Journal of Visualized Experiments

Establishment and detection of sub-acute cerebral microhemorrhages model in rats induced by lipopolysaccharide injection

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58423R2
Full Title:	Establishment and detection of sub-acute cerebral microhemorrhages model in rats induced by lipopolysaccharide injection
Keywords:	blood-brain barrier, cerebral microhemorrhages, LPS, neuroinflammation, rats, protocol
Corresponding Author:	Yonghua Dr. Huang Military General Hospital of Beijing PLA Beijing, Beijing CHINA
Corresponding Author's Institution:	Military General Hospital of Beijing PLA
Corresponding Author E-Mail:	huangyonghua2017@126.com
Order of Authors:	Dandan Li
	Hóngyi Zhào
	Wei Wei
	Nan Liu
	Yonghua Dr. Huang
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TITLE:

Sub-Acute Cerebral Microhemorrhages Induced by Lipopolysaccharide Injection in Rats

AUTHORS:

Dandan Li[1], Hóngyi Zhào[1,2], Wei Wei[1], Nan Liu[2], Yonghua Huang*[1]

Dandan Li and Hóngyi Zhào are co-first authors.

[1]Department of Neurology, PLA Army General Hospital, Beijing, PR China
[2]Department of Neurology, NO 261 Hospital of PLA, Beijing, PR China

yoyo-lidandan-01@163.com
zhaohongyi17@126.com
weiweipagh@qq.com
miemie61@163.com
huangyonghua2017@126.com

Correspondence to:
Yonghua Huang

KEYWORDS:

Blood-brain barrier, cerebral microhemorrhages, lipopolysaccharide, neuroinflammation, rats, protocol

SUMMARY:

We present a protocol to induce and detect CMHs caused by LPS injection in Sprague-Dawley rats, which may be utilized in future research investigations on the pathogenesis of CMHs.

ABSTRACT:

Cerebral microhemorrhages (CMHs) are common in aged patients and are correlated to various neuropsychiatric disorders. The etiology of CMHs is complex, and neuroinflammation is often observed as a co-occurrence. Here, we describe a sub-acute CMH rat model induced by lipopolysaccharide (LPS) injection, as well as a method for detecting CMHs. Systemic LPS injection is relatively simple, economical, and cost-effective. One major advantage of LPS injection is its stability to induce inflammation. CHMs caused by LPS injection could be detected by gross observation, hematoxylin and eosin (HE) staining, Perl's Prussian staining, Evans blue (EB) double-labeling, and magnetic resonance imaging-susceptibility weighted imaging (MRI-SWI) technology. Finally, other methods of developing CMH animal models, including their advantages and/or disadvantages, are also discussed in this report.

INTRODUCTION:

Classical cerebral microhemorrhages (CMHs) refer to tiny perivascular deposits of blood degradation products such as hemosiderin from red blood cells in the brain[1]. According to the

Rotterdam Scan Study, CMHs could be found in nearly 17.8% of persons aged 60-69 years and 38.3% in those over 80 years[2]. The prevalence of CMHs in the elderly is relatively high, and a correlation between the accumulation of CMHs and cognitive and neuropsychiatric dysfunction has been established[3,4]. Several animal models of CMHs have recently been reported, including rodent models induced by type IV collagenase stereotaxic injection[5], APP transgenic[6], β-N-methylamino-L-alanine exposure[7], and hypertension[8], with CMHs induced by systemic inflammation as one of the most well-accepted choices. Fisher *et al.*[9] first used LPS derived from *Salmonella typhimurium* to develop an acute CMH mouse model. Subsequently, the same group reported the development of a sub-acute CMH mouse model using the same approach[2].

LPS is considered as a standardized inflammatory stimulus *via* intraperitoneal injection. Previous studies have confirmed that LPS injection could cause neuroinflammation as reflected by large amounts of microglia and astrocyte activation in animal models[2,10]. Furthermore, a positive correlation between activation of neuroinflammation activation and the number of CMHs has been established[2,10]. Based on these previous studies, we were prompted to develop a CMH rat model by intraperitoneal injection of LPS.

Advances in detection technologies have resulted in an increase in the number of research investigations on CMHs. The most widely acknowledged methods of detecting CMHs include the detection of red blood cells by hematoxylin and eosin (HE) staining, ferric iron detection by Prussian Blue staining[9], detection of Evans blue (EB) deposition by immunofluorescence imaging, and 7.0 Tesla magnetic resonance imaging-susceptibility weighted imaging (MRI-SWI)[10]. The present study aims to develop a method of screening for CMHs.

PROTOCOL:

All methods described here have been approved by the Animal Care and Use Committee (ACUC) of the PLA Army General Hospital.

1. Materials

1.1. Preparation of LPS injection

1.1.1 Add 25 mL of distilled water to 25 mg of LPS powder derived from *S. typhimurium* to a final concentration of 1 mg/mL. Store the injection in a sterile tube at 4 °C.

CAUTION: LPS is toxic.

1.1.2 Prepare a 2% EB solution in normal 0.9% saline solution to keep EB injection at working concentration.

2. Animals

2.1. Administer LPS to male Sprague-Dawley (SD) rats, aged 10 weeks (average weight: 280 ± 20 g) by intraperitoneal injection.

CAUTION: If rats are purchased from another organization, then the adaptive phase should not be less than 7 days.

3. LPS Injections

3.1. Inject LPS intraperitoneally into each rat at a dose of 1 mg/kg, and return the rats to their home cage.

Note: Anesthesia is not necessary. Sucking back the LPS during injection may be necessary to confirm that LPS is not injected into the blood vessels.

3.2. Six hours later, inject the same dose of LPS injection into the rats.

3.3. Sixteen hours later inject the same dose of LPS into the rats.

CAUTION: Injecting SD rats with LPS at a dose of 1 mg/kg may result in a 5% mortality rate. The mortality rate could further increase in younger or older rats or pregnant female rats.

