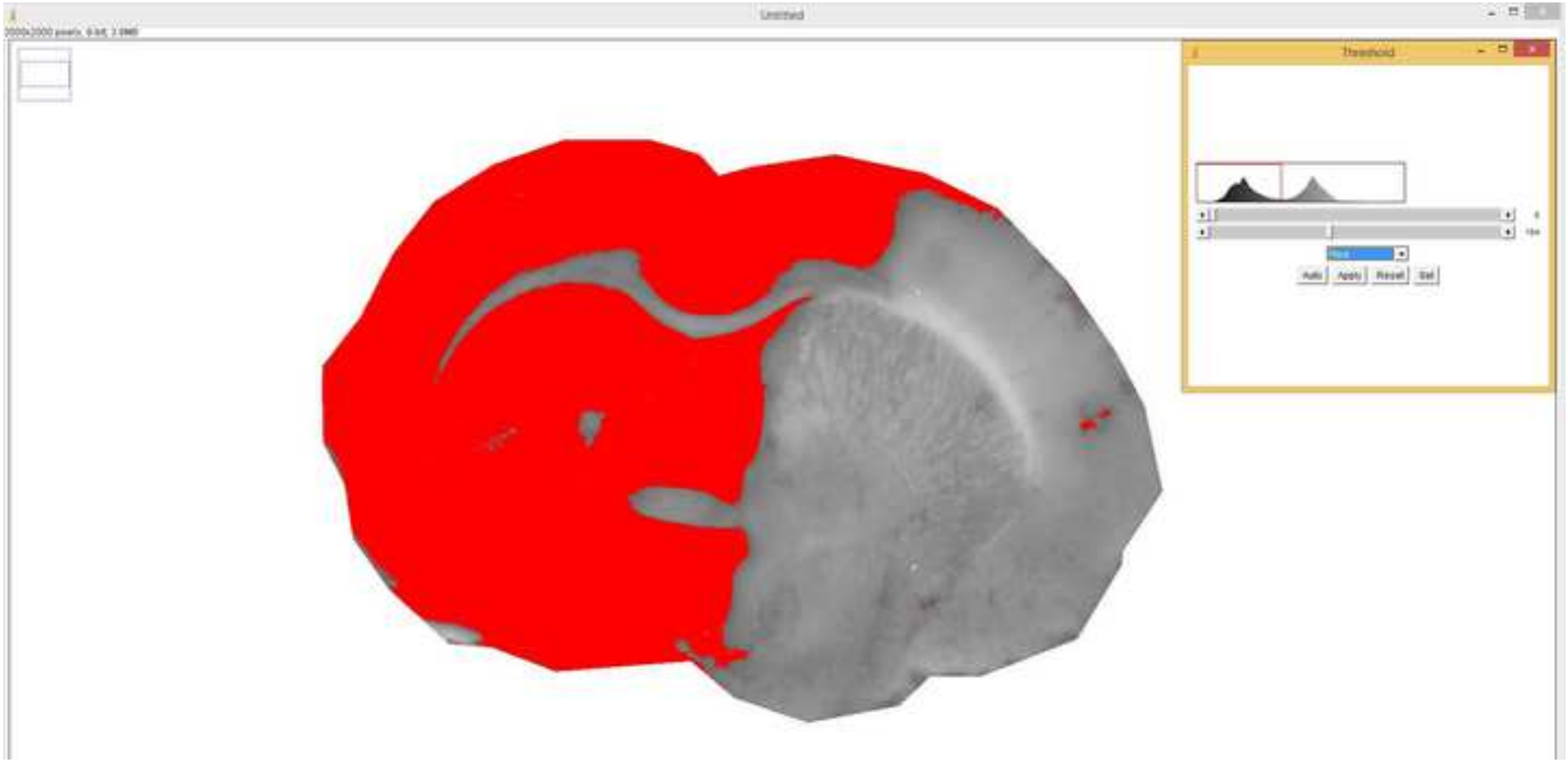
```
close();
open("C:\\ rat 1\\8.jpg");
run("8-bit");
setThreshold(0, 220);
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
run("8-bit");
                        setThreshold(0, 111  );
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
close();
open("C:\\ rat 1\\9.jpg");
run("8-bit");
setThreshold(0, 220);
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
run("8-bit");
                        setThreshold(0, 111  );
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
close();
open("C:\\ rat 1\\10.jpg");
run("8-bit");
setThreshold(0, 220);
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
run("8-bit");
                        setThreshold(0, 111  );
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
close();
open("C:\\ rat 1\\11.jpg");
run("8-bit");
setThreshold(0, 220);
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
run("8-bit");
                        setThreshold(0, 111  );
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
close();
open("C:\\ rat 1\\12.jpg");
run("8-bit");
setThreshold(0, 220);
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
run("8-bit");
                        setThreshold(0, 111  );
```

