Author’s response to reviews

Title: Electroacupuncture at different frequencies (5Hz and 25Hz) ameliorates cerebral ischemia-reperfusion injury in rats by activating p38 MAPK-mediated anti-apoptotic signaling pathways

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Dear Dr. Rowles:

Thank you for your dealing with the manuscript entitled “Electroacupuncture at different frequencies (5Hz and 25Hz) ameliorates cerebral ischemia-reperfusion injury in rats by activating p38 MAPK-mediated anti-apoptotic signaling pathways”, coded as MS: 1767418987155096. The manuscript has been revised and compiled as the reviewers’ suggestions. Our point-by-point reply to the reviewers’ comments is described as follows:

Reviewer: Jakob Troppmair

Previous studies have demonstrated that the cerebral infarct volume increases dramatically between 6 and 24 h following the start of reperfusion in a severe model (90-120 min of ischemia) of transient cerebral ischemia in rats, and that the period of infarct expansion is closely related to the severity of neutrophil infiltration [1, 2]. However, other studies have reported that mild (15-30 min) transient middle cerebral artery occlusion (MCAo) causes gross infarction 1 d after reperfusion, and then aggravates delayed infarct expansion in the entire MCA region in the subacute phase (3-7 d) after reperfusion [3, 4]. In addition, apoptosis has been suggested to play a major role in the development of delayed infarct progression in the penumbra area following mild transient focal cerebral ischemia [3].

Actually, we have conducted surveys of neuroprotection against cerebral ischemia-reperfusion (I/R) injury for a long time. In our previous studies, the extraluminal and intraluminal sutures MCAo models were used and the duration of cerebral ischemia was 90 min [5-8]. In the survey of the effect of electroacupuncture (EA) in cerebral I/R injured rats, we conducted a pilot study prior to the main study and found that EA at acupoints fails to exert neuroprotection against severe cerebral I/R injury (such as 90 min of ischemia). In addition, we found that mild transient MCAo (15 min of ischemia) consistently
caused gross infarction 1 d after reperfusion, and then aggravated delayed infarct expansion 7 d after reperfusion. The percentage cerebral infarct area showed an increasing tendency after 1 (14%), 3 (23%) and 7 d (25%) of reperfusion. The results indicating delayed infarct expansion are in agreement with those of previous studies [3, 4]. Intriguingly, we also found that EA at Baihui (GV20) and Dazhui (GV14) acupoints provides neuroprotective effects against this delayed infarct expansion through various signaling pathways in the ischemic cortical penumbra in the subacute phase (3 and 7 d) after mild (15 min) transient MCAo. These results were presented in articles and have been accepted for publication in peer-reviewed journals [9, 10].

1. In general the value of signaling analysis restricted to day 7 has to be questioned, as most pronounced changes in signaling pathway activity usually are observed at during ischemia and the early reperfusion in other settings of ischemia/reperfusion. Functional impairment is already maximal at day 1 arguing that e.g. underlying cell death causing it already has happened. Also lysates form infarcted and non-infarcted areas may be compared.

Ans.: As mentioned above, in mild (30 min) transient MCAo, infarct progression initiates 1 d after reperfusion and continues for up to 7 d. In the present study, we also found that the percentage cerebral infarct area has a tendency to further increase from 1 to 7 d after reperfusion, whereas EA stimulation at the Baihui (GV20) and Fengfu (GV16) acupoints (EA at acupoints) provides beneficial effects on infarct progression after 30 min of cerebral ischemia followed by 7 d of reperfusion. Therefore, it is reasonable to examine the anti-apoptotic effects and mechanisms of EA at acupoints in the ischemic cortical penumbra 7 d after reperfusion. In addition, functional outcomes were measured using a neurological deficit scale as described previously [11], and we found that the rats had moderate neurological impairment after 30 min of MCAo followed by 1 d of reperfusion and lasted up to 7 d in the ischemic model group. Some of them even got worse on neurological examination at 3-7 d after reperfusion.

2. Figure 1/2A: Since EA treatment proceeds the damage, the question arrives whether EA pretreatment results in smaller infected areas or whether it is somehow linked to better recovery as analysis is only done on day 7.

Ans.: In our laboratory, we conduct the intraluminal suture MCAo model for a long time. During surgery, we carefully checked every step of the process and monitored MCA blood flow using Laser-Doppler flowmetry to confirm the success of the cerebral I/R procedure. Therefore, in the present study, the severity of the initial ischemic insult is similar in experimental groups. In the beginning of the current study, the cerebral infarct area (30 min of ischemia) was measured at 1 and 7 d after reperfusion. The percentage cerebral infarct area also showed an increasing tendency after 1 (about 14%) and 7 d (24%) of reperfusion. The results indicating delayed infarct expansion are consistent with those of our previous study [10] and we have described these findings in the Discussion section from lines 14 p.18 to lines 17 p.18. Based on these results, we propose that a valid comparison between the model and EA at acupoints (EA-5Hz or EA-25Hz group) groups can be conducted at 7 d after reperfusion and the
reduction in infarct size can be attributed to the anti-apoptotic effects of EA at acupoints, but not self-recovery, at 7 d after reperfusion.

