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TITLE:

Assessing Neuroprotective Effects of Glycyrrhizae Radix et Rhizoma Extract Using a Transient Middle Cerebral Artery Occlusion Mouse Model

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KEYWORDS:

Glycyrrhizae Radix et Rhizoma, Ischemia, Reperfusion, Cerebral Injury, Middle Cerebral Artery Occlusion, Stroke

SUMMARY:

In this study, we modify an existing experimental method to obtain more reproducible results, by establishing a middle cerebral artery occlusion (MCAO) mouse model. Oral administration of Glycyrrhizae Radix et Rhizome (GR) methanol extract (GRex), following stroke induction, significantly decreased total infarction volume relative to the untreated control group.

ABSTRACT:

Ischemia followed by reperfusion of cerebral blood flow after a stroke leads to the death of nerve cells and loss of brain tissue. The most commonly used animal model for studying stroke is the middle cerebral artery occlusion (MCAO) model. Previous research studies have reported different infarct sizes even when the same experimental animal species was used under similar MCAO conditions. Therefore, we developed an improved experimental method to address this discrepancy. Mice were subjected to MCAO using a filament as the occlusion material to mimic human stroke conditions and filament thickness was optimized to establish more reproducible

infarction volume. Mice treated with a methanol extract of Glycyrrhizae Radix et Rhizome (GRex) following stroke induction showed a significantly decreased total infarction volume and increased number of surviving cells relative to the untreated control group. This modified experimental protocol successfully and reproducibly demonstrated the beneficial effect of GRex on ischemic stroke.

INTRODUCTION:

Brain damage caused by ischemia and reperfusion of cerebral blood flow leads to the death of nerve cells and loss of brain tissue. This type of brain damage continues to increase with the increasing prevalence of cerebrovascular diseases due to the spread of metabolic diseases such as obesity, hypertension, and diabetes mellitus[1,2]. The absolute number of elderly patients with stroke has dramatically increased worldwide, and the cost of medical care for these patients, who are often left with long-term disabilities, is a major societal burden. Therefore, secondary disabilities should be mitigated as much as possible to reduce the economic burden[1,2].

The most commonly used rodent model of cerebral infarction is the middle cerebral artery (MCA) occlusion (MCAO) model, in which the MCA is occluded with a silicon-coated surgical suturing filament to block blood flow, causing ischemic stroke[3,4]. Using a filament as the occlusion material allows the control of occlusion time and permanence by manipulating the duration of the intra-luminal filament insertion.

Previous studies have shown that even when the same rodent MCAO model is used, the total volume of cerebral infarction varies between experiments, causing low reproducibility of the studies. To improve reproducibility, we optimized the thickness of the filament mint used in the experiment. The results of a preliminary study of the cerebral ischemic period and induced infarction showed that an ischemic period longer than 60 min allowed the volumetric region of damaged brain tissue to be observed and quantified.

Glycyrrhizae Radix et Rhizoma (GR), also known as licorice, consists of the dried roots and rhizomes of *Glycyrrhiza uralensis* and *Glycyrrhiza glabra.* It has been used in Chinese and Korean traditional medicine for various purposes including as a food additive and medicinally[5-7]. In a previous study[8], pre-treatment with GR methanol extract (GRex) showed an anti-apoptotic effect in MCAO mice, including significant prevention of the decrease in the protein expression of B-cell lymphoma 2 (Bcl-2) and Bcl extra-large (Bcl-xL). This study was conducted to improve the reproducibility of the conventional MCAO mouse model by evaluating its efficiency in determining if post-infarct treatment with GRex effectively reduced the infarct volume in MCAO-induced cerebral damage

PROTOCOL:

All procedures involving animals were approved by the ethics committee of Pusan National University (approval number, PNU-2016-1087). A graphical overview of this study is shown in **Figure 1**.

1. Preparation and Administration of GRex

Note: The GR used in this study was purchased from a commercial pharmaceutical company.

1.1. Place 200 g of GR in 2,000 mL of methanol and incubate at room temperature (25 °C) for 5 days.

1.2. Filter the mixture using filter paper with 0.26 mm thickness and 5 µm pore size, and then remove the supernatant. Add 1,000 mL of methanol to the GR residue and filter again.