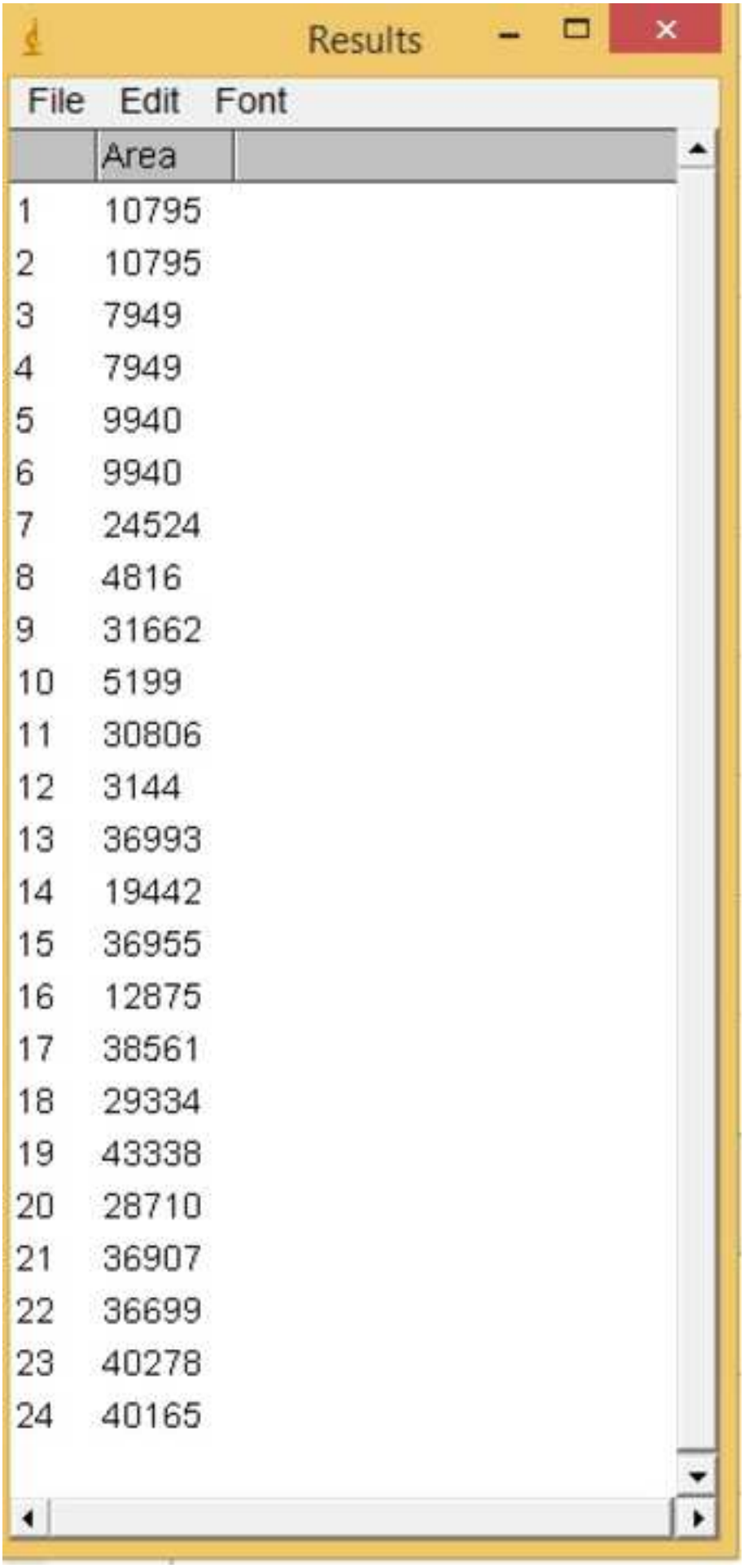
```
//run("Threshold...");
run("Create Selection");
run("Measure");
run("Undo");
close();
```

NOTE: The parameter "cut of 0, 111" was used by us as an example of a specific set.

