Title: Kihi-to, a herbal traditional medicine, improves Abeta(25-35)-induced memory impairment and losses of neurites and synapses

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Author's response to reviews: see over
Dear Editor;
Editorial Office of *BMC Complementary and Alternative Medicine*

Thank you for sending a notice that our paper (MS No. 2142707661194422) entitled “Kihi-to, a herbal traditional medicine, improves Aβ(25-35)-induced memory impairment and loss of neurites and synapses” is acceptable. We considered reviewer’s comments, and added several descriptions. Details revised are shown in next page.

We hope that this revision is sufficient for acceptance.

Yours sincerely,

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Reply comments to Refree 2

Thank you for nice and useful suggestions for improvement of contents. We considered your opinions and revised several points.

1) Kihi-to improved Aβ(25-35)-induced memory impairments and neuronal degeneration by post treatment in vivo. As shown in Fig. 7, Kihi-to also recovered neuritic atrophies by post treatment (4 days after Aβ(25-35) treatment) in vitro experiments. Although Kihi-to was administered simultaneously with Aβ(25-35) in Figs. 8, 9 and 10, these data are not necessary explaining just protective effects of Kihi-to. Aβ(25-35)-induced increase in calpain and decrease in calpastatin sustained at least for 4 days (Fig. 9). As added newly in P.23, L.5 – 7, the increase in calpain and decrease in calpastatin were detected in Aβ(25-35)-injected mice brain, and those were attenuated by Kihi-to treatment (data not shown). In addition, we confirmed that Kihi-to also inhibited calpain enzymatic activity in direct (data not shown). Therefore, it would be possible that Kihi-to might repress the long-lasting expressional changes (and activity changes) of calpain and calpastatin even by post treatment.

Kelly and Ferreira reported that Aβ(1-40) induced sustained Ca^{2+} influx at least 24 h after stimulation [new reference 40] although we observed the Aβ(25-35)-induced Ca^{2+} influx only for 30 sec. If the long-lasting Ca^{2+} entry related to the sustained increase in the calpain expression, blocking the Ca^{2+} influx by Kihi-to may explain in part why Kihi-to was effective to neuritic and synaptic degenerations even by post-treatment. We added those in Discussions (P.23, L.12 – 16).

2) As you kindly suggested, experiments using Aβ(1-42) seem to be appropriate for Alzheimer’s disease study although Aβ(25-35) can be produced in Alzheimer’s disease patients by enzymatic cleavage of the naturally occurring Aβ(1-40) [reference 11]. Of course, we confirmed in previous works that Aβ(25-35) and Aβ(1-42) resulted in similar effects on neuritic atrophy and cell death at 10 μM [ reference 15] (P.4, L.19 – P.5, L.2). However, we are now investigating Kihi-to effects on Aβ(1-42)-induced neuritic atrophy and transgenic mice producing Aβ(1-42). We hope to report those data in a next report.

3) In our preliminary data, Kihi-to influenced glial cells as well as neurons. For example, Aβ(25-35) enhanced slightly numbers of astrocytes and microglia in primary cortical culture. Treatment with Kihi-to reduced those increases glial cells. We also measured the calpain expression level in astrocytes as follows. The calpain level was increased in astrocytes by Aβ(25-35), and Kihi-to inhibited it. We now describe this in Discussion (P.23, L.3 – 5).
Reply comments to Refee 3

Thank you for nice and useful suggestions for improvement of contents. We considered your opinions and revised several points.

1) As we now described in P.16, L.14-15, used dose of Kihi-to, 100 mg/kg/day is similar to human daily dose (approximately 125 mg/kg/day), and was shown as a maximal effective dose by our previous experiment.

2) As you know, oral administration of pure water to mice, the mice suffer from diarrhea. Therefore, we used tap water as vehicle for oral administration. Kihi-to was also dissolved in tap water.

3) Injection volume of Aβ(25-35) was 5 µl. It was added in Methods part (P.8, L.19).

4) Sham group was i.c.v. given a reverse fragment, Aβ(35-25) (25 nmol in 5 µl). It was written in P.6, L.7, P.8, L.19 - P.9, L.1 and figure legends (Figs. 1 – 6).

5) Kihi-to showed slightly enhancing activity of the spatial memory in normal mice in our unpublished data as follows. It was added in P.20, L.14 – 15.

6) Repressing Aβ production and altering vulnerability of neurons against Aβ might be
needed for prevention of Alzheimer’s disease. However, Kihi-to is a medicine, but not a kind of supplements. Traditional medicines are not prescribed easily for healthy human. Therefore, the possibility is very low, which Kihi-to is treated in pre-disease human for protection of Alzheimer’s disease. Although we have now no data of Kihi-to concerning $A_\beta$ production and sensitivity for $A_\beta$, remarkable effects of post-treated Kihi-to are quite important and significant.