3.4. Return the rats to their cages after LPS injection and provide *ad libitum* access to food and drink.

Note: Rats will exhibit a distinct systemic inflammatory response, and thus it is essential that the cages remain clean throughout the experiment.

4. Sample Collection

4.1. EB injection

4.1.1. Seven days after the first injection, anesthetize the rat by intraperitoneally injecting 10% chloral hydrate (1 mL/300 g).

4.1.2. Wait 1-2 min until the rats do not show corneal reflex responses. Perform a 5-8 mm-deep cardiac puncture on each rat; the puncturing point should be 5 mm to the left margin of sternum at the 3rd and 4th intercostals space.

4.1.3. Wait until blood recovery is observed, and then inject EB at a dose of 0.2 mL/100 g body weight into the left heart ventricle.

CAUTION: Cardiac puncture and EB injection should be carefully performed. EB might be injected into thoracic cavity or pericardium instead of the left heart ventricle.

4.1.3.1. Suck back with an empty tube and replace this volume with an EB tube before injection to help reduce the failure rate (*e.g.*, injection to rib cage by error).

4.1.4. Keep the rat in a supine position for 10 min.

Note: If sample is not used to detect EB leakage in immunofluorescence imaging, then this step may be omitted.

4.2. **Gross observation**

4.2.1. Perform cardiac perfusions using ice-cold 1 M phosphate buffer saline (PBS) to clear the cerebral vasculature and brains.

4.2.1.1. Using a scalpel, make an abdominal incision across the entire length of the diaphragm.

4.2.1.2. Cut through ribs just left of the rib cage midline.

4.2.1.3. Open up the thoracic cavity. Use clamps to expose the heart and to facilitate drainage of blood and other fluids.

4.2.1.4. While steadily holding the heart with forceps (the heart should still be beating), directly insert a needle into the protrusion of the left ventricle to extend this to about 5 mm. Secure the needle at that position by clamping near the point of entry.

CAUTION: Do not excessively extend the needle, as it can pierce the interior wall and compromise the circulation of solutions.

4.2.1.5. Release the valve to allow the flow ice-cold PBS solution (200-300 mL) at a slow and steady rate at around 20 mL/min using a perfusion pump. If the animals require fixation instead of gross observation, then use the same dose of ice-cold 0.9% saline solution.

4.2.1.6. Using sharp scissors, make an incision in the atrium, ensuring that the solution continuously flows. If the fluid does not flow freely or is coming from the animal's nostrils or mouth, reposition the needle.

4.2.2. Screen for CMHs by gross observation.

Note: If the sample is not used in calculating the number of CMHs by gross observation, then this step may be omitted.

4.3. Fixation

4.3.1. Perform cardiac perfusions using ice-cold 0.9% saline solution (200-300 mL) to clear the cerebral vasculature and brains.

4.3.2. Perform cardiac perfusions using ice-cold 4% paraformaldehyde for fixation.

4.3.3. Decapitate the rat, isolate the brain, and immerse the brain in 20% sucrose solution for at least 6 h.

4.3.4. Change the solution into 30% sucrose and fix for another 6 h.

4.4. Prepare 10-mm-thick brain tissues sections using a cryostat.

5. HE Staining

Note: This procedure is performed using a HE Staining Kit.

5.1. Wash the slides in distilled water.

5.2. Stain in hematoxylin solution for 8 min. Wash in running tap water for 5 min.

5.3. Differentiate in 1% acid alcohol for 30 s. Wash in running tap water for 1 min.

5.4. Stain in 0.2% ammonia water (Bluing) or saturated lithium carbonate solution for 30 s to 1 min. Wash in running tap water for 5 min.

5.5. Rinse in 95% alcohol (10 dips).

5.6. Counterstain with eosin solution for 30 s to 1 min.

5.7. Dehydrate through 95% alcohol, two changes of absolute alcohol, for 5 min each.

5.8. Clear in xylene for 30 s.

5.9. Mount using Liu's method[9].

5.10. Analyze using a brightfield fluorescence microscope.

Note: Red blood cells released from blood vessels, which are the components of CMHs, appear in red-orange under HE staining.

6. Perl Prussian Blue Staining

Note: This procedure is performed using a Perls Staining Kit.

6.1. Wash slides with distilled water.

6.2. Stain in reaction solution with equal parts mixture of ferrocyanide and hydrochloric acid for 10 min.

6.3. Dehydrate, clear, mount, and analyze the slides as described in steps 5.7-5.10.

7. **EB double-staining**

Note: This procedure follows step 4.1.3.

7.1. Wash slides with PBS three times for 5 min each.

7.2. Incubate slides with 4',6-diamidino-2-phenylindole (DAPI) solution for 15 min at room temperature.

7.3. Wash the slides with PBS solution three times for 5 min each, and mount slides with PBS-glycerol solution.

7.4. Analyze images on a fluorescence microscope. EB deposition is indicated by red fluorescence; nuclei are indicated by blue fluorescence.

8. MRI-SWI

8.1. Perform MRI on a 7-T magnet equipped with an actively shielded gradient system (16 cm inner diameter).

8.2. Seven days after treatment, anesthetize the rats by facemask inhalation of 1.5%-2.0% isoflurane using an isoflurane anesthesia system.

8.3. Apply vet ointment onto the eyes of the rat to prevent dryness while under anesthesia.

8.4. Keep the rats in prone position and perform an MRI-SWI scan.

8.5. Obtain SWI weighted scans using a fast-spin echo sequence using the following parameters: echo time (TE) 8 ms, repetition time (TR) 40 ms, flip angle=12°, field of view (FOV) 35 mm × 35 mm, acquisition matrix 384 × 384, to acquire 1-mm-thick slices.

8.6. Identify CMHs in MRI-SWI according to Greenberg *et al.*[11], which included the following criteria: (1) a diameter of ≤10 mm, and (2) circular, isolated, and low-signal intensity spots.

