Peer Review File

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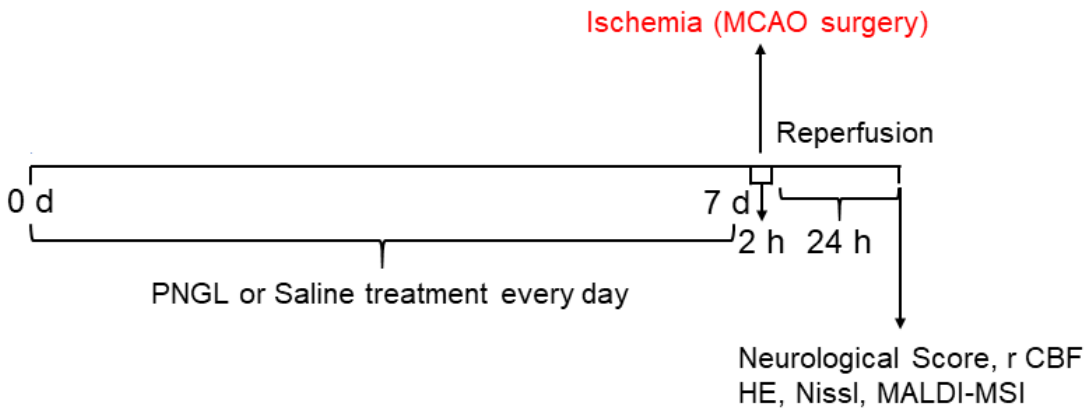
Reviewer Comments

1. The methods part presents many problems. First, there should be a time line of the study because I am not sure whether my brief summary above is actually true with respect to the time line. Second, the Ginseng extract is poorly described. Who was the manufacturer, how was it prepared? The authors show a chromatogram indicating that various ginsenosides are the main ingredients of the extract. They also discuss differences between Ginseng root and ginseng stem and leave extracts (lines 97-118) bit it remains an enigma which extract was actually used. Roots? Stem and leaves?

Reply 1: We are deeply sorry for the trouble caused to you.
1) The correct method is described as follows: Some of the rats were dosed with notoginseng leaf friterpenes (PNGL), 40 mg/kg per day by intraperitoneal injection. After seven days, transient MCAO was performed (for 2 hours), then reperfusion was allowed (for 24 hours). 24 hours later, the authors measured cerebral blood flow, neurological deficits, then prepared brain slices for histopathology and small molecules metabolism detection. The corresponding changes have been presented in the paper (see the method part for details, line 145, 157-165). In addition, the time line of the study is shown below.



2) Notoginseng leaf friterpenes (PNGL) was supplied by Jilin Academy of Chinese Medicine (line 157).
The extract was actually used in Panax notoginseng stem and leaf. The corresponding changes have been presented in the paper:
a. Currently, PNGL, as the total saponins of Panax notoginseng stem and leaf, are believed to have the functions of regulating blood lipid, sedative and hypnotic, analgesic, anti-inflammatory and anti-aging (line 102-104).
b. Additionally, chemical fingerprinting data have found that the content of total saponins in Panax notoginseng stems and leaves are mainly protopanaxadiol-type saponins, and contains almost no protopanaxatriol-type saponins (line 114-117).

2. The authors mention ketamine/xylazine as anesthetic but no treatment for post-operative pain.

Reply 2: With regard to the wound pain in rats after operation, we take the following methods:
a. After MCAO surgery, the wound was disinfected with iodine, and then the wound was sutured with sterile surgical suture to reduce the bleeding.
b. The body temperature of the rats was maintained at 37 ± 0.5 °C during the entire procedure using a heating blanket.
c. We also injected tramadol (2.5 mg·kg^{-1}) by tail intravenous to relieve the pain caused by the operation.
All these methods can relieve post-operative pain and avoid post-operative infection in rats to different degrees (see the method part for details, line 142-150).

3. It is amusing that the authors refer to the Declaration of Helsinki (line 122) which is clearly a document dealing with clinical (human!) studies.