Supplemental File 3

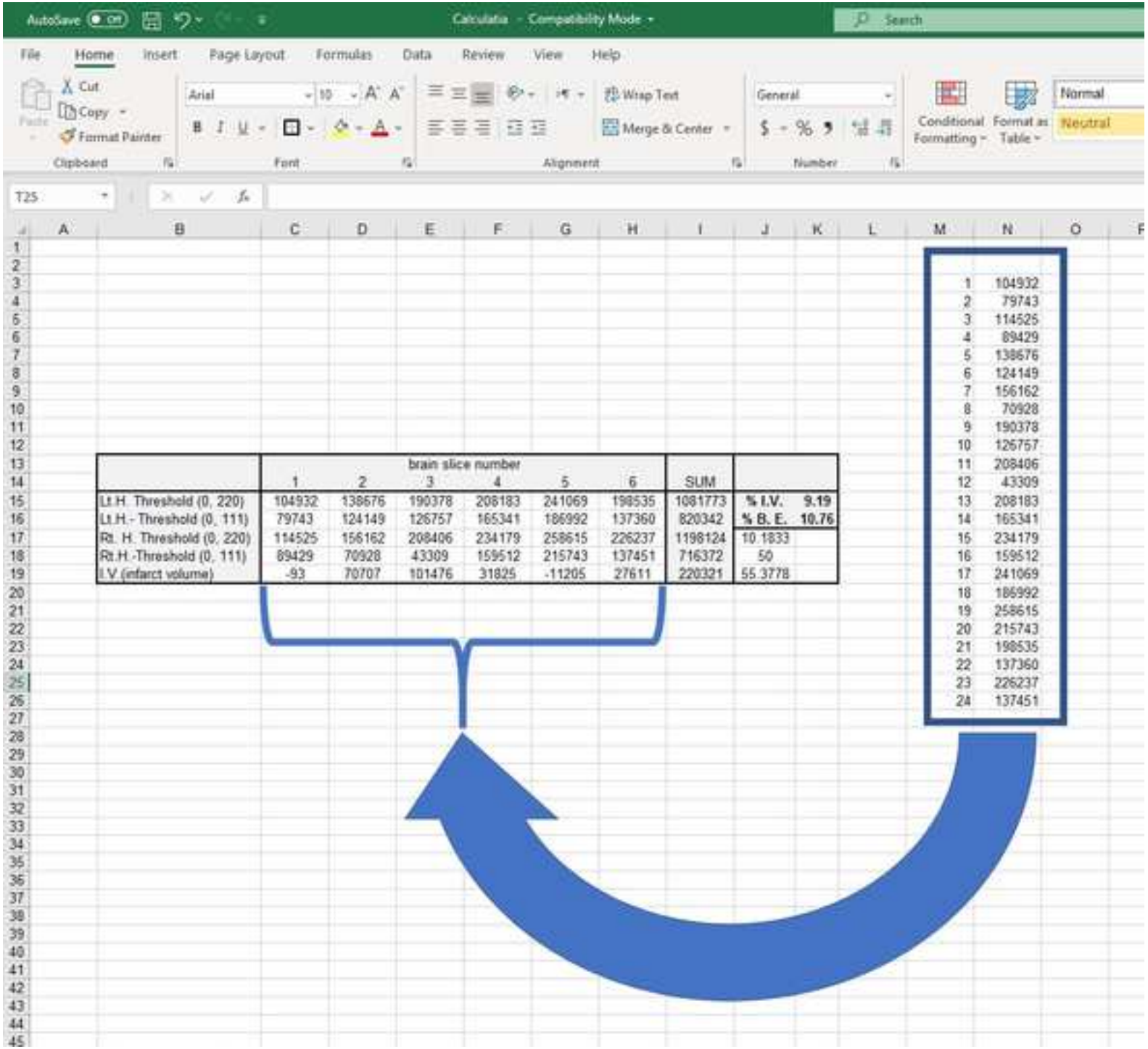
Click here to access/download;Supplemental File (Figures, Permissions, etc.);Sup3.jpg





Supplemental File 5

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Sup5.jpg



	brain slice number							
	1	2	3	4	5	6	SUM	
Lt.H. Threshold (0, 220)	104932	138676	190378	208183	241069	198535	1081773	% I.V. 9.19
Lt.H.- Threshold (0, 111)	79743	124149	126757	165341	186992	137360	820342	% B. E. 10.76
Rt. H. Threshold (0, 220)	114525	156162	208406	234179	258615	226237	1198124	10.1833
Rt.H.-Threshold (0, 111)	89429	70928	43309	159512	215743	137451	716372	50
I.V (infarct volume)	-93	70707	101476	31825	-11205	27611	220321	55.3778

1	104932
2	79743
3	114525
4	89429
5	138676
6	124149
7	156162
8	70928
9	190378
10	126757
11	208406
12	43309
13	208183
14	165341
15	234179
16	159512
17	241069
18	186992
19	258615
20	215743
21	198535
22	137360
23	226237
24	137451