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Original Article

ULTRAFILTRATED FRACTION OF KOREAN RED GINSENG EXTRACT IMPROVES MEMORY IMPAIRMENT OF TG2576 MICE VIA INHIBITION OF SOLUBLE AB PRODUCTION AND ACETYLCHOLINESTERASE ACTIVITY

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ABSTRACT

Objective: The goal of this study was to research for an effective fraction on memory improvement of Korean red ginseng.

Methods: In this study, 80 % ethanol red ginseng extract (RE) was divided into inner fluid (REUI) and outer fluid (REUO) by the ultrafiltration and then REUO was further separated into four fractions namely, REUO-00, REUO-30, REUO-50 and REUO-70, respectively, by Diaion HP-20 column chromatography.

Results: REUO has protected more significantly the H_2O_2-induced SHSY-5Y cell death than REUI. Interestingly, the hydrophobic parts of the REUO (REUO-EtOHs) such as REUO-30,-50 and-70 decreased more significantly the H_2O_2-induced cell death than its hydrophilic part (REUO-00) in a dose-dependent manner. Then, we focused on the activity of a candidate for cholinergic functions, because memory deficits of neurodegenerative diseases are closely associated with cholinergic dysfunctions. The REUO-EtOHs (1.25 mg/ml) inhibited the activity of the acetylcholinesterase and its half maximal inhibitory concentration (IC_{50}) was about 2.358 mg/ml. Additionally, we investigated whether the intake of the REUO (50 mg/kg/d) during 12 w could improve memory impairment of 12-month old Tg2576 mice and decrease total soluble amyloid-β (Aβ) proteins in the mouse brain cortex. The REUO alleviated significantly the memory impairment and successfully reduced the levels of the soluble Aβ proteins in the mouse cortex.

Conclusion: We finally suggest that the REUO, including majorly its hydrophobic part that may be considered as more effective for memory improvement, will be highly considered as valuable candidate for the memory-enhancing ingredients against cholinergic dysfunctions and cognitive impairments of neurodegenerative diseases including Alzheimer's disease.

Keywords: Ginseng, Alzheimer's disease, Acetylcholinesterase, Ultrafiltration, Memory

INTRODUCTION

Alzheimer's disease (AD), the most common age-related neurodegenerative disorder, is characterized by the formation of senile plaques including amyloid-β (Aβ) peptides in the brains [1]. AD also manifests cognitive deficits and impairment of activities of daily living [2]. The cholinergic hypothesis states that the cholinergic dysfunction in the brain is related to the cognitive decline in the elderly and AD [3]. Inhibition of acetylcholinesterase (AChE) is currently taken as a primary therapeutic strategy for AD [4]. Although four AChE inhibitors have been currently approved for the treatment of AD, the drugs have produced undesirable side effects such as diarrhea, nausea, vomiting and bradycardia [1]. Therefore, a search for novel molecules from natural products has gained much attention by the researchers worldwide. As a result, a number of botanicals as memory enhancers have been tested for anticholinesterase activity [5].

Ginseng (*Panax ginseng* Meyer) is frequently used in Asian countries as a traditional herbal medicine. Many researchers have demonstrated various preventive effects of ginseng on memory impairment [6] and oxidative stress [7]. Especially, the improved cognition has been reported with the treatment of ginsenosides Rb_1 and Rg_1 in animal models [8]. Despite the attractive features of ginsenosides as potential nutraceuticals for AD, their use has been limited for several reasons, including its high production cost and poor bioavailability [9].

In the course of the search for the effective fraction of the red ginseng as a functional food supplement with enhancing memory improvement and more safety applications, we found that an effective memory enhancing materials of Korean red ginseng might be not ginsenosides but an unidentified component. In this study, we investigated the inhibitory effects of ginsenoside included or not included fraction by ultrafiltration system and Diaion HP-20 column chromatography on the H_2O_2-induced cell death and checked whether a potential fraction could improve memory deficits of Tg2576 mice and decrease total soluble Aβ levels in the mouse brain cortex, compared with the AIE (extract of *Artemisia iwayomogi*).