In addition, in the Grouping section, we have described the EA at acupoints (EA-5Hz group) method designed in our current study from line 1 p.8 to lines 5 p.8 and the description is “The rats in the EA-5Hz group were subjected to MCAo and simultaneously received EA at acupoints at a frequency of 5 Hz (EA-5Hz). The rats were received the first EA at acupoints for 25 min during MCAo. They were subjected to 30 min of ischemia followed by reperfusion. After 1 d of reperfusion, the rats received EA-5Hz (25 min) once daily for 6 (total 7) consecutive days, and were sacrificed 7 d after reperfusion”. Therefore, the EA method designed in our present study is posttreatment, but not pretreatment.

3. (1) Figure 3: The increase in p38 is striking (A), but the blot shown does not seem to be truly representative if compared to (D). To gain insights into the activity of signaling pathways the important parameter is the ratio of pMAPK/MAPK, pMAPK/actin ratios do not provide any relevant information. Thus to arrive at conclusions that p38 signaling is activated in these samples p-p38/p38 ratios have to be calculated.

(2) Also in the analysis of intracellular (signaling) proteins a possible effect of EA in the SHAM setting has to be considered.

Ans.: (1) In Figure 3, we have recomposed the bar graphs to show the ratios of pMAPK/MAPK according to the reviewer’s comments.

(2) The purpose of the present study is to evaluate the effects of EA at acupoints, at a frequency of 5Hz or 25Hz, after 30 min of cerebral ischemia followed by 7 d of reperfusion, and to evaluate the involvement of MAPK cascades in the ischemic cortical penumbra. The rats were randomly divided into Sham, Model, EA-5Hz, Non-acup-5Hz, EA-25Hz, and Non-acup-25Hz groups. Comparison between the sham and model groups, and we could clarify the differences between non-ischemia and I/R injury. Comparison among the model, EA at acupoints and EA at non-acupoint groups, then we could evaluate the effects and mechanisms of EA stimulation at real acupoints in the ischemic cortical penumbra at 7 d after reperfusion. Therefore, the research designs exactly meet the needs of the present study.

4. Figure 4: In the case of pCREB the ratio pCREB/CREB would be informative. A rationale should be provided why these proteins were analyzed.

Ans.: (1) We performed quantitative immunoblot of total CREB according to the reviewer’s suggestion. The representative western blot image of CREB was shown in Figure 4(A). We have recomposed the bar graphs to show the ratio of p-CREB/CREB in Figure 4(D).

(2) The reasons why p-CREB and CREB were evaluated have been described in detail in the Background section from lines 18 p.3 to lines 20 p.3 and the Discussion section from lines 16 p.19 to lines 4 p.20.

5. The data on the Bcl-2 family proteins suggest effects of the procedure on the Bcl-2 protein rheostat. These data also suggest that an intrinsic cell death pathway may be activated. However, this is not supported by data shown in
Figure 7 (e.g. Cytochrome c release). Thus also remains elusive how activation of caspase 3 may have been achieved.

Ans.: Evidence has suggested that Bax activation during apoptosis involves translocation to the mitochondria and insertion into the mitochondrial outer membrane, and that these processes disrupt mitochondrial membrane integrity and consequent release of apoptogenic proteins, including cytochrome c, Smac/DIABLO, and AIF [12-14]. The release of cytochrome c facilitates the formation of the apoptosome and then initiates cytochrome c-mediated apoptosis. The release of Smac/DIABLO binds to XIAP and prevents XIAP-mediated caspase suppression, and then induces caspase-3-mediated apoptosis [15]. Translocation of AIF from mitochondria to the nucleus initiates apoptosis through a caspase-independent apoptotic pathway [16].

As shown in Figure 7 and 8, we found that EA-5Hz and EA-25Hz effectively reduced the extent of cytosolic and mitochondrial Smac/DIABLO and cytosolic cleaved caspase-3 upregulation, and simultaneously restored cytosolic XIAP expression in the cortical penumbra 7 d after reperfusion. Nonsignificant differences in the cytosolic and mitochondrial cytochrome c and AIF among the model and treatment groups indicated that EA-5Hz and EA-25Hz exert nonsignificant effects on cytochrome c- or AIF-mediated apoptosis. Our results thus strongly suggest that EA-5Hz and EA-25Hz are neuroprotective against Bax-mediated apoptosis by inhibiting the translocation of Smac/DIABLO from mitochondria to the cytosol, thereby restoring XIAP-mediated suppression of caspase-3 activity, but not inhibiting cytochrome c or AIF release, in the ischemic cortical penumbra 7 d after reperfusion. Therefore, data from Figure 7 and 8 support our conclusion.