1.3. Combine the two supernatants, filter through filter paper, concentrate under vacuum, and then freeze-dry the residue to produce GRex.

1.4. Dissolve the GRex in dimethyl sulfoxide (DMSO), dilute with 0.9% physiological saline, and filter through a 0.45 µm syringe filter. Then, adjust the final concentration of DMSO to < 5%.

1.5. Administer GRex (300 mg/kg body weight) 1 h after the reperfusion of MCAO *via* oral gavage. Administer DMSO diluted in physiological saline (10 mL/kg body weight) only to the normal group and control groups, respectively.

NOTE: The concentration of GRex used in this experiment was determined according to the concentration that was active through our previous study[8].

2. Mouse Model of MCAO

2.1. Use male C57BL/6 mice aged 6 weeks and weighing 22-25 g. Provide all animals with free access to standard chow and water, and house them in an environment with controlled temperature (22 ± 1 °C) and a 12 h light/dark cycle.

2.1.1. Divide the mice into groups of six mice each, which should consist of sham-operated normal, control, and GRex treatment groups.

2.1.2. Perform MCAO surgery (modification of the method of Koizumi *et al.*[9]) on the control and GRex treatment groups using a stereo-microscope.

2.2. Induce inhalation anesthesia in the mice using 2% isoflurane in 70% N_2O and 30% O_2. Anesthesia is considered sufficient when the mouse becomes unresponsive to mechanical stimulus applied to its tail. Maintain the body temperature of the mice at 36.5 ± 0.5 °C using a body temperature-holding blanket connected to a thermometer.

2.3. Remove all the hair on the chests and necks of the mice by shaving followed by use of hair removal cream, and then make an incision of approximately 2 cm long with iris surgical scissors in the center of the neck. Carefully isolate the left common carotid artery (LCCA),

external carotid artery, and the branch of the internal carotid artery from surrounding connective tissues.

2.4. Ligate the external carotid artery and the common carotid artery with a surgical suture (4-0 silk suture, half hitch knot) to temporarily block the blood flow into the internal carotid artery during the operation.

2.5. Insert a silicon-coated nylon suture (8-0 monofilament, 11 mm long) through the internal carotid artery to the origin of the left MCA. Adjust the thickness of the silicon-coated part of the filament to a range of 0.10-0.12 mm.

2.6. Measure the decrease in relative cerebral blood flow (rCBF) in the MCA using a laser Doppler flowmeter. MCAO will be confirmed when the rCBF is maintained at < 20% of the resting condition values during the entire ischemic period.

2.7. Fix the inserted filament to the blood vessel for 2 h while the cerebral artery is occluded, and then carefully withdraw the filament to restore the blood flow for 22 h of reperfusion. Suture the skin by sewing at 5 places (3-0 silk suture, two half hitches knot) and allow each mouse to awaken from the anesthesia.

2.8. In the normal group, perform a sham operation following the same procedure above (until 2.4), with the following exception. Ligate the common carotid artery and suture the incised muscle and skin.

3. **Measurement of Volume of Damaged Brain Tissue**

3.1. After euthanasia of the mice for brain damage measurement with CO_2 inhalation, excise the mouse brains 24 h after the onset of MCAO using iris surgical scissors and angled forceps.

3.1.1. After removing the head using scissors, make an incision in the midline skin of the head to flip over the skin from the skull.

3.1.2. Break the parietal bones with angled forceps, peeling off dura matter at the same time, and then isolate the brain carefully from the skull.

3.2. Cut the excised tissue into sections (1 mm thick) using a mouse brain matrix, and then stain the sections for 17 min in a solution of 2% 2,3,5-triphenyltetrazolium chloride (TTC).

3.3. Fix the sections in 10% formalin for at least 2 h and then photograph them using a digital camera. TTC will be observed to stain viable tissue red while the necrotic areas will be white.

