Author's response to reviews

Title: Electroacupuncture-like stimulation at Baihui and Dazhui acupoints exerts neuroprotective effects through activation of the brain-derived neurotrophic factor-mediated MEK1/2/ERK1/2/p90RSK/Bad signaling pathway in mild transient focal cerebral ischemia in rats

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Author's response to reviews: see over
Dear Mr. Prozenko:

Thank you for your dealing with the manuscript entitled “Electroacupuncture-like stimulation at Baihui and Dazhui acupoints exerts neuroprotective effects through activation of the brain-derived neurotrophic factor-mediated MEK1/2/ERK1/2/p90RSK/Bad signaling pathway in mild transient focal cerebral ischemia in rats”, coded as MS: 1195288503102738.

The manuscript has been revised to comply with the reviews’ suggestions. Our point-by-point reply to the reviewers’ comments is described as follows:

Reviewer: Stephanie Tjen-A-Looi

1. The observations are interesting finding on the biochemical changes induced with EA-like stimulation. How do the changes interact with reperfusion associated reactive oxygen species should be discussed.

Ans.: The purpose of our present study was to evaluate the effects of EA-like stimulation at Baihui and Dazhui acupoints (EA at acupoints) after mild ischemia (15 min) followed by 3 d of reperfusion, and to elucidate the mechanisms associated with BDNF-mediated neuronal survival. It’s well known that reactive oxygen species (ROS) play a key role in various mechanisms of cerebral ischemia-reperfusion (I/R) injury. Previous studies have demonstrated that enzymatic reactions using oxygen in the mitochondria generate large amounts of ROS, which cause oxidative stress and cytochrome c-mediated apoptosis, eventually leading to infarct expansion during cerebral I/R period [1,2], whereas EA exerts neuroprotective effects against cerebral I/R
injuries through suppression of oxidative stress [3,4]. In the present study, EA at acupoints provides neuroprotective effects through activation of the BDNF-mediated MEK1/2/ERK1/2/p90RSK/Bad signaling pathway in mild transient focal cerebral ischemia. Based on these findings, we reasonably deduce that EA at acupoints exerts beneficial effects against infarct expansion through modulation of cytochrome c-mediated caspase 3 activation pathways. Therefore, it is our goal to elucidate the exact mechanism of action of EA at acupoints involved in the modulation of ROS during reperfusion in the next study.

References:

Reviewer: Zhi-Ling Guo

1. The rationale for selection of both Baihui (GV20) and Dazhui (GV14) acupoints was not clearly elucidated. Did application of electroacupuncture (EA) at either of these acupoints alone have any effect on cerebral ischemia-reperfusion injury?

Ans.: (1) We have recomposed the introduction section from lines 14 p.4 to lines 21 p.4 according to the reviewer’s suggestion. The revised description is “According to traditional Chinese medicine, Baihui (GV20) and Dazhui (GV14) are both acupoints on the “Du meridian”, which travels into the brain, and are commonly used to treat stroke. Experimental studies in rats have shown that EA stimulation at acupoints (such as Baihui and Shuigou acupoints) can attenuate cerebral infarction and improve neurological outcome after transient middle cerebral artery occlusion (MCAo) [20,21]. Kim et al. have reported that pretreatment with EA at Baihui and Dazhui acupoints elicit neuroprotection through increased BDNF and stromal cell derived factor-1α (SDF-1α) expression 1 day after MCAo [22].”

(2) Previous studies have demonstrated that a single EA stimulation at Baihui acupoint can reduce cerebral edema and infarct size during the acute phase [1,2] and exert protective effects against caspase-3-dependent neuronal apoptosis during the subacute phase [3], of cerebral ischemia. In the present study, the electrode, which consisted of two stainless steel wires, was implanted in Baihui and Dazhui acupoints, and then connected to the electrical stimulator (Trio 300, ITO Co., Germany) for EA stimulation. Therefore, in this study, the neuroprotective effects were elicited by EA at Baihui and Dazhui acupoints, not Baihui or Dazhui acupoint alone.
References:


   neuroprotection may be mediated by glutamate transporter type 2. Neurochem Int 63: 302-308.

3. Zhou HP, Wang MS, Shi F, Ma SL, Li HO, et al. [Effects of acupuncture pre-conditioning on 
   apoptosis in hippocampal neurons following ischemia-reperfusion injury in aged rats]. Zhonghua 
   Yi Xue Za Zhi 91: 1203-1206.

2. In Figure 5, the images showing co-labeling of nuclei (NeuN) and caspase-3 were quite poor. 
   Neurons containing a single- or double-labels were not shown clearly. Thus, these staining 
   results could not strongly support the statement derived from this experiment.

Ans. In this study, we carefully performed the immunohistochemical costaining step-by-step 
   procedures described on p.10. As shown in Figure 5 (B), numerous NeuN-positive cells (blue) 
   were present in the sham and EA groups (the expression of active caspase-3 was absent or weak), 
   whereas the NeuN/active caspase-3 double-labeled cells (purple) were scattered in the ischemic 
   cortex in the model, non-acup, and U0126+EA groups. One of the NeuN/active caspase-3 
   double-labeled cells was present at higher magnification and it clearly showed colocalization of 
   NeuN (blue) and active caspase-3 (red) proteins. In our laboratory, the immunohistochemical 
   costaining method has been used in studies of colocalization for a long time. Our previous
immunohistochemical costaining results have been accepted for publication in peer-reviewed journals [1,2].

References:


3. In Figures 3 and 4, there were positive staining appeared on representative panels of images obtained from “negative control stain”. What were they? In addition, for comparisons, the size of images showing “negative control stain” should be the same to others produced from routine staining.

Ans. (1) Negative control stain was prepared using non-immune serum in place of the primary antibody and it was described in the Immunohistochemical (IHC) analysis section from lines 4 to lines 6 on p.10. The purpose of the negative control stain is to exclude the possibility that some property of the brain tissue causes non-specific staining with the detection reagent applied and then it can prevent the false positive results. In the present study, negative controls
are necessary to evaluate non-specific background staining, and we can perform rigorous quantitative immunohistochemical analysis of BDNF, pRaf-1, pMEK1/2, pERK1/2, and pp90RSK proteins.

(2) In Figures 3 and 4, negative controls for staining were prepared using adjacent serial sections from the EA group incubated without primary antibodies and partly embedded in the brain section images of the EA group, and these made it simple and easy for readers to understand the difference between negative control staining and routine staining.

4. In Table 1, compared to sham control, were there any significant changes in other groups in addition to the model group? It needed to be indicated.

Ans.: We have corrected the analytic presentation of Table 1 on p.32 according the reviewer’s suggestion.

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