8.7. At the end of the experiment, euthanize the rats using the overdose carbon dioxide (at a flow of 6 L/min) method.

REPRESENTATIVE RESULTS:

CMHs can be detected using various approaches. The most well-accepted methods include the following: (1) gross observation and assessment of surface CMHs (shown in **Figure 1**, upper panel); (2) HE staining for the detection of red blood cells (shown in **Figure 2A**, upper panel) or Prussian staining detecting ferric iron derived from lysis of red blood cells (**Figure 2A**, lower panel); (3) EB doubled staining for the detection of EB deposition derived from BBB leakage (**Figure 3**, left panel); (4) MRI-SWI detection of CMH hypointense signals (**Figure 4**, left panel).

Figure 1. Gross observation of surface CMHs. Upper panel (A): Rat model; Lower panel (B): Control animal. Red arrows signify surface CMHs. Modified and reused with permission[10].

Figure 2. HE staining and Prussian staining images. (A) Reflected rat model. Red blood cells were found outside the capillaries in upper panel, and ferric iron points derived from lysis of red blood cells were detected in lower panel; (B) Reflected control animal. Neither red blood cells nor ferric iron points were found. Scale bars in left panel of (A) and (B): 100 μm. Scale bars in left panel of (A) and (B): 50 μm. Modified and reused with permission[10].

Figure 3. Double labeled EB deposition fluorescence imaging. Left panel: Rat model. Amount of EB molecules leaked from injured BBB were detected (in red); Right panel: Control animal. No EB molecules were detected. DAPI in blue was used as mount medium. Scale bars: 100 μm. Modified and reused with permission[10].

Figure 4. MRI-SWI images. Left panel (A): Rat model. Black arrows signify hypointense signals of CMHs; Right panel (B): Control animal. No hypointense signals were found. Modified and reused with permission[10].

DISCUSSION:

Research studies on CMHs have increased in the past few years. However, the mechanism of CMHs remains unclear, prompting scientists to establish animal models that simulate this particular condition. For example, Hoffmann *et al.* developed a hypoxia-induced CMH mouse model that shows that CMHs are caused by hypoxia and disruption of cerebrovascular autoregulation[12]. Reuter *et al.*[5] established a CMH model in APP23-transgenic mice, which showed that cerebral amyloid angiopathy (CAA) plays a major role in the etiology of CMHs. Fisher *et al.* reported a CMH mouse model that was induced by LPS injection[9], which revealed that CMHs are caused by inflammation[2,13,14]. Similarly, we have successfully developed a sub-acute CMH model in SD rats using LPS injection and have found that nitric oxide synthase (NOS), especially neural NOS and endothelial NOS, are involved in the etiology of CMHs, which result in inflammation[10].

The critical step of our protocol is the LPS injection, which has been extensively used in developing murine models of neuropsychiatric disorders such as depression[15], schizophrenia[16], Parkinson's disease[17], Alzheimer's disease[18], and Prion disease[19]. To our knowledge, our use of a single dose (1 mg/kg) is much higher than that was applied in other studies[15-19]. This might be a plausible reason for the mortality in the current study. LPS dose modification for CMH induction may be an interesting research topic, as different doses or injection schedule have been shown

to influence the number, size, distribution, and progression of CMHs[2]. A previous study has shown that the administration of LPS to mice at a dose of 3 mg/kg induces acute CMHs[2].

Although the development of different animal models has facilitated research investigations on CMHs, we have to admit that those animal models do not simulate the process of clinical CMHs. For example, in our rat model, as well as Fisher's mice model, CMHs were observed in the cerebellum, although most were observed in lobar regions (mainly CAA-related) and deep- or infra-tentorial locations (mainly hypertension-related)[20,21]. We have no explanation for this discrepancy in distribution, although both Fisher and our team attribute this observation to the vulnerability of cerebellar blood vessels to inflammation.

In most clinical cases, CMHs result from more than one etiological factor, although inflammation plays an important role in its etiology. Studies focusing on multiple factors that induce CMHs, instead of pure inflammation-induced CMHs, can thus be more helpful in simulating this condition. The LPS injection model could be used with other underlying factors to investigate the mechanism of CMHs. For example, Fisher *et al.* have conducted a preliminary, yet interesting step with aging mice injected with LPS, and demonstrated that aging is a key factor that could make the brain more susceptible to inflammation-induced CMHs[14]. In our opinion, the significance of the present model is its compatibility with other factors in animal models for aging[14], trauma[22], as well as chronic diseases such as hypercholesterolemia[23], or transgenic models such as hypertension[24] because of the simplicity, time-effectiveness, and stability of this protocol.

Patients with CMHs show cognitive declines, neuropsychiatric manifestations, and vertigo, which are associated with the distribution of CMHs. One limitation of the present protocol is that after LPS injection, we could not rule out the effects of peripheral inflammation on the behavior of the rats, although a decrease in social behavior and burrowing (intrinsic natural activity of rodent reflecting the impairment of daily activities) were observed. Further studies on ways to improve our method of generating a CMH animal model, *e.g.,* method of injecting LPS or schedule of observation, are warranted.

Nevertheless, this simple, cost-effective, and stable CMH murine model induced by LPS injection may be utilized by researchers in elucidating the etiology of CMHs.

ACKNOWLEDGMENTS:
We thank Teacher Jian Feng Lei and colleagues from Capital Medical University for guidance during MRI. We also thank Jing Zeng from the Department of Neurology, the Second People's Hospital of Yichang for providing technical support.

DISCLOSURES:
The authors have nothing to disclose.