3.4. Analyze and quantify the cerebral infarct area of each section using ImageJ.

4. Hematoxylin and Eosin (H&E) and Cresyl Violet Staining of Histological Sections

4.1. Euthanize the mice for histological study by CO_2 inhalation and perfuse them transcardially with 10 mL of phosphate-buffered saline (PBS), followed by 10 mL of 4% paraformaldehyde (PFA). Isolate the brain using the same procedure as above (3.1) and immerse the brain in 10 mL of 30% sucrose overnight.

4.2. Embed the brain tissue in optimal cutting temperature (OCT) compound and slice it coronally into 15-µm-thick sections using a cryostat. Mount the sections on glass slides, followed by staining with hematoxylin and eosin (H&E) or cresyl violet.

4.3. Immerse the glass slides in 80% ethanol for 1 min followed by staining in hematoxylin solution for 5 min.

4.3.1. Dip the slides in 1% acid alcohol twice, immerse in saturated lithium carbonate solution for 30 s, wash with tap water for 30 s, and then counterstain in eosin solution for 30 s.

4.3.2. Rinse the slides in tap water, soak in 95% and absolute ethanol consecutively.

4.3.3. Air-dry the slides, clear them in xylene for at least 10 min, and then mount the coverslips using mounting medium.

4.4. Place the glass slides on a slide warmer for at least 1 h, followed by immersion in 50% ethanol diluted with chloroform overnight.

4.4.1. Stain the slides with 0.1% cresyl violet for 10 min in a 40 °C dry oven.

4.4.2. Immerse in 95% ethanol for 30 min, then dehydrate in absolute ethanol for 2 times.

4.4.3. Clear 2 times in xylene for 5 min, then mount with mounting medium after air drying.

4.5. Using a microscope, observe the histological changes that occurred after MCAO-induced brain injury.

5. Statistical Analysis

5.1. Express the experimental results as means ± standard deviation and determine the statistical significance between the groups using a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* analysis using a data analysis software.

5.2. Set the statistical significance at a p-value < 0.05.

REPRESENTATIVE RESULTS:

In the sham-operated normal group, no cerebral infarct is observed whereas in the control group, a relatively wide range of damaged areas is observed. In the mice administered 300 mg/kg GRex in the MCAO model group, a statistically significant reduction in damaged area is observed (**Figure 2**).

The histological changes are investigated by staining ischemic brain sections with H&E or cresyl violet. H&E staining provides structural information and specific functional information about cells[10], whereas cresyl violet staining is used to estimate the total number of hippocampal neurons[11]. Thus, H&E or cresyl violet intensity, as measured using ImageJ software (**Figure 3A**), provides an index of cell survival. H&E and cresyl violet staining intensities significantly decrease in the control group relative to the normal group (**Figure 3B, 3C**). The GRex-treated group shows greater histological integrity, implying less neuronal cell death, than the control group (**Figure 3C**). These results indicate that GRex has potent neuroprotective effects against ischemia/reperfusion-induced brain injury.

Figure Legends

Figure 1. Scheme of the middle cerebral artery occlusion (MCAO) model and treatment with the methanol extract of Glycyrrhizae Radix et Rhizome (GRex). Mice were treated with 300 mg/kg of GRex 1 h after MCAO reperfusion, which was maintained for 2 h. Mice were euthanized 24 h after the MCAO commenced, and then the harvested brain slices were stored in a deep freeze for protein assay or stained with TTC solution for infarct measurement.

Figure 2. Representative images of (A) brain sections showing the effects of methanol extract of Glycyrrhizae Radix et Rhizome (GRex) treatment on post-middle cerebral artery occlusion (MCAO)-induced brain infarct volumes and (B) single treatment with 300 mg/kg GRex 1 h after MCAO reperfusion significantly suppressed infarct volumes. Results are presented as means ± SDs. ###p<0.001 vs normal group, **p<0.01 vs control group; n=6 per group.