MATERIALS AND METHODS

Materials

The red ginseng (Panax ginseng C. A. Meyer) were purchased from local market namely, Geumsaninsam cooperative association (Geumsan, Korea). The ginseng specimen was deposited in the International Ginseng and Herb Research Institute (No.; GS201105). All other chemicals were of analytical grade and were purchased from Sigma.

Extraction and fractionation

Dried red ginseng was extracted with 500 ml of 80 % ethanol at 80 ℃ for 6 h using a round-bottom flask fitted with a cooling condenser. The obtained 80 % ethanol extract (RE) was concentrated and dried *in vacuo*. RE was dissolved with 1 l of water and then concentrated up to 20 % of extraction volume by ultrafiltration system with Hollow Fiber cartridge (pore size; 3 kDa, membrane area; 6 m^2). The concentrated inner fraction (REUI) and the filtrated outer fraction (REUO) prepared by ultrafiltration system, were further dried in a freeze dryer. REUO was finally fractioned by Diaion HP-20 column chromatography using H_2O, 30 % EtOH, 50 % EtOH and 70 % EtOH in a sequential elution process, yielding four fractions namely of REUO-00, REUO-30, REUO-50, and REUO-70, respectively.

Measurement of cell viability

SH-SY5Y cells were seeded in a 96 well plate at a density of 7 x 10^3 cells/well. The collected fractions (50 μg/ml) and DHED (50 μM) were

introduced into the media for 4 h before treatment with H_2O_2 (750 μM). Cell viability was determined using WST-1 metabolizing activity according to the manufacturer's instruction (Roche, Indianapolis, IN, USA). The absorbance of the reaction product was finally measured with an ELISA reader (Bio-Rad, Munich, Germany) at a wavelength of 450 nm.

Acetylcholinesterase assay

The acetylcholinesterase (AChE) assay was carried out by the colorimetric method using acetylthiocholine iodide as a substrate [10]. Absorbance was measured at 410 nm immediately after adding 100 μl of enzymes to the reaction mixtures. Reading was repeated at 30 s intervals for 5 min. AChE activity was calculated using absorption coefficient 1.36 l/mmol/min. The half maximal inhibitory concentration (IC_{50}) of the sample was calculated from a linear estimate of the enzyme inhibition dose response curve.

Animals

Tg2576 mice were purchased from Taconic Laboratories that over-express a mutated form of the human amyloidal precursor protein (APP) 695 [11]. All experiments were carried out in accordance with the Guidelines for Animal Experiments of Ethics Committee of Seoul National University in Korea. The outer fraction of the ultrafiltrated red ginseng extract (REUO, 50 mg/kg) or the *Artemisia iwayomogi* extract (AIE, 50 mg/kg) mixed with the standard laboratory chow, was administered to the 9 mo old mice for 12 w. Food intake and mouse weight were recorded weekly at proper manner.

Passive avoidance test

A step-through type passive avoidance test apparatus (Model PACS-30, Columbus Instruments Int., USA) was used to evaluate the effects of the REUO or the AIE on learning and memory, essentially as described by Shen *et al.* [12].

Collection of brain tissues and western blot

After the behavioral test, mice were terminated at 13 mo old age. The brains were removed from the skull and the brains were further used for molecular works. The protein concentrations were quantified using Bio-Rad Protein Assay Reagent (Bio-Rad, USA). Brain tissues were homogenized with 10 volumes of homogenization buffer (12.5 mM sodium phosphate pH 7.0, 400 mM NaCl) and centrifuged at 1,000g for 10 min. After adding homogenization buffer containing 0.5 % Triton X-100 to the supernatant, the mixtures were continuously stirred for 30 min and centrifuged again at 10,000g for 10 min. Protein was resolved in 16.5 % tris/glycine gel, electrophoresed at 30~50 mg of protein/lane, and transferred onto a nitrocellulose membrane (Amersham Pharmacia, Buckinghamshire, UK). The protein blot was confirmed with 6E10 primary antibody for total soluble Aβ peptide and detected using horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia, Buckinghamshire, UK). Immunoreactive bands were visualized using an ECL enhanced chemiluminescence system (ECL; Amersham Pharmacia, Buckinghamshire, UK). The protein loading control was checked with GAPDH antibody.