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Figure1

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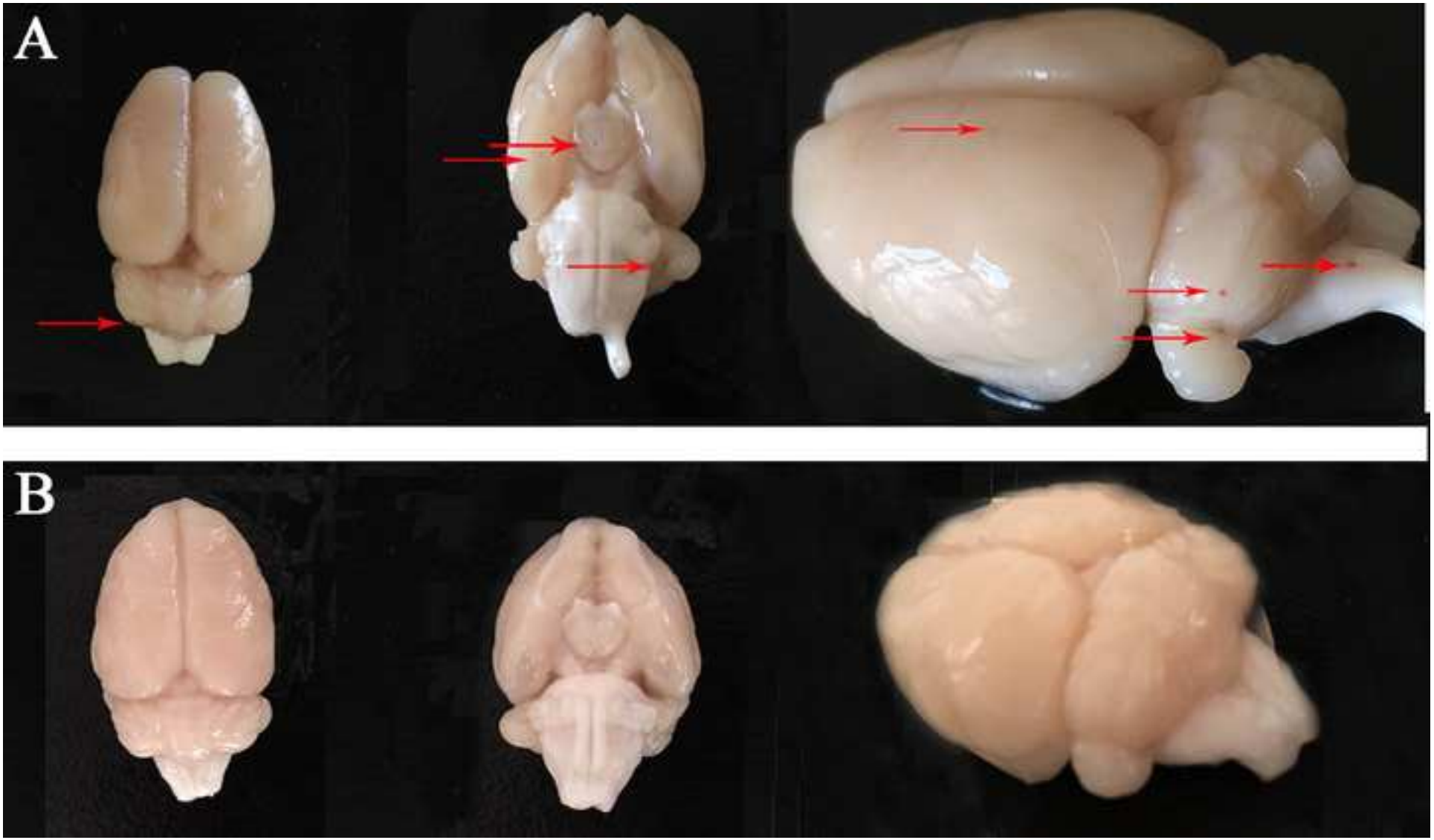

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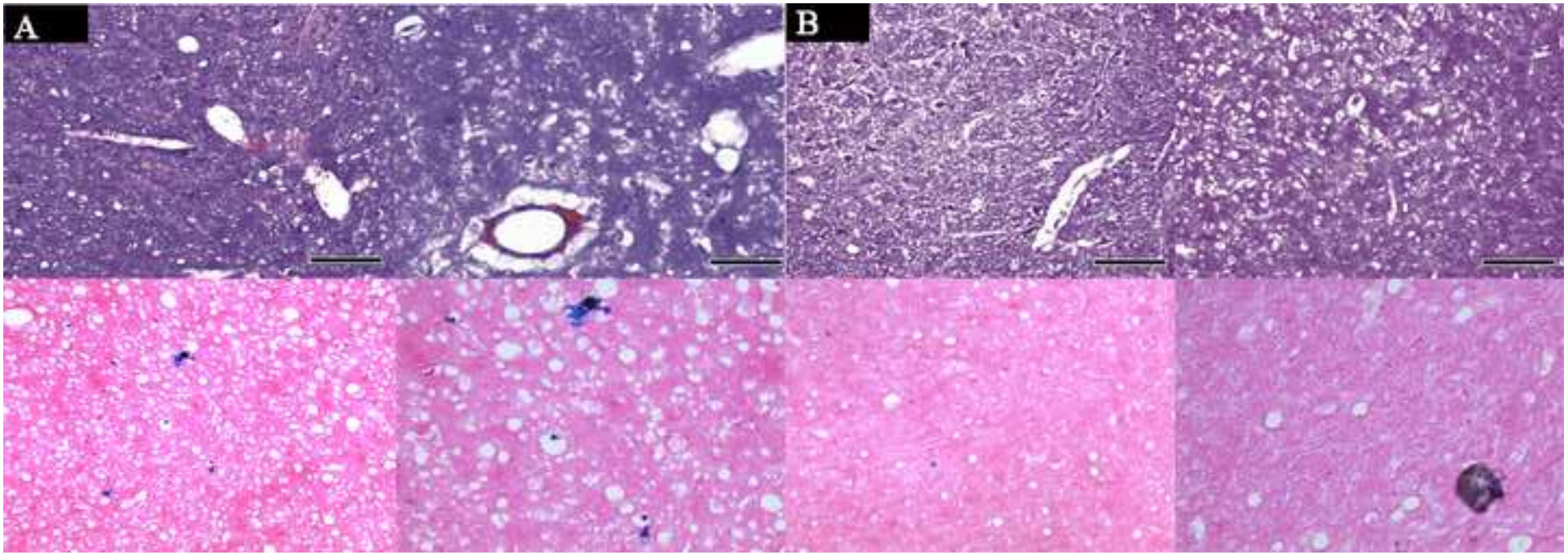

