Author's response to reviews

Title: Danggui-Jakyak-San ameliorates memory impairment and increase neurogenesis induced by transient forebrain ischemia in mice

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Author's response to reviews: see over
Dear Editor,

We are pleased to submit a revised version of our manuscript titled “Danggui-Jakyak-San ameliorates memory impairment and increase neurogenesis induced by transient forebrain ischemia in mice”. We investigated the mechanisms the effect of Danggui-Jakyak-San on ischemic restoration. We thank the reviewers for their constructive and helpful suggestions and we have responded to each of their comments as follows:

Reviewer: Yukiya Suzuki

1. In Figure 1, the authors started this study with the results that DJS increased learning and memory under physiological condition. Please explain why the authors chose this design.

Re: We wanted to know the physiological effect of DJS on memory and neurogenesis, and set the proper range of dose for further experiment. In this experiment, the most effective dose of DJS was 100 mg/kg and we chose this for further experiments. We specified the animals and described this issue in the Background section in more detail (Page 5, line 21 to Page 6, line 1).

2. In the figure legends section, be specific about the number of animals in each experiment and which tests were used to assess which experiments.

Re: According to reviewer’s suggestion, we clarify the number of animals used and explained the purpose of each experiment in the figure legends (Page 23, lines 3-4, lines 6-7, line 8, line 9, line 11, lines 15-17, line 20, lines 21-23; page 24, lines 2-3, lines 7-8, line 12, lines 13-14, lines 16-17, line 20, line 21).

3. In Figure 1A, the authors showed the significant decrease of the effects of DJS at 400 mg/kg. In this context, the authors should write possible limitations of this study in the discussion.

Re: In vivo condition, bell-shaped dose-response curve is often observed [1, 2]. It is maybe due to the interactions of several molecules or due to the activation of feedback loop. In this study, prior interpretation might be correct since DJS contains various compounds. Therefore, attention should be paid for choosing optimal dose for treatment. This issue and limitation of this study were included in the revised manuscript (Page 17, lines 6-8).

4. In the methods section (surgery and drug administration), the authors should describe the vehicle administration in detail.

Re: DJS, which was dissolved in 10% Tween 80 solution, was administered from 7 days to 35 days after BCCAO (100 mg/kg, p.o., once daily) in the BCCAO + DJS group. BCCAO + vehicle group was administered with the same schedule with vehicle (10 % Tween 80 solution) instead of DJS. According to the reviewer’s
suggestion, we clarified the method of vehicle administration in detail in the ‘Methods’ section (Page 9, lines 5-9).


1. In Abstract-Methods section; the experimental groups and statistical methods should be added.

Re: Transient forebrain ischemia was induced by bilateral common carotid artery occlusion (BCCAO). Animals were divided into three groups (sham, BCCAO + vehicle, and BCCAO + DJS). To test the effect of DJS on learning and memory, Morris water maze or passive avoidance test was conducted. To test neuroprotective and neurogenic effect, immunohistochemistry and Western blot analysis were used. Statistical significance was analyzed with Student t-test, one-way or two-way analysis of variance test. According to the reviewer’s suggestion, we added the experimental groups and statistical methods in ‘Abstract-Methods’ section (Page 3, lines 9-14).

2. In Methods-Animal section; the experimental groups and animal numbers of each group should be added. What is performed to BCCA + vehicle group as vehicle?

Re: According to the reviewer’s opinion, we mentioned the experimental groups and animal numbers of each group (Page 6, lines 13-15; page 10, line 13; page 11, line 19). Moreover, we added method for vehicle treatment in ‘Methods-Surgery and drug administration’ section (Page 9, lines 7-9).

3. In Methods-Materials section; Catalog numbers of antibodies should be added.

Re: According to reviewer’s mention, we included catalog number of all antibodies (Page 7, line 2-10).

4. In Methods- BrdU treatment and tissue preparation section; mice in ischemia group are given DJS for 28 days, why normal naive mice are given for 14 days

Re: While DJS showed its memory enhancing effect at 14-day treatment in normal naive mice, it showed its effect at 28-day but not at 14-day treatment in ischemic mice. The reasons of the differences are unclear, but we speculate that the effective compounds, which are contained in DJS, are different in depends on conditions. This issue was included in the revised manuscript (Page 16, Lines 9-13).