Figure 3. Representative images of (A) hematoxylin and eosin (H&E)- and cresyl violet-stained brain sections and (B, C) color intensities, which were used to evaluate the effects of the methanol extract of Glycyrrhizae Radix et Rhizome (GRex) on the brains of middle cerebral artery occlusion (MCAO)-injured mice. Histological integrity and tissue damage in mouse brains were assessed using (B) H&E or (C) cresyl violet staining 1 h post-MCAO reperfusion. Red staining in H&E-stained sections indicates nuclear damage. Neurons dyed with cresyl violet were stained purple. The GRex-treated group showed better histological integrity than the control group did, indicating less neuronal cell death. **a**, H&E-stained; **b**, cresyl violet-stained; **c** and **d**, enlargements of **a** and **b**, respectively. Results are means ± SDs. ###p < 0.001, and *p<0.05 vs normal and control groups; n = 6 per group.

DISCUSSION:

With the increasing prevalence of metabolic diseases such as chronic hypertension, diabetes, and hyperlipidemia, which are major risk factors for stroke, stroke prevention and treatment have become an important area of medical research[12,13]. Deficits in language and movement after a stroke are strongly correlated with the degree of damage to brain tissue[14] and result in a

poor quality of life for patients and their families[15]. It is important to use an appropriate animal model of stroke that involves the same pathological changes as those that occur in human disease to study the efficacy of drug treatments. The MCAO model mimics thrombotic strokes by obstructing cerebral arterial vessels. It is commonly used because it is relatively reproducible and minimally invasive[16-19].

However, a comparison of the area of cerebral infarction induced for the same resting time reported by several researchers, reveals that the total infarction volume varies between studies. We concluded that this was due to differences in the occlusion materials used and the surgical procedure. Therefore, although the MCAO rodent model is considered highly reproducible, it is not always possible to obtain such reproducibility. Therefore, we optimized the thickness of filaments used in mouse MCAO model through our preliminary study and previous report[8].

The most distinctive result of our preliminary study compared to that of other studies is that TTC staining did not reveal any cerebral infarction when the ischemia was induced for 60 min (data not shown). Even following 90 and 120 min of MCAO in the mice, our result showed a lower infarction volume than that of other research studies. One limitation of this study is that we have not yet determined the exact cause of these results; however, we are planning to explore this phenomenon in further studies.

Numerous studies have recently reported that GR or its components have pharmacological activities including antitumor, antimicrobial, and anti-inflammatory effects[20-22]. A previous study reported that GRex pre-treatment effectively inhibited the activation of caspase-9 by upregulating the protein expression of Bcl-2 and Bcl-xL[8]. However, preventative treatments for stroke are less clinically relevant than post-stroke treatment.

In this study, which was based on a previous study[8] evaluated the effectiveness of GRex post-treatment in an MCAO mouse model. As depicted in the representative results section, GRex post-treatment showed beneficial effects in reducing total infarction volume and ameliorating damages to cellular structures in MCAO-induced brain injury in mice. The specific action mechanisms of GRex on post-ischemic brain injury lacks in this study, but the experimental protocols used in this study successfully demonstrated the effects of this herbal remedy by mimicking human effects of a stroke.

Although the experimental results are not observed in this study, the neuronal deficit score (NDS) was measured in our preliminary experiment and no significant difference was noted between the control and the GRex-treated groups, which is presumed to have been due to the short observation time compared to the severity of the stroke. We are planning to observe the effects of GRex treatment on the NDS over a long period after causing moderate damage.
In conclusion, the neuroprotective effect of GRex treatment in a mouse MCAO model was demonstrated in this study with good reproducibility. The proteins involved in the underlying mechanism should be examined in future studies.

DISCLOSURE:

The authors have nothing to disclose.

ACKNOWLEDGMENT:

Not applicable.

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Fig. 1.