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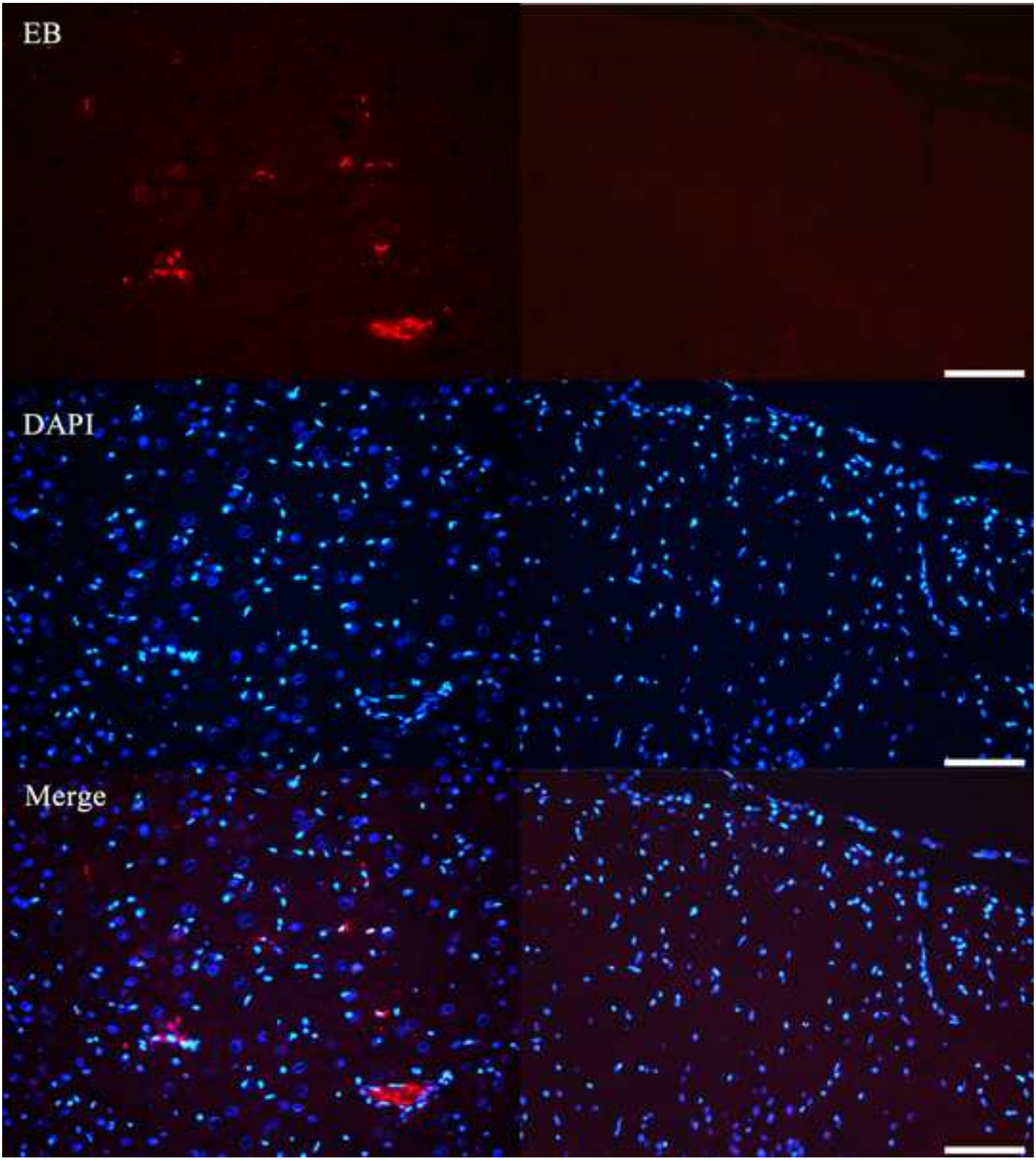

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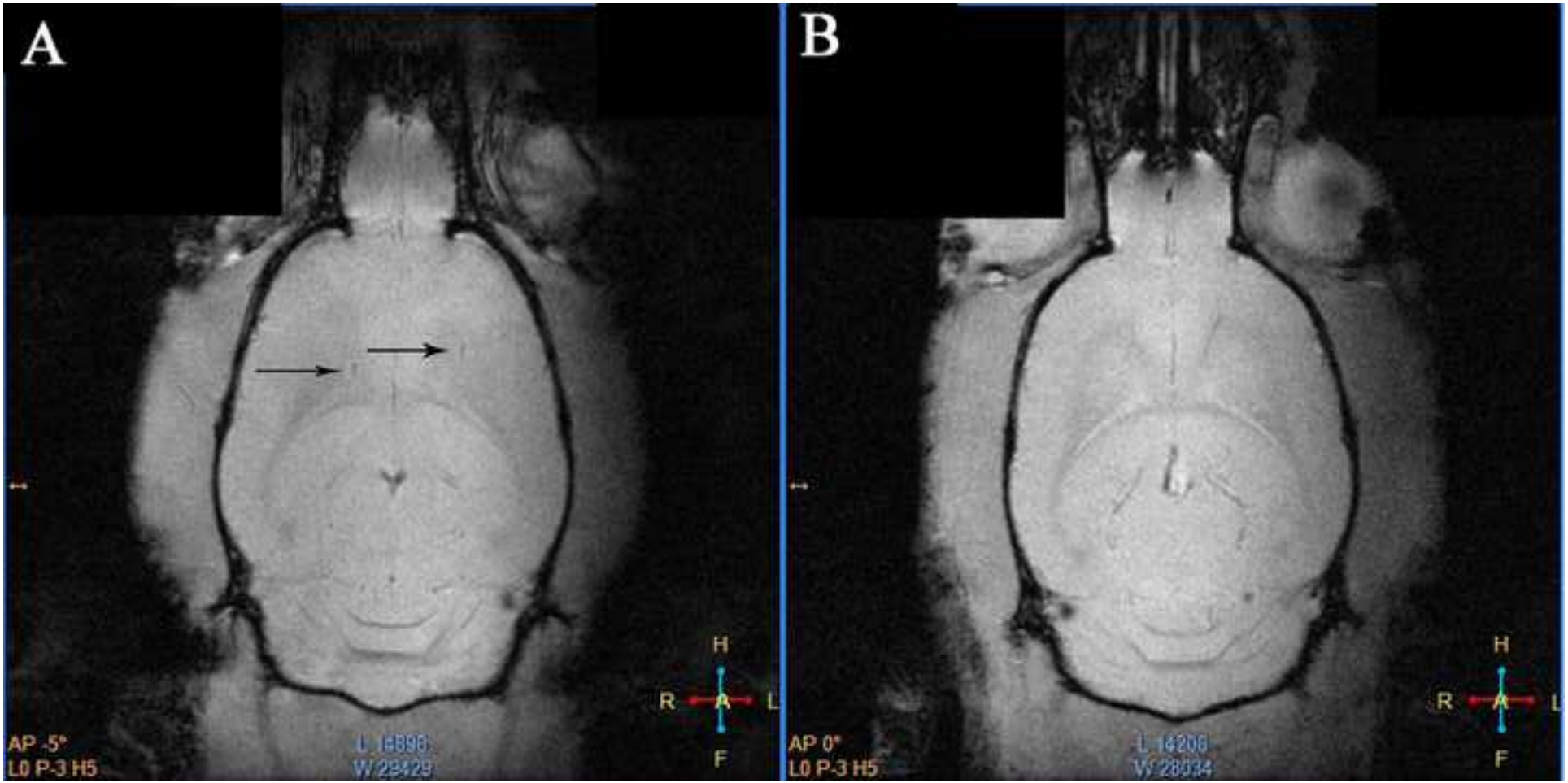

