Reviewer’s report

Title: Hemopexin promotes angiogenesis via up-regulating HO-1 in rats after cerebral ischemia-reperfusion injury

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Reviewer: Tadahiko Ishiyama

Reviewer’s report:

The authors attempt to address whether hemopexin improves angiogenesis after cerebral ischemia-reperfusion injury in rats.

They found that hemopexin promoted angiogenesis in rats via up-regulating heme oxygenase-1 after cerebral ischemia-reperfusion injury.

There are several issues that need to be addressed.

Background

1. In clinical settings, thrombolytic therapy is not the main cause of ischemia/reperfusion injury. Please check this.

Methods

2. What were the exclusion criteria? How was the mortality? How many mice died and excluded? How did the author confirm the successful MCAO procedure?

3. How many rats were used for the neurobehavioral evaluation? Did the authors use the same mice at 24h and 7 d? If so, ordinary one-way ANOVA should not be used.

4. Rats were given saline or study drugs immediately after the cerebral ischemia-reperfusion. Authors evaluated the behavior of rats after awakening. How long did it take from the drug administration to behavior evaluation? Please give time interval.

5. Authors described that the model was deemed successful when the score was between 1 and 3 using the method of Longa. However, no data were presented in the text.

6. How was the "penumbra" defined in this study? Cerebral infarction would be completed at the timing of 7 days after ischemic insult. Because authors evaluated blood vessel density in the ischemic penumbra, assessment method should be clarified.

7. Infarct volume should be evaluated. Did HPX treated rats have smaller infarct volume?
8. The method used for PCR should be clarified. How were the penumbra and ischemic core detected? How was the penumbra tissue obtained? How large were the obtained samples? Did they include cortex, striatum, and white matter? Please specify.

9. Treatment: 10 L of saline or 10 L of sodium azide cannot be given to a rat.

10. In neurobehavioral evaluation, authors defined that score 3-7 showed severe neurological dysfunction, 8-11 moderate, and 12-18 were divided into mild neurological dysfunction. However, those assessments did not appear in results.

Statistical analysis

11. I do not think Least Significant Difference (LSD) test is suitable for multiple comparison of 5 groups, because LSD is not sufficiently controlling for Type I error.

12. Please state how the n value was determined for each experiment.

Results

13. Authors described that "The NBS scores of rats in each group at 24h after I/R were 15.5±2.43, 5.17±1.60, 5.00±2.10, 5.80±1.47, 5.33±1.47, and 5.33±1.97 respectively". Six data are presented. However, authors assigned rats to 5 groups. In addition, please give n. Parenthesis is not necessary.

14. Authors described that "The NBS scores of MCAO + HPX + ZnppIX group were significantly lower than that of MCAO + HPX + ZnppIX group (P < 0.05).(Figure 1) The same group is compared.

15. Figure 1: Chinese letters in the figure should be translated into English. Please give n. NBS scores: Because NBS is neurological behavioral score, scores should be deleted.

16. Table 1: Data of MCAO + HPX + ZnppIX group is lacking.

Discussion

17. First paragraph: Authors described that "NBS scores at 24 h and 7 d after I/R reduced to normal levels before HPX treatment. Therefore, we demonstrated that HPX can effectively improve neurologic deficits after I/R in rats, HO-1 May be the key molecule that was related to the neuroprotection effect of HPX". Did authors want to mention that NBS at 24 h and 7 d after I/R reduced to normal levels with HPX treatment? If so, however, NBS in the MCAO+HPX group was significantly lower than that in the Sham group. Figure 1 shows that NBS at 24 h and at 7 d in the MCAO+HPX group was
significantly higher than that in the MCAO, the MCAO+Vehicle, and the MCAO+HPX+ZnppIX groups. "HO-1 May be" should be "HO-1 may be".

18. Third paragraph: Authors discussed CD31 and vWF (probably von Willebrand factor. Please clarify). However, CD31 and vWF were not investigated in this study. Do those factors relate to eNOS?

19. Third paragraph: Authors described that "It was reported in animal models that angiogenesis associated gene expression began to rise in 2h after ischemia reperfusion, protein expressions of growth factors which were associated with endothelial cell hyperplasia and their receptors also began to increase within 1h after ischemic stroke". Why did authors not evaluate the blood vessel density at 24 hours after ischemia-reperfusion?

20. Fourth paragraph: Authors described that "in comparison with sham group, HO-1 mRNA level in ischemic penumbra irritably increased at 24 h after I/R and decreased to the normal level at 7 d after I/R in rats". However, Table 1 shows that HO-1 mRNA levels at 7 d did not decrease to normal level (the values may be at the Sham group level). In addition, HO-1 mRNA level was still high in the MCAO+HPX group. Please rewrite. "in comparison" should be "in comparison".

21. Fourth paragraph: Authors described that "In comparison with sham group, the angiogenesis density in the ischemic penumbra and the level of serum eNOS diminished notably". However, Figure 2 and Table 2 show that in comparison with the Sham group, the angiogenesis density in the ischemic penumbra and the level of serum eNOS increased but not diminished significantly. "In comparison" should be "In comparison".

22. The discussion section includes a lot of repetition of the results. Authors should discuss the relationship between angiogenesis after stroke and neurological outcomes. Authors also discuss the clinical importance of this study. In addition, limitation should be mentioned.

23. There are several misspellings (e.g. Carcia, exposd, eal-time, anaesthia, anethsia, penumbr, comparision, etc.) and grammatical errors. Please check entire manuscript and correct.

24. List of abbreviations: Authors used a lot of abbreviations that did not appear in this section. Please list all abbreviations.

Are the methods appropriate and well described? 
If not, please specify what is required in your comments to the authors.

No

Does the work include the necessary controls? 
If not, please specify which controls are required in your comments to the authors.
Yes

**Are the conclusions drawn adequately supported by the data shown?**
If not, please explain in your comments to the authors.

No

**Are you able to assess any statistics in the manuscript or would you recommend an additional statistical review?**
If an additional statistical review is recommended, please specify what aspects require further assessment in your comments to the editors.

I recommend additional statistical review

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