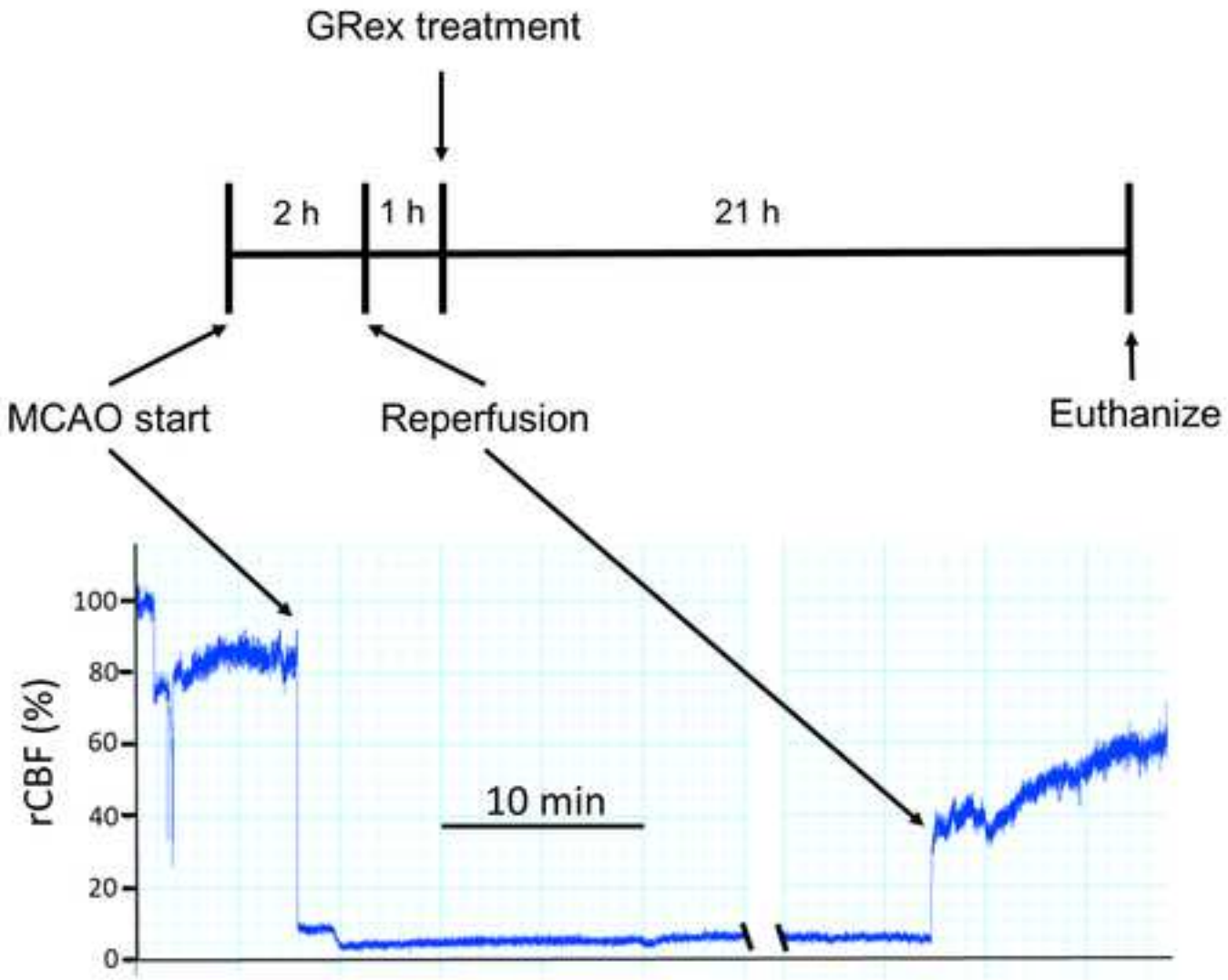
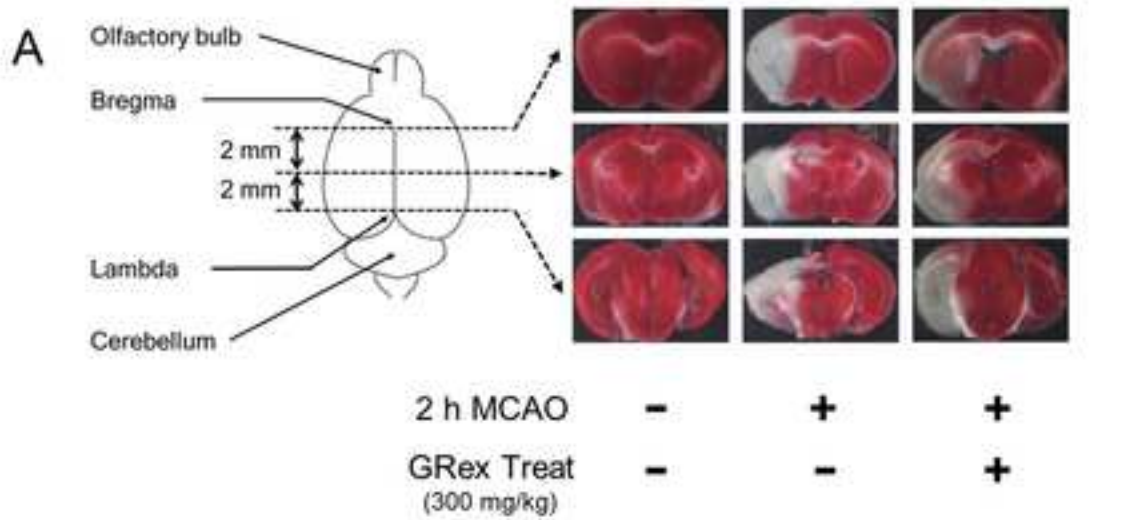