Table of Materials

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Name of Material/ Equipment	Company
LPS	Sigma-Aldrich
EB	Sigma-aldrich
DAPI dying solution	Servicbio
Perl's Prussian staining	Solarbio
HE staining	Solarbio
chloral hydrate	Sigma-Aldrich
phosphate buffer saline (PBS)	Solarbio
0.9% saline solution	Hainan DonglianChangfu
paraformaldehyde	Sigma-Aldrich
20% sucrose solution	Solarbio
30% sucrose solution	Solarbio
vet ointment	Solcoseryl eye gel, Bacel, Switzerland

Catalog Number	Comments/Description
L-2630	for inflammation induction
E2129	for EB leakage detection
G1012	count medium for IF
G1424	Kit for Prussian staining
G1120	Kit for HE staining
47335U	For anesthesia
P1022	a kind of buffer solution commonly used in solution for perfusion
158127	a kind of solution commonly used for
G2461	a kind of solution commonly used for
G2460	a kind of solution commonly used for for rat's eyes protection

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CORRESPONDING AUTHOR:

Name: Yonghua Huang

Department: Neurology

Institution: PLA Army General Hospital

Article Title: Sub-Acute Cerebral Microhemorrhages Induced by Lipopolysaccharide Injection in Rats

Signature: Yonghua Huang Date: 7/3/2018

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Dear Dr. Huang,

Your manuscript, JoVE58423 Establishment and detection of sub-acute cerebral microhemorrhages model in rats induced by lipopolysaccharide injection, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.

Your revision is due by **Jun 28, 2018**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Nam Nguyen, Ph.D.
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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Reply:

Thank you for your advice, we have improved the manuscript by native English speakers. And we can confirm the absence of spelling or grammar issues.

2. Please print and sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

Reply:

We have print and sign the Author License Agreement, and upload it within new-version manuscript file.

3. Please submit the figures as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300dpi.

Reply:

We ensure that the resolution of images, and submit the figures as a .psd.

4. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Reply:

We have changed form of the references according to the example.

5. Please include volume and issue numbers for all references.

Reply:

Yes, they are all included.

6. Please define all abbreviations before use.

Reply:

Yes, we confirm this.

7. Please use focused images of uniform size/resolution (at least 300 dpi).

Reply:

Thank you for your suggestions, we changed our figures at a resolution at 300dpi.

8. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

Reply:

Yes, we have all the figures included a title and a description. Of course, the legends are listed as isolated paragraphs following Representative Results section in new-versioned manuscript.

9. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

Reply: Yes, we have revised it and rebuilt a table accordingly.

10. Figure 1: Please label the panels. What do the red arrows signify?

Reply:

The red arrows signify CMS in gross observation. We have mentioned this in the legend and description in new version manuscript.

11. Figure 2: Please label the panels. Please provide scale bars.

Reply: We have labeled the panels and provided scale bars.

12. Figure 3: Please increase the size of the scale bars and the font. Please label the panels.

Reply: We have increased the size and labeled the panels.

13. Please include a Summary that clearly describes the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Reply:

We have added a Summary (29 words) in Abstract section that describes the protocol and its applications. Thank you for your advice.

14. Please provide an institutional email address for each author.

Reply:

Thank you for your advice, we have provided the email address for each author.

15. For in-text formatting, corresponding reference numbers should appear as numbered

superscripts after the appropriate statement(s).

Reply:

Yes, we confirm these.

16. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

Reply:

We have mentioned the statement in new version manuscript (in section 9).

b) Please specify the euthanasia method.

Reply:

Overdose carbon dioxide method was used for euthanasia (CL-1000-S1, Yuyan Instruments). We have specified that.

c) Please mention how animals are anesthetized and how proper anesthetization is confirmed.

Reply:

Thank you for your advice. Actually, in our experiment, only those rats for MRI needed anaesthesia with gas. The number of rats is 6 for each group, and the rats were anaesthetized with facemask inhalation of 1.8% isoflurane by an isoflurane anesthesia system (JD Medical Distributing Co., Inc., Phoenix, AZ). The rats for perfusion, intraperitoneal injection of 10% chloral hydrate (1 mL/300g) was chosen. All these methods have been mentioned in new-versioned manuscript.

d) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

Reply: Thank you for your suggestion, we used vet ointment and specified it in new version manuscript.

e) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

Reply:

Thank you. In our experiment, animals would not suffer from surgery operation. As a result, we did not discuss post-surgical treatment.

f) Discuss maintenance of sterile conditions during survival surgery.