Reply 3: I am deeply sorry for my ignorance. I have deleted the relevant description from the manuscript (line 124) and carefully studied the contents of the Declaration of Helsinki. I promise that I will try my best to enrich the subject knowledge in the future scientific research and will never make similar mistakes.

4. In line 134, "previously described by. "By whom???

Reply 4: The inappropriate description here has been changed (see line 136-138).

5. What is "NBP", the "positive drug" (line 147)? If this is supposed to be a positive control, could the authors kindly identify what they used? It was given "at 7 d prior to MCAO" (line 148). Just once? or daily?

Reply 5: NBP was used as a positive drug in this study. For drug administration, PNGL (40 mg·kg-1), NBP (20 mg kg−1) or vehicle (0.9 % normal saline) was exposed to continuous intraperitoneal administration (once per day) for 7 d prior to MCAO surgery. The corresponding changes have been presented in the paper (see line 157-165).

6. In line 153, "published previously by. "By whom???

Reply 6: The inappropriate description here has been changed (see line 168).

7. In the Methods, the authors write "means ± SD for ten animals" (line 174). Most figure legends state N=4-6 animals.

Reply 7: Sorry, this is a writing error caused by my carelessness. The inappropriate description here has been deleted (see line 189).

8. Line 200, “PNGL administration for 7 days after MCAO”: did I completely misunderstand the time line? Was the treatment post stroke? Please clarify. Line 233, “rats subjected to MCAO for 24 hours”. Now I am completely confused. I thought it was a 2 hours’ transient ischemia? I don´t think this manuscript can be properly evaluated if nobody understands what was being done.

Reply 8: Once again, I am deeply sorry for the unnecessary errors caused by my carelessness, and I will seriously reflect on myself to ensure that I will not make similar mistakes in future papers.
1) It should be changed to “PNGL administration for 7 days prior to MCAO” (see line 218).
2) It should be changed to “rats subjected to MCAO for 2 hours” (see line 244).

9. The results from the neurological score are relatively disappointing. How was the score calculated?

Reply 9: Neurological performances were performed after ischemic reperfusion by two blinded investigators using a 5-point scale. This method involves five tests assessing spontaneous activity, that is, 0, normal walk; 1, inability to walk straight and mild forelimb weakness; 2, circling toward the paretic side and severe forelimb weakness; 3, fall down to the paretic side; 4, no spontaneous walking with depressed consciousness level; and 5, death.
The scoring data is shown in the following table:

	sham			PNGL
group 1	0		group 1	2
group 2	0		group 2	3
group 3	0		group 3	3
group 4	0		group 4	3
group 5	0		group 5	1
group 6	0		group 6	1
	mcao/r			NBP
group 1	4		group 1	2
group 2	3		group 2	1
group 3	2		group 3	2
group 4	3		group 4	2
group 5	4		group 5	3
group 6	3		group 6	2

10. What I cannot understand is why the authors apparently scanned the whole brain slice instead of comparing ipsilateral (ischemic) and contralateral (healthy) hemispheres. It would have been much more interesting to compare the two hemispheres in the same animals (paired analysis) instead of comparing individual rats that underwent different extents of ischemia.

Reply 10:
Thank you for your suggestion. Your comments are really meaningful.
In fact, our quantitative method is:

Firstly, the ratio of ischemic hemisphere to whole brain tissue was calculated; secondly, the ratio of model group and drugs group to control group was calculated respectively. This quantitative method is helpful to balance individual differences and inter-group differences and obtain more accurate data. In addition, the ischemic hemispheres produce edema after cerebral ischemia, the ischemic hemispheres will undergo slight morphological deformation and there is a great difference between the left and right brain areas. If we compare ischemic hemispheres with non-ischemic hemispheres at this time, it will bring statistical error artificially. Therefore, we compared the ischemic hemisphere with the whole brain tissue.

11. The authors present comparisons between striatum and cortex and indicate that these differences may reflect region-specific differences of local metabolism. I think that metabolic pathways are identical in striatum and cortex, but the extent of ischemia may be different.