6. Figure 9: also changes in total CREB have to be considered.

Ans.: CREB is a selective nuclear transcription factor. CREB phosphorylation (p-CREB) triggers the translocation from the cytosol to the nucleus, and then regulates the expression of genes (such as Bcl-2 and Bcl-xL) for cell survival [17]. Our immunoblot analysis results indicate that cytosolic p-CREB was effectively restored by EA-5Hz and EA-25Hz 7 d after reperfusion (Figure 4). We performed p-CREB/DAPI (nuclear staining) costaining and further examined p-CREB immunoreactivity within the nuclear compartment in the cortical penumbra. Results from p-CREB/DAPI costaining (Figure 9) indicated intense p-CREB immunoreactivity within cytosolic and nuclear compartments in the cortical penumbra. These results are consistent with those of a previous study, which indicated that cytosolic p-CREB expression is positively associated with nuclear p-CREB expression and reflects the activation and nuclear translocation of CREB following hypoxic preconditioning [18]. Therefore, we propose that EA-5Hz and EA-25 Hz both provide neuroprotection against cerebral I/R injury through the activation of CREB signaling in the ischemic cortical penumbra 7 d after reperfusion. The design of the immunofluorescent costaining (p-CREB/DAPI) (Figure 9) meets the needs of the present study.

References


Reviewer: Toru none Kawada

1a. What is the definition of penumbra? Was the averaged penumbra region defined based on the ischemic injury in the Model group and was the anatomically same area selected from other groups to perform the cellular analysis? Or otherwise did the authors define the penumbra in each animal? In the latter case, the selection of penumbra can be somewhat arbitrary and may need some criteria such as the distance from the ischemic core region.

Ans.: (1) The definition of penumbra is that the ischemic cerebral tissue potentially destines for infarction, but not yet irreversibly damaged. The tissue is still viable and is the target of therapies [1].

(2) For quantitate analysis of immunoblot, the rats were sacrificed 7 d after reperfusion, and their brains were removed and then coronally sectioned from -4.3 to +1.7 mm bregma. The right ischemic cortex was carefully separated into its penumbra (frontoparietal cortex chosen between 3 mm and 8 mm to the ischemic core) and ischemic core fractions (Figure 1). We have recomposed the Western Blot Analysis section of the present study from lines 18 p.9 to lines 20 p.9. The revised description is “The right ischemic cortex was carefully separated into its penumbra (frontoparietal cortex chosen between 3 mm and 8 mm to the ischemic core) and ischemic core fractions”.

Figure 1: A representative coronal brain section shows the penumbra and ischemic core fractions.

1b. Page 6, lines 14-16. After 30 min of MCAo, the suture was carefully removed to restore blood flow, and the right distal MCA was exposed through a cranial burr hole. Was the burr hole made after MCAo? Please describe the procedure along the time line.

Ans.: We have recomposed the Methods section of the present study from lines
13 p.6 to lines 16 p.6 and lines 19 p.6 to lines 2 p.7 according to the reviewer’s suggestion. The revised descriptions are “Briefly, the rats were anesthetized with a 5% isoflurane-oxygen mixture, maintained on a 2% isoflurane-oxygen mixture, and the right distal middle cerebral artery (MCA) was exposed through a cranial burr hole (2.5 mm lateral and 2.0 mm posterior to the bregma). After dissection, the right common carotid artery (CCA) and internal carotid artery (ICA) were exposed,” and “Blood flow in the MCA was monitored by Laser-Doppler flowmetry (DRT4, Moor Instruments Inc, Wilmington, USA) through the cranial burr hole in the preischemia (>500 units), ischemia (<100 units), and reperfusion (>300 units) periods.”

1c. Page 7. The authors applied electroacupuncture to the Baihui and Fengfu acupoints. Does this mean that the current was passed between the Baihui and Fengfu acupoints? Can the polarity of stimulation affect the results? The electrodes consisted of 0.5-mm stainless steel wires. Does 0.5 mm indicate the diameter of the wire? Were the animals tethered to the stimulator during electroacupuncture? How was it possible for animals be freely moving (Page 8, line 15)? We do not usually use the words “freely moving” if the animals are tethered.

Ans.: (1) Yes, the current was passed between the Baihui and Fengfu acupoints.
(2) Actually, at the beginning of the present study, we have taken into account the effects of EA at acupoints with different polarity. Therefore, EA at acupoints was performed with the same polarity throughout the study, and the anode was connected to the Fengfu acupoint and the cathode was connected to the Baihui acupoint (Figure 2).