Fig. 2.



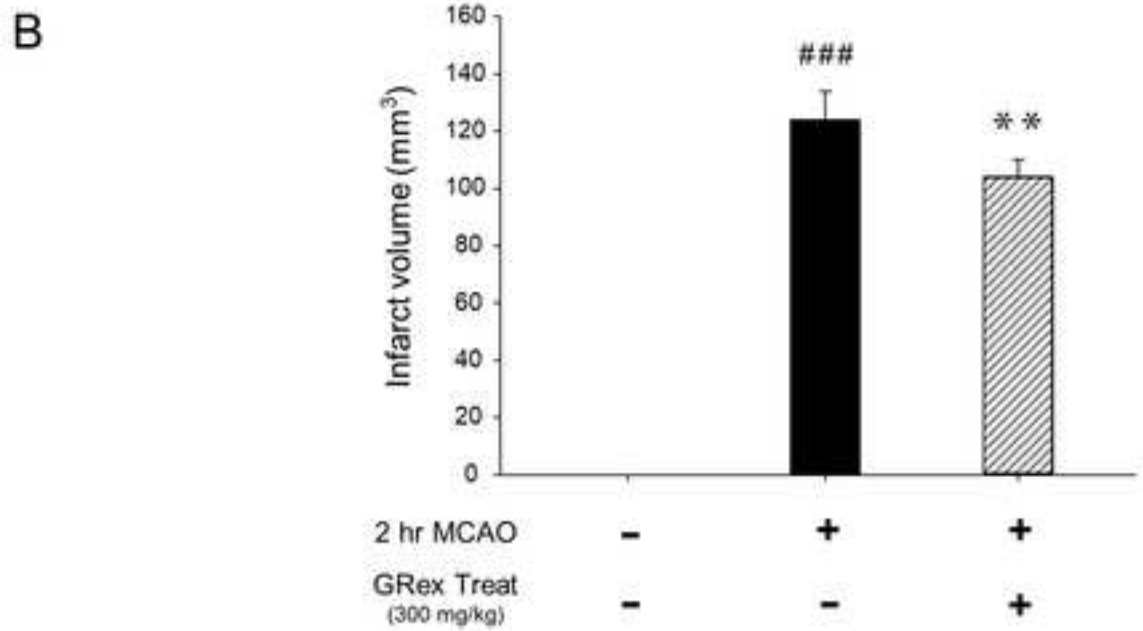


Fig. 3.

