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 reviewers’ suggestions. Our point-by-point reply to the reviewer’s comments is described as follows:

**Reviewer: Zhi-Ling Guo**

1. 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.** We carefully re-performed the immunohistochemical co-staining for NeuN and active caspase-3 according to the reviewer’s suggestion. Representative images of NeuN-active caspase-3 expression in the experimental groups were shown in Figure 5. As shown in Figure 5 (B), numerous NeuN-positive cells (blue) were present in the sham group. In addition, NeuN-positive cells were highly expressed in the ischemic cortex in the EA group (the expression of active caspase-3 (red) was relatively weak), whereas NeuN-active caspase-3 double-labeled cells (purple) were predominant in the model, non-acup, and U0126+EA groups (the expression of NeuN was relatively weak). One of the NeuN-active caspase-3 double-labeled
cells was present at higher magnification and it clearly showed colocalization of NeuN and active caspase-3 proteins. Thus, our NeuN-active caspase-3 co-staining results could support the statement from lines 17 p.18 to line 1 p.19, and the statement is “In our study, double staining for active caspase-3 and NeuN revealed that active caspase-3-labeling colocalized with relatively weak NeuN labeling, and markedly increased in the ischemic cortex after 3 d of reperfusion, consistent with changes in apoptosis. However, EA at acupoints markedly suppressed any increases in active caspase-3-labeling. In contrast, EA at acupoints effectively restored NeuN labeling through antigen retrieval”.

2. 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 Figure 3, 4 and 5, the representative negative control images of BDNF, pRaf-1, pMEK1/2, pERK1/2, and pp90RSK were shown the same size as those obtained by routine staining.

3. 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.33 and added the descriptions from lines 15 to lines 18 p.14 and line 1 to lines 2 p.16. The descriptions are “After 3 d of reperfusion, we observed a greater number of BDNF-, pRaf-1-, pMEK1/2-, pERK1/2-, and pp90RSK-positive cells in the ischemic cortex in the model, EA, non-acup, and U0126+EA groups compared to the sham group (all $P < 0.05$; Figures 3B, 4A, 4B, 4C and 5A; Table 1)” and “We observed increased TUNEL positivity in the ischemic cortex in the model, EA, non-acup, and U0126+EA group ($P < 0.05$ vs. sham group; Figure 5C; Table 1) after 3 d of reperfusion”.

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

Sincerely yours,

Chin-Yi Cheng