Reply:

Thank you. In our experiment, animals would not suffer from surgery operation. As a result, we did not discuss sterile conditions.

g) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

Reply: Thank you. In our experiment, animals would not suffer from surgery operation. As a result, we did not live them unattended.

h) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

Reply:

Thank you. In our experiment, animals would not suffer from surgery operation.

i) Please do not highlight any steps describing euthanasia.

Reply:

Thank you for your reminding.

17. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Reply: Thank you, we have added more details to our protocol steps.

18. 4.1.2: How was the incision done? What was used? How large is the incision?

Reply:

Thank you. In our experiment, animals would not suffer from incision or surgery operation.

19. Back puming?

Reply:

Thank you for the reminding. We need to back puming during intraperitoneal injection. And we have specified this.

20. How is the perfusion done? This should be made clear.

Reply:

Thank you for your suggestion, and we have added perfusion steps to make it clear. Please find them in new-versioned manuscript.

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Reply:

Thank you for your reminding, we have got the permission from Journal of Stroke and Cerebrovascular Diseases for reuse some figures. And we have mentioned the permission in legends.

22. Please expand the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

Reply:

We have added a detailed lengends section and tried to describe how to analyze the outcome and the relevant meanings. Please find them in new version manuscript.

23. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Reply:

Thank you very much for your suggestions, we have added details to discuss the critical steps, modifications, limitations, etc. Please find in new-versioned manuscript.

24. Please do not abbreviate journal titles.

Reply:

OK. We will not abbreviate journal titles.

Reviewers' comments:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.

Reviewer #1:

The methods article presented by Zhao et al is a concise and well-written work regarding the induction of sub-acute cerebral microhemorrhages (CMHs) in rats by way of LPS injection. Although other methods to induce CMHs are established in other animal models, there is clear justification for creating this model in rats. Please cite previous attempts to induce cerebral microhemorrhages in rats with a micro collagenase injection (G McAuley, M Schrag, S Barnes, A Obenaus, A Dickson, W Kirsch. In vivo iron quantification in collagenase-induced microbleeds in rat brain. Magnetic resonance in medicine 67 (3), 711-717); the model proposed by the Zhao and colleagues in my view is an improvement over the technique previously employed. The article elaborates on methods to detect CMHs in rats and the inclusion of SWI images is a particular strength of the current study as histological techniques can easily overlook areas of microhemorrhage. A complete table of all materials and equipment was references in the paper, but appears to be missing -- this would be useful for future investigators utilizing this method and should be added prior to publication. It might also be useful to also include how controls were obtained (i.e. were the negative control rats only treated with vehicle?). This group did a great job demonstrating the efficacy of the methods used as highlighted in the figures provided but it might be advisable that the figures be annotated more definitively so readers can verify results without having to make assumptions. Still, this group has proven their methodology to be reasonable and effective.

Reply

Thank you very much for your suggestions!

The model reported by Prof McAuley and colleagues has gained much attention from researchers. They introduced not only how to induce collagenase-induced microbleeds, but also the method to detect microbleeds by MRI-SWI in vivo. We have cited this helpful article.

We have added a complete table of all materials and equipment. Please find it in new-versioned manuscript.

Control group rats were only treated with PBS solution, it’s quite simple.

Also, we have added a complete legends section to explain the figures. Thank you very much!

Reviewer #2:

Major Concerns:

I think the manuscript is sound, but it requires extensive editing for language. I believe it will need re-review by the authors after editing is completed, to assure fidelity. After the authors have check it, I would like to reread it.

Reply:

Thank you for your advice, we have improved our manuscript by native English speakers. Thank you very much.

Reviewer #3:

Manuscript Summary:

The manuscript presented by Zhao and colleagues titled "Establishment and detection of sub-acute micro-haemorrhages model in rats induced by lipopolysaccharide injection", outlines a method of the production and detection of cerebral micro-haemorrhages (CMH) in adult Sprague-Dawley rats, as a result of multiple intraperitoneal injections of LPS.

Major Concerns:

This is a quick and relatively cheap method of producing cerebral micro-haemorrhages. What is not clear from the manuscript as it currently stands is what the normal micro-haemorrhage burden is in each animal or the reproducibility of the method. The authors acknowledge that this model does not replicate the clinical distribution of cerebral micro-haemorrhages; an apparently common reality in models of inflammation-induced CMH. Given the lack of clinical relevance, and the abundance of other models, it is not clear whether this model adds anything to the field, despite the easy and cost-affordability it offers experimenters. A more detailed introduction/discussion of the other methods in the field and the nature of the CMHs produced in the model might help.

Reply:

Thank you for your comments, the method has good reproducibility.

The method has its own limitations, meanwhile, it indeed has advantages despite of simplicity and cost-affordability. For example, more than one etiological factors take part in the formation of CMHs in most clinical cases, as a result, studies focusing on multiple factors induced CMHs, instead of pure inflammation induced CMHs, can be more helpful to mimic the reality. LPS injection model could be used with other underlying factors to investigate the mechanism of CMHs in future application. The significance of the present model is the compatibility with other factors in animal models, such as aging, trauma[1], especially with chronic models such as hypercholesterolemia[2], or transgenic models such as hypertension[3], because short time-consuming and stability of this protocol.

We have mentioned all these points in discussion in new-versioned manuscript.

Minor Concerns:

For a methods paper, the details in the protocol are quite superficial. For instance, the serotype of LPS is not given, nor the batch number. However, there is substantial awareness in the field that the response of animals to LPS is highly dependent on serotype, batch and storage.

More minor details that are missing or unclear include:

i) Is mounting media used for cryosectioning, if so, which one?