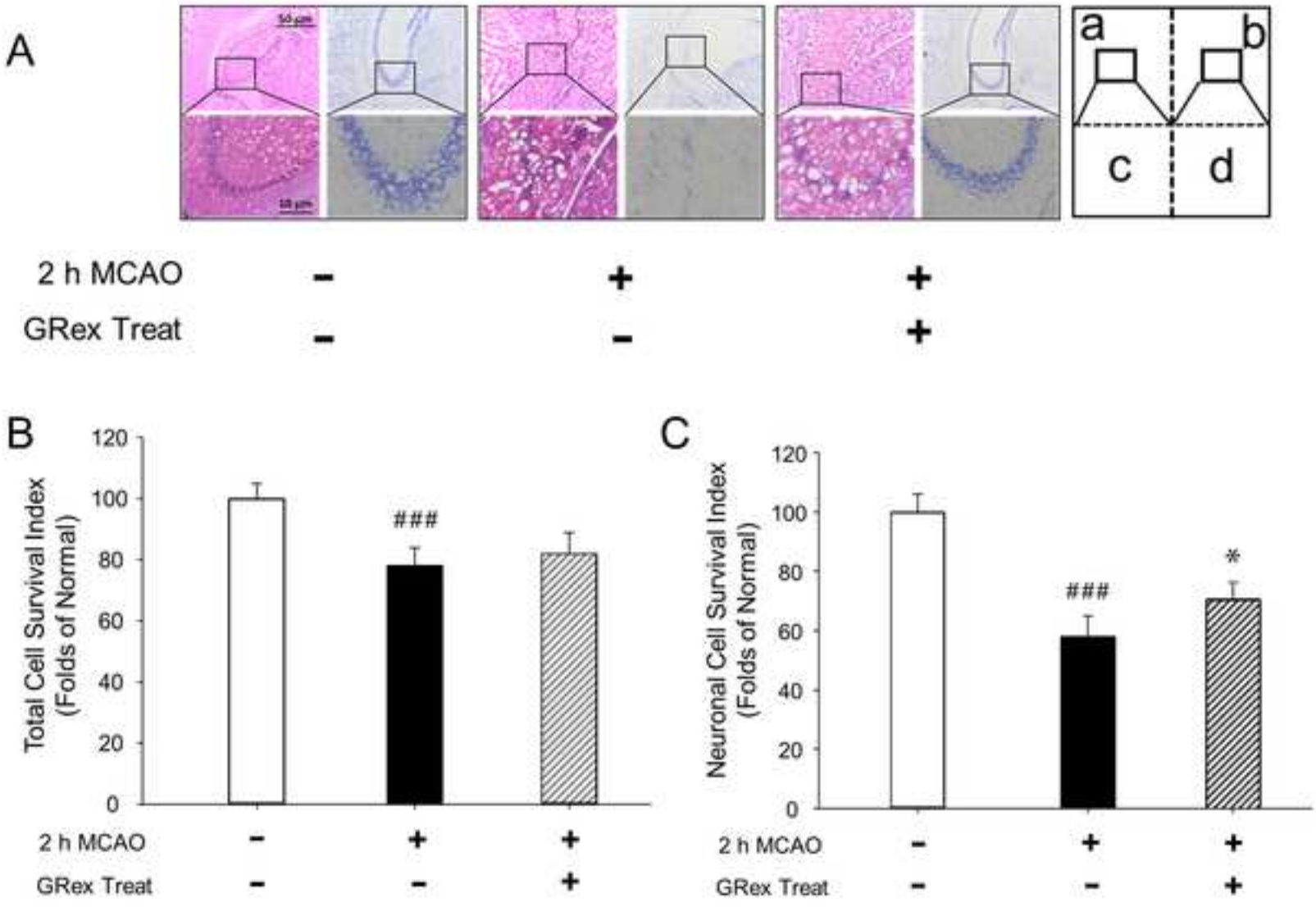


Table of Materials

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Name of Material/ Equipment	Company
	Gwangmyoung Pharmaceuticals Co., Korea
	Advantec
	Sigma
	Sigma
	Leica
	Nikon
	Moor Instrument
	Harvard Appratus
	Harvard Appratus
	Canon
	Leica
	Carl Zeiss
	Systat Software Inc.
	NIH Image
	Doo Yeol Biotech
	JOONGWAE
	TROIKAA
	AILEE
	Baekjae
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JUNSEI