Reply 11: Thank you for your new insights. I think your opinion is really reasonable.

According to previous study (doi: 10.1186/s12918-018-0644-0), many biochemical differences were observed among the various brain regions, illustrating the diversity of global metabolism corresponding to specialized regional brain function.

In my study, the functions of cortex and striatum are similar, but not completely consistent. We believe that both regional specificity and the degree of ischemia can be used as the reasons for the difference in the level of small molecule metabolism. If we need to further clarify which reason is dominant, we also need to use other detection methods for further analysis and discussion.

12. Some considerations in the discussion makes sense, e.g. glucose and citrate levels may be increased because of lower glucose consumption and a slowing down of the TCA cycle. On the other hand, it would be expected that ischemic cells use up all the glucose they have (extracellular glucose falls to zero during ischemia). Does MALDI measure glucose phosphates?

Reply 12: In principle, in order to achieve the detection of a substance, it is usually necessary to realize the ionization of this substance. There are two conditions to realize the ionization of a substance. One is that the substance has an ionizable structure, and the other is that there is a suitable matrix to ionize it by electron transfer.

Next, we summarize the selection of substrates:

1) 1,5-dihydroxybenzoic acid (DHB) and α-cyanocinnamic acid (CHCA) have better detection effect on phospholipids and peptides.

2) Erucic acid (SA) has better detection effect for proteins with high molecular weight.

3) 1,5-naphthalene diamine, quercetin and other substrates can be used to detect phospholipid molecules in positive and negative ion mode.

4) 9-aminoacridine (9-AA), 1,5-naphthalene diamine hydrochloride and naphthalene ethylenediamine hydrochloride are suitable for the detection of metal ions, oligosaccharides, acidic metabolites and phospholipids in negative ion mode.

5) For some molecules which are difficult to ionize or have low content, it is necessary to add derivatization reagents to improve the detection sensitivity.

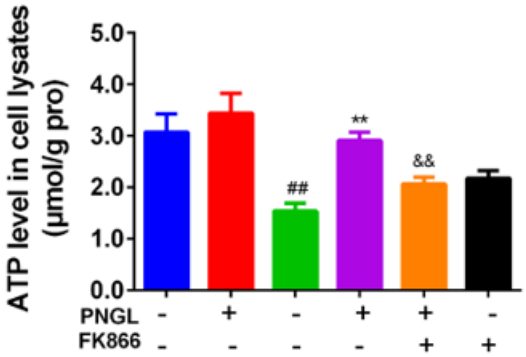
Glucose phosphates have ionizable structure. Therefore, as long as the matching matrix is found to realize the ionization of this substance, glucose phosphate is expected to be detected.

13. ADP and AMP may decrease, and xanthine increase, because of ATP breakdown. Can the authors also measure ATP levels? Oxidative stress may be responsible for the reduction in glutathione and ascorbic acid, but can the authors distinguish between the oxidized and reduced forms of ascorbic acids?

Reply 13:

1) According to previous study (doi:10.1021/ac5034566), ATP level detection can be achieved through MALDI-MSI. However, we did not detect the level of ATP in our study. Fortunately, in our previous study, we detect ATP level at the cellular level by ATP detection kit. The results showed that remarkable decreases in ATP level was observed in the model group (green) compared with control group (blue). However, the level of ATP was significantly increased after

PNGL treatment (purple). In addition, the red histogram represents only PNGL treatment, no modeling damage. In our future research, we will continue to detect the level of ATP in brain tissue in order to more effectively clarify the relationship between ATP level and cerebral ischemia, and further explore the effect of PNGL on ATP changes.



2) Thank you for your question. Your question provides a new idea for our further research. We reviewed the literature related to the detection of ascorbic acid by MALSI-MSI. So far, we have not found any studies on the detection of ascorbic acid oxidation and reduction states by MALDI-MSI. However, as answered in question 12, as long as the substance meets the conditions for ionization, relevant research is just around the corner.

