Author's response to reviews

Title: Rg3-enriched ginseng extract ameliorates scopolamine-induced learning deficits in mice

Authors:

Jiyoung Kim (jiyoungkim1107@snu.ac.kr)
Jaesung Shim (digichart11@naver.com)
Siyoung Lee (lsy_prime@naver.com)
Woohyun Cho (kingfisher85@naver.com)
Eunyoung Hong (eyhong@cj.ne)
Jin Hee Lee (jhlee81@cha.ac.kr)
Jung-Soo Han (jshan06@konkuk.ac.kr)
Hyong Joo Lee (leejho@snu.ac.kr)
Ki Won Lee (kiwon@snu.ac.kr)

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Author's response to reviews: see over
Point-by-point response to referees’ comments

Comments to the Author:

Reviewer #1:
In the manuscript entitled “Rg3-enriched ginseng extract ameliorates scopolamine-induced learning deficits in mice”, Kim et al. demonstrated that Rg3-enriched ginseng extract administration significantly suppresses the scopolamine-induced learning deficits by reducing the inhibition of acetylcholinesterase activity and NF-κB signaling in the hippocampus. I basically agree with the author’s opinion. Unfortunately, I cannot recommend for the publication in the current form due to following problems. These are some serious problems require the authors’ attention:

Major comments:
Author addressed Rg3-enriched ginseng extract administration can ameliorate memory-related performance by two possible mechanisms. The first one is to modulate cholinergic neurons and the second one is to regulate NF-κB signaling. What is the connection between these two mechanisms? Author should clarify it.

; We thank the reviewer’s comment. It has been reported that muscarinic acetylcholine receptors in the central nervous system inhibit systemic inflammation and activation of muscarinic cholinergic transmission in the central nervous system lowers serum tumor necrosis factor (TNF) levels [1]. Since scopolamine is a non-selective muscarinic receptor antagonist, blockage of muscarinic receptor by scopolamine might promote inflammation and TNF. Previous studies have shown that scopolamine not only impairs cholinergic system, but
also induces expression of pro-inflammatory cytokines and mediators such as TNF-α, interleukin-1β (IL-1β), cyclooxygenase-1 (COX-1), and COX-2 [2-8]. It was found that scopolamine modulates the stimulation of NF-κB pathway (i.e., phosphorylation of IκBα and p65), which plays a key role in regulating the inflammatory responses [4, 6, 7]. Furthermore, chronic treatment of non-steroidal anti-inflammatory drugs (NSAIDs) reversed the cognitive deficits in scopolamine-treated mice [3]. Thus, scopolamine might cause learning and memory deficits through cholinergic neuronal injury complicated by NF-κB-mediated inflammation. Rg3-enriched ginseng extract (Rg3GE) might ameliorate memory-related performance through protecting cholinergic system by inhibiting NF-κB signaling.

*During the study, the Rg3-enriched ginseng extract administration was applied before the scopolamine injection. Why not apply it after the injection? Dose the order affect the conclusion? The author shall discuss it.*

; We thank the reviewer’s comment. Since scopolamine causes temporary cognitive deficit [9, 10], animal models for amnesia using scopolamine have been developed by injecting scopolamine 30 to 60 minutes before measuring memory deficit. To investigate the anti-amnesic activity of compounds, the protocol which applies test compounds before the scopolamine injection is being used [4, 11-19]. Whether the administration order of Rg3GE and scopolamine does affect the conclusion was not investigated. We will consider your comments for future research.

*Did treatments improve the visual capacity of the animals in the MWM task?*
We thank the reviewer’s comment. We have not examined the visual capacity of the animals in the MWM task. However, no differences in swimming speed between groups were found, which is accepted that sensorimotor function is intact between groups [20]. Research on pharmacological effects of ginseng has been continued since 1950. There is no evidence that ginseng extracts or ginsenosides improve the visual capacity.

In the "Material and Methods" part, the authors should clearly explain how they selected the four mice hippocampi analyzed by ELISA. According to their cognitive performances in the Morris Water Maze or just randomly?

; We thank the reviewer’s comment. Four or three mice hippocampi were randomly selected for acetylcholinesterase activity or western blot analysis, respectively. As suggested, the “Methods” part was revised.

The number of animals per group and the statistical test used should be specified in all Figure legends.

; We thank the reviewer’s comment. As suggested, the number of animals per group and the statistical test used were specified in all Figure legends.

In the text “These results suggested that Rg3-enriched ginseng extract might be useful for the treatment of failure of memory function by Alzheimer's disease.” Alzheimer model is etiologically different from scopolamine-induced memory impairment. Can you conclude this statement? Is this repeated SCO treatment an established AD model?
We thank the reviewer’s comment. As pointed out, Alzheimer model is etiologically different from scopolamine-induced memory impairment. As described in the “Background” part, scopolamine, a nonselective muscarinic acetylcholine receptor antagonist, interferes with the processes of learning acquisition and short-term memory [21, 22], induces amnesia, and has been used to generate experimental animal models for the screening of anti-amnesic drugs [23]. The mention of Alzheimer’s disease and relevant text in the manuscript has been removed.

Why did authors administer the scopolamine to the animal once a day on days 9-14? Is this experiment a "scopolamine-induced" neuronal impairment? Rg3-enriched ginseng extract, negatively charged polysaccharide, can bind nitrogen containing scopolamine, and reduce the free scopolamine. Higher dose of Rg3-enriched ginseng extract results in reduced scopolamine concentration, which does not exert enough neuronal impairment in the model animals. Although memory impairment was induced by injecting scopolamine 60 min after test compounds oral administrations, this procedure is to compensate the time gap between the i.p. and oral administration. I think scopolamine should be administrated before test compounds injection.

