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](http://www.editorialmanager.com/jove) and log in as an author. You will find your submission under the heading "Submission Needing Revision".  
  
Best,  
  
Nam Nguyen, Ph.D.  
Manager of Review  
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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.  
21. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

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.