Figure 2: A representative image shows that the rat was received EA at acupoints in the cage. The red electrode indicated anode and was connected to the Fengfu acupoint; the black electrode indicated cathode and was connected to the Baihui acupoint.

(3) Yes, the diameter of the wire is 0.5 mm. The electrodes consisted of two stainless steel wires with 0.5 mm diameter (Figure 3). We have recomposed the Electrode Implantation section from lines 5 p.7 to lines 7 p.7. The revised description is “The electrodes consisted of two stainless steel wires (diameter 0.5 mm) used for acupoint (or nonacupoint) stimulation.”

Figure 3: A representative image shows the electrode used in the present study.

(4) Yes, the rats were tethered to the stimulator during electroacupuncture (Figure 2). We have deleted the worlds “and freely moving” in lines 20 p.8 and the revised description is “the rats were fully awake in the cages.”

1d. The rats in the EA-5Hz group were subjected to MCAo and simultaneously received EA at acupoints at a frequency of 5 Hz (EA-5Hz) for 25 min. They were then subjected to 30 min of ischemia followed by reperfusion. "simultaneously" can mean that EA was performed during MCAo. "then" can mean that the ischemia and reperfusion was performed after EA. Was EA performed on the same day of MCAo? In that case, what was the time difference between MCAo
and the first EA? The descriptions are difficult to understand and may need to be improved with the time line in mind.

Ans.: In the present study, the rats were subjected to MCAo and simultaneously received the first EA at acupoint. The first EA was performed during MCAo. We have recomposed the Grouping section from line 1 p.8 to lines 5 p.8 and the revised description is “The rats in the EA-5Hz group were subjected to MCAo and simultaneously received EA at acupoints at a frequency of 5 Hz (EA-5Hz). The rats were received the first EA at acupoints for 25 min during MCAo. They were subjected to 30 min of ischemia followed by reperfusion. After 1 d of reperfusion, the rats received EA-5Hz (25 min) once daily for 6 (total 7) consecutive days”.

2. It seems that the neurological deficit scores decreased with time in the EA group. Did the 7-day treatment provide the maximum improvement? In other words, is it possible to further reduce the neurological deficit scores by continuing the EA treatment? Some discussion may be added.

Ans.: During the pilot study evaluations, the rats with cerebral I/R injury received EA treatment once daily for 10 consecutive days, and the results revealed that the effects of 7-day treatment on neurological deficit scores were similar with those of 10-day treatment. Therefore, EA treatment was scheduled for 7 d in the present study. We couldn’t be sure whether the 7-day treatment provides the maximum improvement; however, it is our goal to elucidate the long-term (such as 1 month) therapeutic effects of EA at acupoints after mild transient MCAo (30 min) in rats in the next study.

Discretionary revisions
1. Page 5, line 13. The rationale for the use of 5 Hz and 25 Hz stimulation may need to be discussed because in the background section the cited papers used lower frequencies (2/15 Hz).

Ans.: In the Background section, we have cited the paper which used EA at higher frequencies (30/50 Hz) after transient global cerebral ischemia in rats. We have added the description from lines 11 p.5 to lines 14 p.5, and the description is “Tian et al. (2013) demonstrated that EA stimulation at the Baihui, Mingmen (GV4), and Zusanli (S36) acupoints (30/50Hz) provided neuroprotection against brain edema through the activation of Na+, K+-ATPase in rats after transient global cerebral ischemia”.

2. Page 12, line 4. All variables were approximately normally distributed ... Is it possible to talk about distribution with only 5 data points?

Ans.: The data obtained from the study have been fully discussed with the professional statistician in our department and confirmed that all variables were approximately normally distributed. Therefore, we used one-way ANOVA followed by the Scheffe’s test which is one of the most conservative post hoc tests.

3. Figures seem to be small even when I enlarged them on the screen. Labels are illegible. Could the resolution be improved?
Ans.: In the present study, the figure files have been produced at high resolution to meet submission requirements.

4. Possible minor corrections are as follows.
Page 11. RT (room temperature?) needs to be defined. Page 20, line 2. expression was marked[ly] downregulated. Page 21, line 19. where it interacts with
Ans.: We have corrected these errors in the article.

5. Figure 1 legends.
Sham, Sham group; Model, Model group; EA-5Hz, EA-5Hz group; Non-acup-5Hz, Non-acup-5Hz group; EA-25 Hz, EA-25 Hz group; Non-acup-25 Hz, Non-acup-25 Hz group. These descriptions add nothing and can be omitted.
Ans.: We have deleted these descriptions in the Figure 1 legend.

References

The authors carefully examined this manuscript and corrected the errors in words, sentences, units and symbols in the manuscript according to the reviewer’s commend.

Please handle our manuscript at your convenience. Thank you for your kindly help.

Sincerely yours,

Chin-Yi Cheng