High-performance liquid chromatography (HPLC) analysis of ginsenoside

Ten mg of ginseng extract or fraction was melted by 1 ml of methanol (MeOH) and filtered out by 0.45 μm membrane filter after extraction of ultrasonic waves for 2 h, then analyzed in HPLC. The HPLC system was Waters 1525 (Waters, USA) with PDA detector (Water, 2998). Waters Xbridge™ C18 column (250 mm × 4.6 mm, 5 μm, Waters, USA) was also used. The detection wavelength, flow rate, injection volume, and column oven temperature were set at 203 nm, 1.0 ml/min, 20 μl, and 40℃, respectively. The mobile phase consisted of purified water (A) and acetonitrile (B) using the following gradient program: 0 min 18 % B, 0-42 min 24 % B, 42-46 min 29 % B, 46-75 min 40 % B, 75-100 min 65 % B, 100-135 min 85 % B, and 135-180 min 18 % B.

Statistical analysis

Data were expressed as mean±SE (standard error of mean). One-way ANOVA followed by Dunnett's post hoc test (SPSS version 21) was applied to study the relationship between the different variables. $p<0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Ginsenoside content in red ginseng extracts and each fraction

As shown in table 1, the contents of ginsenoside, including protopanaxadiol (PPD) type ginsenoside such as Rb_1, Rb_2, Rb_3, Rc, Rd, Rg_3, Rk_1, and Rg_5 and protopanaxatriol (PPT) type ginsenoside such as Rg_1, Re, Rf and F_1, were measured separately in RE, REUI, REUO, and REUO-30. The total ginsenosides contents of RE, REUI, and REUO were 24.57, 106.94, and 10.74 mg/g, respectively. The total ginsenosides in REUI of an inner fluid fraction after ultrafiltration, was about 4.4 and 9.9 times higher than those of RE of ginseng extract and REUO of an outer fluid fraction of ginseng extract. The main ginsenoside of REUO was PPT-type ginsenosides such as Rg_1, Re, and Rf, while REUI contained PPD-type ginsenosides such as Rb_1, Rb_2, Rb_3, Rc, Rd, Rg_3, Rk_1, and Rg_5, more hydrophobic ginsenosides than PPT-type ginsenoside. The ratio of Rb_1 to Rg_1 (Rb_1/Rg_1) and PPD-to PPT-type ginsenoside (PPD/PPT) of RE, REUI, and REUO were found to be 1.77 and 1.75, 15.90 and 11.22, and 0.23 and 0.13, respectively. Generally, ultrafiltration is a technique for separating dissolved molecules in solution on the basis of a size which means that molecules larger than the membrane pore size rating will be retained at the surface of the membrane [14]. The molecular weight of ginsenoside was below 1,110, even though the molecular weight of PPD-type ginsenoside was slightly larger than those of PPT-type ginsenoside. The ginsenoside molecular size is smaller than pore size of membrane filters used for these experiments. Nevertheless, relatively hydrophobic PPD-type ginsenosides in RE did not pass through the hydrophilic membrane with 3 kDa pore size [14]. Only relatively hydrophilic PPT-type ginsenosides, Re and Rg_1 slowly passed through the membrane in continuous solution flow system. So, REUO included PPT-type ginsenosides and smaller dissolved micromolecules, passed through the membranes. However, any ginsenoside was not detected in the REUO-30 fraction by Diaion HP-20 column chromatography from REUO.

Some subfractions of the red ginseng extract decrease cytotoxicity induced by H_2O_2