5. In Methods- Immunohistochemistry section; how the hippocampus sections were prepared? Is any brain atlas used, if used please give the reference. For Immunohistochemistry, how brains were used; whole or half? If whole brains were used, which brains were used for western blot? Please give the distribution of mice for Immunohistochemistry and western blot analysis.

Re: We used brain atlas [1] for hippocampal sectioning, and mentioned this in Method section (Page 10, line 17). In biochemical experiments, six and four mice in each group were used for immunohistochemistry and Western blot analysis, respectively. And whole brains were used for each experiment. We added the
distribution of mice for immunohistochemistry and Western blot in ‘Methods’ section
(Page 10, line 13; page 11, line 19). Although the group size was big, we endeavored
to minimize the animal number. Our experimental protocols were approved by
IACUC.

6. In Methods-Quantification and statistical analysis section; it is unclear how
the numbers of the dentate gyrus cells were counted? Was counting frame used?

Re: Because the cell distribution was not even, we counted all of immune-positive
cells in each slice without using counting frame as described in the ‘Quantification
and statistical analysis’ section.

7. In Methods-Quantification and statistical analysis section; while the study
focused on dentate gyrus why was the damage scoring performed for CA1
region? Damage scoring should be done for dentate gyrus. In Nissl staining, what
are the criteria for damage scoring? Such as pyknotic nuclei?

Re: In this model, delayed CA1 neuronal loss, which is closely related with memory
impairment, is well established in BCCAO brain [2-4] However, dentate granule cells
are reported to have almost no effect following transient ischemia [5,6], and no
damaged cells were observed in the dentate gyrus in the present study. Therefore, we
did not include the damage scoring in the dentate gyrus region. In this study, although
DJS has facilitatory effect on neurogenesis, we wanted to test if the effect of DJS on
BCCAO-induced memory impairment is related with protecting effect on neuronal
loss in this region. But it was not the case. We mentioned this in ‘Result’ section
(Page 14, lines 9-13). For scoring damage range, we counted pyknotic cells as dead
cells in Nissl staining section. And this was semi-quantitatively scored from 0 to 3 (0,
normal; 1, < 30% of the neurons were irreversibly damaged; 2, 30–60% of the
neurons were irreversibly damaged; 3, 60-100% of the neurons were irreversibly
damaged), as described elsewhere [7].

8. In Figure 1B; the arrows should be added for BrdU-positive cells

Re: According to reviewer’s suggestion, we added arrows to mark BrdU-positive
cells.

9. In Figure 2B; Days that have significance should be marked on the figure.

Re: According to reviewer’s suggestion, we added significance mark on Figure 2B.

10. In Figure 3A; Immunopositive cells cannot be observed clearly because of
background staining not made. Arrows should mark Immunopositive cells in
higher magnificance. Nissl micrographs should be replaced with dentate gyrus
and arrows should be added for damaged cells.

Re: According to reviewer’s suggestion, we included subsets with higher magnificent
one and marked immune-positive or damaged cells with arrows. Because dentate
granule cells are reported to have almost no effect following transient ischemia [5,6],
we placed the Nissl micrographs of CA1 region rather than those of DG to show
effects of DJS more clearly.
11. Discussion section has focused on neurogenesis; discussion about DJS should be added.

Re: DJS has shown neuroprotective effect in various in vivo and in vitro conditions [8-14]. However, functional mechanisms of the effect of DJS on various neurological deficits are still not clarified. Previous reports indicated that DJS regulates neurotransmitters level including acetylcholine and monoamines, and reduces oxidative damage in various brain disease model [10, 13, 15]. In this study, we found that delayed long-term treatment of DJS improves ischemic damage-induced spatial memory impairment. In the present study, DJS significantly increased transient forebrain ischemia-induced hippocampal neurogenesis and attenuated memory impairment. Moreover, because delayed treatment with DJS failed to rescue ischemia-induced neuronal cell death, we suggest that DJS-induced increase of neurogenesis may be a possible mechanism for functional improvement. However, it was found that DJS did not affect neuronal differentiation. Further research should be required to clarify this issue. These discussions were included in ‘Discussion’ section (Page 16, line 1-9).