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MUTO PURE CHEMICALS

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Sigma-Aldrich

JUNSEI

Scigen

Fine Science Tools

Fine Science Tools

Fine Science Tools

Fine Science Tools

Fine Science Tools

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Catalog Number

Filter paper No. 2

D8418-250ML

CLS431220

M50

SMZ745

moorVMS-LDF

34-1352

55-7020

Eos-M2

CM3050S

Zeiss Axio

SigmaPlot version 12

ImageJ

Standard rodent chow

A02104781

ISOTROY 100

SK47510

57

NB825

T8877-25G

69360-1263 20KG

EasyStain

3200-2

C5042-10G

P6148-1KG

31365-0350 1KG

4583

10055-12

11241-30

11254-20

11260-20

11274-20

14001-12

14084-08

15371-92

18153-11

Comments/Description

Glycyrrhizae Radix et Rhizoma

Qualitative filter paper

Dimethyl sulfoxide (DMSO)

Syringe filter (0.45 μm)

Stereo Microscope

Stereo Microscope

Laser Doppler

Anesthesia Tabletop Bracket with N_2O&O_2 Flowmeter System

Homeothermic Monitoring System

Digital Camera

Cryostat

Microscope

Data Analysis

Data Analysis

Mouse diet

Isoflurane

Isoflurane

Silk suture (4-0 Black silk)

Silk suture (3-0 White silk)

Nylon suture (8-0 monofilament)

2,3,5-triphenyltetrazolium chloride (TTC)

Formalin (Formaldehyde solution)

Hematoxylin (Harris Hematoxylin)

Eosin (1% Eosin Y Solution)

Cresyl violet (acetate)

Paraformaldehyde

Sucrose

Optimum cutting temperature (OCT) compound

Disecting Knife

#4 Forcep

#5 Forcep

#6 Forcep

#7 Fine Forcep

Surgical Scissors

Extra Fine Bonn Scissors

Moria Pascheff-Wolff Spring Scissors

Vessel Dilating Forcep

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Article Title: Assessing Neuroprotective Effects of Glycyrrhizae Radix et Rhizoma Extract Using a Transient Middle Cerebral Artery Occlusion Mouse Model

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Response to Editorial and Reviewers' comments

Editorial and Reviewers' comments and Changes to be made by the Author(s):

2.2 How is sufficient depth of anesthesia determined?

Response: Corrections done in the manuscript rephrasing more detailed explanation.

2.3 How? Shaving or depilatory cream? What is used for the incision? How large of an incision?

Response: Corrections done in the manuscript rephrasing more detailed explanation.

2.4 What type of suture is used? What type of knot?

Response: Corrections done in the manuscript rephrasing more detailed explanation.

2.7 Suture how?

Response: Corrections done in the manuscript rephrasing more detailed explanation.

2.8 What steps?

Response: Corrections done in the manuscript rephrasing more detailed explanation.

2.9 Is this supposed to be a substep of 2.8?

Response: Yes it is. Corrections done in the manuscript.

3.1 How is euthanasia done?

Response: The sentence was rephrased in the manuscript.

3.1 If this is to be filmed, how is the excision done?

Response: Corrections done in the manuscript rephrasing more detailed explanation.

3.1 For how long?

Response: The sentence was rephrased in the manuscript.

4. Is step 3 and 4 performed on the same mouse? Or is step 3 or step 4 performed on the same mouse? This is unclear.

Response: No, authors conducted separate sets of experiment for brain damage measurement (step 3) and for histological study (step 4). Related sentences were rephrased in the manuscript to avoid confusion.

4.1 Euthanize the mouse again? Mice or mouse?

Response: As depicted above, the related sentence was rephrased.

4.2 How?

Response: As depicted above, the related sentence was rephrased.

4.3 Please break up these run on sentences.

Response: Corrections done in the manuscript.

Additional change made by the Authors:

1. Several additional materials used in this study were added in accordance with corrections in the manuscript.
2. The authors invited Dr. Chang-Hyun Kim who assisted Dr. Chiyeon Lim conducting design experiment using animals to revise this manuscript together. Dr. Chang-Hyun Kim also gave us many advises on writing terms on animal experiment. Thus, the authors concluded to add Dr. Chang-Hyun Kim as the co-author.