The red ginseng extracted by 80 % ethanol was divided into the inner fraction (REUI) and the outer fraction (REUO) by the ultrafiltration membrane. Dehydroevodiamine (DHED) was used as a positive control. Exposure of SHSY-5Y neuroblastoma to H_2O_2 (750 μM) during 24 h, induced significant neurotoxicity compared to control ($p<0.01$, fig. 1). DHED or each fraction of the red ginseng extract was pretreated to cells for 4 h before H_2O_2 treatment. Both DHED (50 μM) and REUO (50 μg/ml) treatment decreased more significantly the H_2O_2-induced cell death than the vehicle and the REUI (50 μg/ml) treatment compared to the control (fig. 1A). Ginsenoside content of REUI was about 10.7 %, while its content of REUO was about 1.1 % (table 1). These results revealed that the inhibitory effect of H_2O_2-induced cell death was due to an unidentified component of REUO but not ginsenosides of REUI. In order to find out neuroprotective ginseng component, REUO was further separated into several different fractions such as, REUO-00, REUO-30, REUO-50 and REUO-70, by Diaion HP-20 column chromatography using H_2O, 30 % EtOH, 50 % EtOH and 70 % EtOH in a sequential elution process, respectively. The hydrophilic fraction of REUO-00 eluted with H_2O included the hydrophilic component such as sugar. Any ginsenosides were not detected in REUO-30 eluted with 30 % EtOH (table 1). The hydrophobic fractions (50 μg/ml) of the REUO (REUO-EtOHs) such as REUO-30, REUO-50, and REUO-70 also declined more significantly the H_2O_2-induced cell death than the vehicle and the hydrophilic fractions (50 μg/ml) of the REUO such as REUO-00 (fig. 1B). Therefore, REUO-EtOHs including REUO-30, REUO-50 and REUO-70 fractions, were collected in order to compare with the red ginseng extract (RE) along with its ultrafiltrated outer fraction (REUO). As shown in fig. 1C, the REUO-EtOHs (50 μg/ml) decreased more significantly the H_2O_2-induced cell death than the RE (50 μg/ml) and the REUO (50 μg/ml). Additionally, REUO-EtOHs has also inhibited the H_2O_2-induced cell death significantly in a dose-dependent manner (fig. 1D).

The subfractions of the red ginseng extract inhibit AChE activity

Amyloid-β (Aβ) represents the underlying cause of the cognitive deficits observed in AD, leading to cell death through the induction

of oxidative stress [15]. Therefore, memory-enhancing foods on memory are considered to be effective for cholinergic functions [16]. The characteristic roles of Aβ on cholinergic function reported recently that AChE activity was increased more around amyloid plaque [17]and vulnerable cholinergic neuronal loss in AD was closely related to inhibition of high-affinity choline uptake and ACh release by Aβ [18]. Owing to the cholinergic hypothesis, strategies for increasing synaptic levels of ACh have been widely explored in the development of anti-dementia drugs [19]. To test a cholinergic action, the acetylcholinesterase (AChE) activity assay was carried out. DHED and the *Artemisia iwayomogi* extract (AIE) were used as the positive controls. The inhibitory percentage of 37.8 μM DHED, the 0.5 mg/ml AIE and 1.5 mg/ml REUO-EtOHs were noted as % of inhibition (fig. 1E). The REUO-EtOHs inhibited more significantly the AChE-induced activity than 250 μM Rb_1, 250 μM Rg_1, 1.25 mg/ml RE and 1.25 mg/ml REUO, respectively. The REUO-EtOHs also inhibited the AChE activity in a dose-dependent manner (fig. 1F). The concentration required for 50 % enzyme inhibition (IC_{50}) was found to be 2.358 mg/ml. In this study, we also demonstrated that some candidates including REUO blocked the AChE activities, and the REUO inhibited the AChE activity in a dose-dependent manner. It indicates that those plant extracts might contain an efficient unidentified anticholinesterase component. Because the REUO is a crude extract, its inhibitory activity (IC_{50}=2.358 mg/ml) could not be compared to that of known single compounds, such as donepezil [20] and DHED [21]. Among the possible strategies aimed at increasing cholinergic neurotransmission, the AChE inhibitor would be valuable candidates and easily accessible therapeutic agent for maintaining ACh levels in the brain as well as improving cognitive ability [22].

The ultra filtered outer fraction of the red ginseng extract improves the memory impairment and decreases the levels of total soluble Aβ peptides in Tg2576 mice

To investigate whether the impairments of learning and memory in Tg2576 mice could be improved by the intake of the REUO, the passive avoidance test was carried out.