We thank the reviewer’s comment. This experiment is a "scopolamine-induced" memory impairment model that is non-degenerative and shows cholinergic dysregulation. Since scopolamine causes temporary cognitive deficit [9, 10], animal models for amnesia using scopolamine have been developed by injecting scopolamine 30 to 60 minutes before measuring memory deficit. To investigate the anti-amnesic activity of compounds, the protocol which applies test compounds before the scopolamine injection has been used [4, 11-19]. Since our study purpose was to investigate the preventive anti-amnesic effects of
Rg3GE, we administered Rg3GE once a day on days 1-14 and injected scopolamine once a day on days 9-14 and Morris water maze test was used to assess scopolamine-induced learning deficits on days 9-14. The anti-amnesic activities of methanol extracts of wild ginseng and cultivated ginseng, and ginsenosides Rg5 and Rh3, negatively charged polysaccharides as well, were described in an animal model by which these test compounds were applied before the scopolamine injection [16, 24]. Your point that “Rg3-enriched ginseng extract, negatively charged polysaccharide, can bind nitrogen containing scopolamine, and reduce the free scopolamine. Higher dose of Rg3-enriched ginseng extract results in reduced scopolamine concentration, which does not exert enough neuronal impairment in the model animals.” is very important and carries conviction. We will consider your comments for future research.

**Minor comments:**

1. There are some mistakes in grammar, and some sentences are confusing. 2. The general language needs revision, and it would be strongly advisable for the authors to have the manuscript checked by a native English speaker.

; We thank the reviewer’s comment. The manuscript was checked by a native English speaker and revised.

3. They need to describe the analysis protocols of HPLC in more detail.

; We thank the reviewer’s comment. The analysis protocol of HPLC was described in more detail in the “Methods.”
4. In my opinion, one-way ANOVA using the Turkey’s post hoc test is more suitable than one-way ANOVA using the LSD post hoc test. Furthermore, please confirm that significance statistical difference between all groups, as they are described in the text.

; We thank the reviewer’s comment. As you commented, one-way ANOVA using the Turkey’s post hoc test must be more suitable than one-way ANOVA using the LSD post hoc test. Data for acetylcholinesterase activity and p-p65 were re-analyzed by one-way ANOVA followed by Turkey’s post hoc test. Morris water maze data were analyzed by one-way repeated ANOVA followed by Fisher’s LSD test. Fisher’s LSD post hoc test has been used to test differences between different treatment groups in spatial memory tasks [25-27].

Reviewer #2:

This study clearly demonstrated that Rg3GE recovered scopolamine-induced memory deficits and the related mechanisms. This study was well-designed and the manuscript is overall described. I would like to ask authors to deal with following issues before it is published in this journal.

Comments:

1. I was just wondering how to choose the concentration of RGE and Rg3GE. Authors only included one concentration for 100 mg/kg in SCO+RGE group. Could you say that Rg3GE (100 mg/kg) is comparable to RGE (100 mg/kg)?

; We thank the reviewer’s comment. It has been reported that methanol extracts of wild ginseng at doses of 50, 100 and 200 mg/kg in a dose-dependent manner improve learning impairments induced by scopolamine in mice [16]. Standardized ginseng extract at doses of
10 and 30 mg/kg in rats showed improved learning and memory [28]. Red ginseng at dose of 100 mg/kg ameliorated place navigation deficits in young rats with hippocampal lesions and aged rats [29]. RGE at dose of 200 mg/kg has cholinesterase inhibitory activity and cholinergic function in scopolamine-induced amnesic mice [30]. Thus, we investigated the anti-amnesic activities of Rg3GE at 50 and 100 mg/kg, and RGE at 100 mg/kg was used as the positive control to confirm the anti-amnesic effects of Rg3GE. The inhibitory effects of Rg3GE at 100 mg/kg on scopolamine-induced learning deficit, acetylcholinesterase activity, and the level of p-p65 were similar or better compared to RGE at 100 mg/kg (Figure 4-6).

2. Regarding the Morris water maze, authors only tested the learning phase for 6 days, but not conducted probe test without target. If you did not conduct this, it would be only involved in the learning phase, but not in the memory-retrieval. Authors should describe this issue.

; We thank the reviewer’s comment. We found that Rg3GE improved the learning deficit induced by scopolamine in Morris water maze test, but not memory-retrieval in Probe test, suggesting that Rg3GE is beneficial in learning acquisition, but not in memory-retrieval. As suggested, it was described in the “Discussion” part.

3. In terms of NF-κB pathway (i.e., phosphorylation of p65), oral administration of Rg3GE even more suppressed phosphorylation of p65 in the hippocampus, compared to the Non-induction+VEH group. What is the interpretation of this suppression? It would be better discuss on it in the discussion.

; We thank the reviewer’s comment. It has been reported that ginseng extracts and ginsenosides suppress NF-κB activation and inflammatory responses in various cells and
tissues [31-39]. We observed phosphorylation of p65 in the hippocampus of non-induction+vehicle group and Rg3GE even more suppressed phosphorylation of p65, compared to the non-induction+vehicle group. Thus, the strong inhibitory activity of Rg3GE on NF-kB activation and inflammatory responses might have even more suppressed phosphorylation of p65, compared to the non-induction+vehicle group. As suggested, this suppression was indicated in the “Results” part.

References


36. Son HY, Han HS, Jung HW, Park YK: Panax notoginseng Attenuates the Infarct Volume in Rat