Reply:

Yes, DAPI was used as mounting media for cryosectioning. We have specified it in new-versioned

manuscript.

ii) The manuscript states that action 4.2.1 will only be performed for gross observation of CMHs, but surely this is a standard element of any perfusion fixation protocol?

Reply:

4.2.1 is introduced to perform gross observation, but if the perfusion solution changed from PBS solution into 0.9% saline solution, this step, as well as 4% paraformaldehyde consists of fixation procedure. We have mentioned and detailed this in new-versioned manuscript. Hopefully, readers would not have ambiguity. Thank you very much!

iii) What does "the environmental cleanliness need to be maintained" actually mean in reality, in action 3.3? Likewise, what is the "certain but definite systemic inflammation response" that occurs in this model. Given the potential 10% death rate, should researchers be monitoring animals for signs of distress? How frequently? For what features? Is it necessary to have a humane end-point to these experiments?

Reply:

After the first dose of LPS injection, some rats would suffer from diarrhea, vomit and other digestive symptoms, as well as lack of motivation characterized as lack of hair tidy-up behaviors. All these factors resulted in the environmental untidy, which will do harm to rats survival rates. Researchers need to take care of the rats every 2 hours and move the rats into new clean cages.

10% death rate is for acute mode, in which a higher dose of LPS injection per time was chosen (3mg/ kg). It's a mistake, for sub-acute model of rats (1mg/ kg LPS for single injection), the mortality rate is less than 5%. However, researcher still needed to monitor the signs of distress for digestive symptoms every 2 hours.

Overdose carbon dioxide system was used as humane end-point apparatus in these experiments, we have mentioned these in new-versioned manuscript. Thank you very much!

iv) What stage of anaesthesia should be reached in section 4.1.1 before section 4.1.2 can be started?

Reply:

The stage is characterized as the corneal reflex disappearance. Thank you!

v) The MRI method appears to be out of sequence. Is the information provided really sufficient for the method to be successfully reproduced by other researchers?

Reply:

Thank you very much for your suggestions, we have notice that this section is difficult for the readers to understand. After the improvement by MRI expert, we have re-arranged the expression of MRI-SWI protocol, in order that the method will be sufficient for successfully reproduced by other researchers. Please find them in new-versioned manuscript.

vi) Is perfusion fixation, without further fixation by immersion, sufficient for good tissue preservation?

Reply:

Apart from perfusion fixation, 20% and 30% sucrose solution immersions by step are needed for good tissue preservation. We have mentioned this point in manuscript, thank you.

The whole manuscript needs extensive editing by a native English speaker.

Reply:

Thank you very much, and we have improved our manuscript by native English speakers.

References:

1. Robinson, S., et al. Microstructural and microglial changes after repetitive mild traumatic brain injury in mice. J Neurosci Res. 95(4), 1025-1035 (2017). doi: 10.1002/jnr.23848.

2. Kraft, P., et al. Hypercholesterolemia induced cerebral small vessel disease. Plos One. 12(8), e0182822 (2017). doi: 10.1371/journal.pone.0182822.

3. Schreiber, S., Bueche, C.Z., Garz, C., Baun, H. Blood brain barrier breakdown as the starting point of cerebral small vessel disease? - New insights from a rat model. Exp Transl Stroke Med, 5(1), 4 (2013). doi: 10.1186/2040-7378-5-4.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

   Reply: Thank you for your advice, we have had our manuscript improved by native English speakers.

2. Please print and sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

   Reply: We have printed and signed ALA, and uploaded it. Thank you very much.

3. Where are the figure legends? Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

   Reply: We have uploaded figure legends, please find them in Representative Results section in new-versioned manuscript.

4. Figure 1: Please label the panels. What do the red arrows signify? Having a border between the panels would greatly improve the Figure.

   Thank you, we have labeled the panel and added a border between the upper and lower panel. The red arrows signify CHMs, which have been explained in legends in new-versioned manuscript.

5. Figure 2: Please use a different color for the panel labels.

   Thank you for the suggestion, we chose black color.

6. Figure 4: Please label the panels.

   Reply: Thank you for your advice, we have labeled the panel.

7. Additional details are needed in the manuscript. Please see the comments in the attached manuscript.

Reply: Thank you for your suggestions, we have improved our manuscript according to the comments one by one.

8. Please provide an institutional email address for each author. Please avoid the use of 126.com accounts.

   Reply: Thank you, we have provided the email address of other authors.

   Dandan Li--; Hóngyi Zhào--zhypla17@163.com;
   Weiwei—Weiweipagh@163.com; Nan Liu—miemie61@163.com

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   Reply: Yes, we have obtained the permission from the “Journal of Stroke and Cerebrovascular Diseases”, and uploaded it. Each figure legend has the citation.

10. Please do not abbreviate journal titles.

    Reply: Yes, we can confirm this. Thank you.

11. Please highlight 2.75 pages of protocol text for inclusion in the protocol section of the video.

    Reply: Thank you very much, we have highlighted it in yellow background, please find them in new-versioned manuscript.

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Reviewers' comments:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.

Reviewer #1:

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More minor details that are missing or unclear include:
i) Is mounting media used for cryosectioning, if so, which one?
ii) The manuscript states that action 4.2.1 will only be performed for gross observation of CMHs, but surely this is a standard element of any perfusion fixation protocol?
iii) What does "the environmental cleanliness need to be maintained" actually mean in reality, in action 3.3? Likewise, what is the "certain but definite systemic inflammation response" that occurs in this model. Given the potential 10% death rate, should researchers be monitoring animals for signs of distress? How frequently? For what features? Is it necessary to have a humane end-point to these experiments?
iv) What stage of anaesthesia should be reached in section 4.1.1 before section 4.1.2 can be started?
v) The MRI method appears to be out of sequence. Is the information provided really sufficient for the method to be successfully reproduced by other researchers?
vi) Is perfusion fixation, without further fixation by immersion, sufficient for good tissue preservation?

The whole manuscript needs extensive editing by a native English speaker.