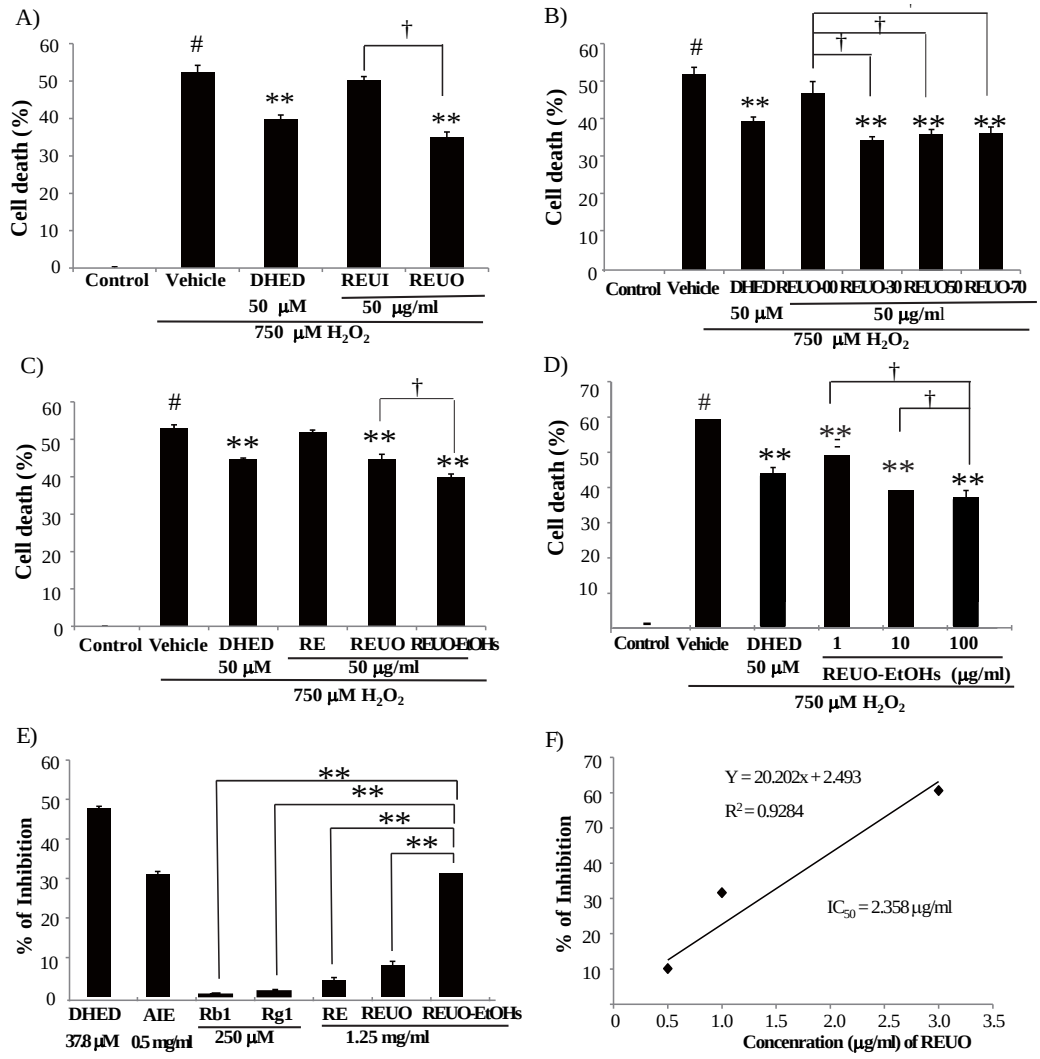

Fig. 1: Effects of the sub fractions of the red ginseng extract on cytotoxicity induced by H_2O_2 (750 μM). A) The H_2O_2-induced cell death was inhibited by the 50 μg/ml REUO but did not by the 50 μg/ml REUI in SH-SY5Y cells. The level of the REUO is similar with the level of the positive control (50 μM DHED-treated cells). B) The H_2O_2-induced cell death was decreased by the 50 μg/ml REUO-30, 50 μg/ml REUO-50, and 50 μg/ml REUO-70 but did not by 50 μg/ml REUO-00 in the SH-SY5Y cell, respectively. The levels of the REUO-30, REUO-50 and REUO-70, are similar with the level of the DHED-treated cells. C) The H_2O_2-induced cell death was prevented by the 50 μg/ml REUO and the 50 μg/ml REUO-EtOHs but did not by the 50 μg/ml RE in SH-SY5Y cells. The levels of the REUO-EtOHs were more effective than that of the REUO in the inhibition of the H_2O_2-induced cell death. D) The REUO-EtOHs prevented the H_2O_2-induced cell death as a dose-dependent manner. #p<0.05 compared with the control, **p<0.01 compared with the H_2O_2-treated control, and †p<0.05 compared between two samples linked by the line in one-way ANOVA with a *post hoc* Dunnett's test. Each value represents the mean±SE. E) The AChE activity was inhibited by 37.8 μM DHED, the 0.5 mg/ml AIE, the 1.25 mg/ml REUO, and the 1.25 mg/ml REUO-EtOHs but did not by 250 μM Rg_1, 250 μM Rb_1 and the 1.25 mg/ml RE. F) The concentration required for 50 % enzyme inhibition (IC_{50}) was 2.358 mg/ml. The inhibitory efficacy was expressed as the percentage of the inhibition of enzyme activity compared to the control value (100 %). Each value represents the mean±SE (n =5)