14. The only interesting result for possible translation to humans is shown in Fig. 1A – PNGL improves cerebral blood flow. All the other findings, while interesting, are a direct consequence of the fact that after PNGL, cerebral blood flow was improved. And the improvement of cerebral blood flow leads to the recovery of stroke, accompanied by the improvement of the level of small molecular metabolites.

Reply 14:

I think your opinion is really reasonable.

The main goal of stroke treatment is to remove occlusive blood clots and restore cerebral blood supply in the shortest possible time. *Panax notoginseng* has the effect of promoting blood circulation and removing blood stasis. PNGL, as the total saponins of *Panax notoginseng* stem and leaf, can significantly improve cerebral blood flow. And the improvement of cerebral blood flow leads to the recovery of stroke, accompanied by the improvement of the level of small molecular metabolite.

15. Lines 281-191 and 335-336: Authors should realize that (a.) aspartate and glutamine are NOT neurotransmitters; (b.) glutamine is typically present in astrocytes whereas NAA is mainly a neuronal molecule and (c.) NAA is not an indicator of neuronal damage.

Reply 15:
Thank you for your comments.
We read a lot of relevant literature and found that there were professional deficiencies in our understanding of glutamine and NAA. And we have made changes in the corresponding position of the manuscript (see line 317-329). However, we have different opinions on the understanding of aspartate. After consulting a large number of literatures (DOI: 10.1126/science.6121375; DOI: 10.1111/j.1471-4159.2009.06187.x and so on), we believe that aspartate is an excitatory neurotransmitter in the central nervous system.
Academia becomes wonderful because of its argumentative nature. Thank you for giving us the opportunity to express our opinion. Please forgive me if our words have offended you.

16. In the discussion, the authors fail to mention many previous publications from experimental stroke research which already described metabolic changes similar to the ones described here. Changes of ionic distribution, with sodium entering cells and potassium leaving, were described in detail many years ago. Moreover, the authors do not mention the many previous studies with Ginseng extracts in cerebral ischemia. The authors should do some serious reading of the literature and discuss previous findings on metabolites together with their own data. As the findings that the authors present were largely known, I do not see how these results help to "explore new mechanisms for the treatment of stoke". It would make more sense if the authors discuss the pros and cons of the novel MS imaging method they describe.

Reply 16: Thank you for your valuable advice. We have made changes in different paragraphs of the discussion as requested.

In addition, as you said, these findings are well known and do not serve as a new mechanism for exploring stroke. After our discussion, we revised this to "explore new mechanisms for the PNGL treatment of ischemic/reperfusion injury by ischemic stroke."

On the one hand, the reason is that there are few studies on PNGL in neuropharmacology, especially in ischemic stroke. Previous studies have shown that PNGL has a potent anti-ischemic stroke effect, the mechanism is associated with inhibiting inflammatory response. In view of the multi-site and multi-target characteristics of traditional Chinese medicine, we will continue to study the new mechanism of PNGL against cerebral ischemia and provide a more comprehensive and in-depth study on the mechanism of PNGL in the treatment of ischemic stroke.

On the other hand, this study is not just about studying the mechanism of cerebral ischemic disease and exploring new technologies for detecting small molecule metabolism. We performed PNGL treatment on the ischemic model, the main purpose of which is to detect the effect of PNGL on small molecule metabolism after cerebral ischemia.

17. Accordingly, while many references about earlier work are missing, many others do not relate to the content of the article, e.g. Refs. 9 (clozapine?), 16, 25 (kidney?), 26 (oxiracetam?), 28 (melatonin?) and so on.

Reply 17: Thank you for your carefulness and patience. We have corrected the references you pointed out (Refs. 9, 16, 25, 26 and 28). The modified references are marked with a green background. In addition, we have added several new references and marked them with a yellow background (Refs., 21 and 29).

18. Line 238: Typo, “infract area”.

Reply 18: Thank you for your suggestions. We have been revised as "infarct area" (see line 249).

19. Figure Legends: Fig. 2, what is (F)? Fig. 3, what is (G)? Fig. 4, what is (F) and so on.

Reply 19: The answer to this question shown in the original manuscript (see line 590, 596 and 602-603).