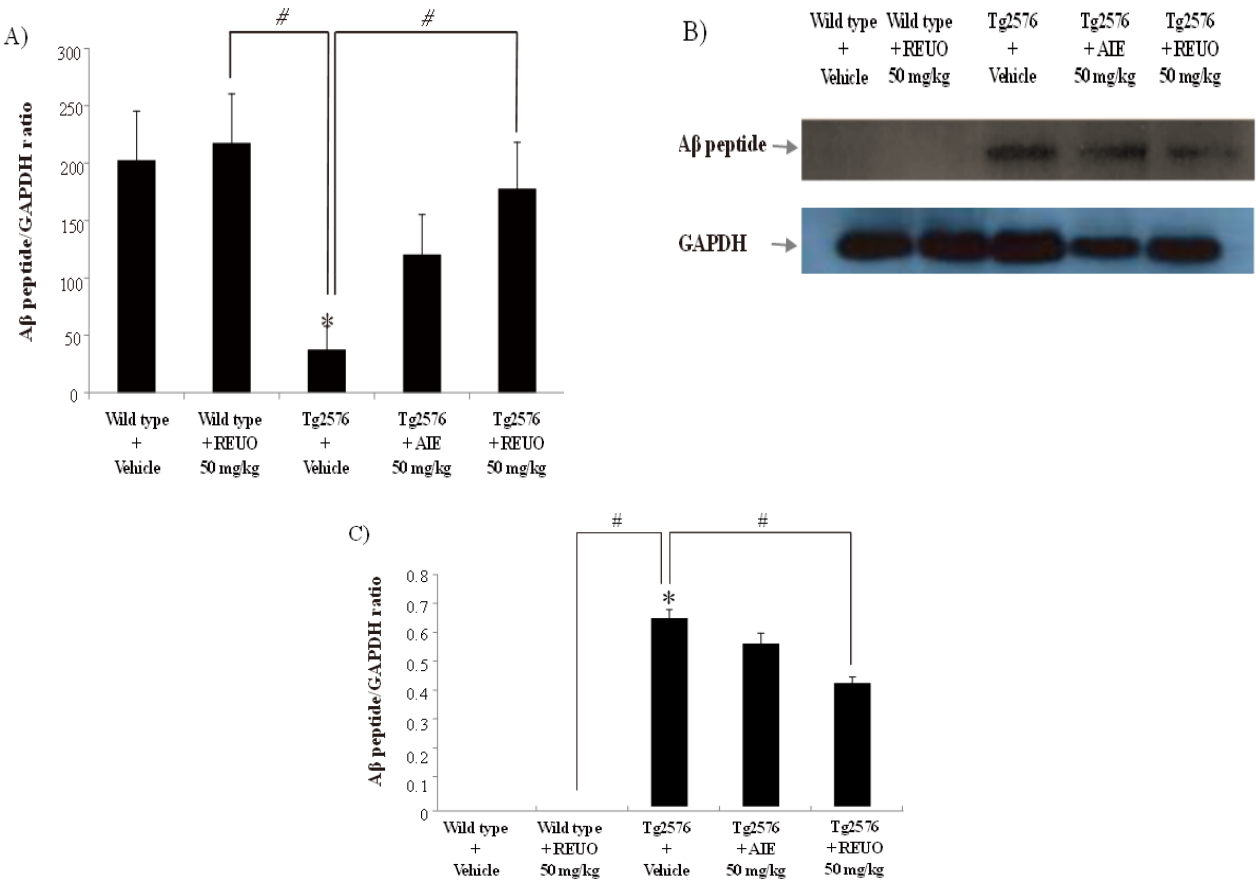

Fig. 2: Effects of the ultrafiltrated outer fraction of the red ginseng extract on memory impairment total Aβ levels in Tg2576 mouse brains. In 12 mo-old Tg2576 (Tg) and wild-type (Wt) mice, the passive avoidance test was performed after intake of the REUO (50 mg/kg) or the AIE (50 mg/kg) for 12 w. A) Note that latency was decreased significantly in the vehicle-treated Tg2576 mice compared to the vehicle-treated and the REUO-treated wild-type mice. However, the short latency time was increased significantly by the REUO intake compared to non-treatment in the Tg2576 mice. B) Each representative blot of total soluble Aβ peptides or GAPDH was shown. C) The production of total soluble Aβ peptides was highly increased in the cortex of the vehicle-treated Tg2576 mice compared with the vehicle-treated and the REUO-treated wild-type mice. Note that the high levels of total soluble Aβ in the vehicle-treated Tg2576 mice was decreased by the intake of the REUO. Data represents mean±SE. *p<0.05 and #p<0.05 compared with the vehicle-treated wild-type mice and the vehicle-treated Tg2576 mice, respectively, one-way ANOVA with a *post hoc* Dunnett's test (n=5~9)

The AIE (50 mg/kg) or the REUO (50 mg/kg) mixed with the standard laboratory chow was administrated to 9 mo-old Tg2576 and wild-type mice during 12 w. As shown in fig. 2A, the latency of the non-treated Tg2576 mice (37.86±20.53 s) was more shortened than that of non-treated (202.70±42.57 s, p = 0.004) and the REUO-treated (217.56±42.99 s, p = 0.004) wild-type mice. Interestingly, the latency of the non-treated Tg2576 mice was more increased than that of the REUO-treated Tg2576 mice (177.83±40.35 s, p = 0.012).

However, the AIE compared with REUO did not improve the memory impairment of Tg2576 mice. After completing the behavioral test, neurochemical changes in the brain were analyzed to investigate the inhibitory effect of the REUO on the Aβ production after repeated intake of the REUO (50 mg/kg) mixed with the standard laboratory chow to Tg2576 mice for 12 w. The AIE (50 mg/kg) or the REUO (50 mg/kg) mixed with the standard laboratory chow was administrated to 9 mo-old Tg2576 and wild-type mice during 12 w. As shown in fig. 2A, the latency of the non-treated Tg2576 mice (37.86±20.53 s) was more shortened than that of non-treated (202.70±42.57 s, p = 0.004) and the REUO-treated (217.56±42.99 s, p = 0.004) wild-type mice. Interestingly, the latency of the non-treated Tg2576 mice was more increased than that of the REUO-treated Tg2576 mice (177.83±40.35 s, p = 0.012). However, the AIE compared with REUO did not improve the memory impairment of Tg2576 mice. After completing the behavioral test, neurochemical changes in the brain were analyzed to investigate the inhibitory effect of the REUO on the Aβ production after repeated intake of the REUO (50 mg/kg) mixed with the standard laboratory chow to Tg2576 mice for 12 w.

Levels of total Aβ peptides in the brains of the mice were shown in the fig. 2B and 2C. Levels of total soluble Aβ peptides were increased more in vehicle-treated Tg2576 mice than the vehicle-treated or the REUO-treated wild-type mice. Interestingly, increasing levels of total soluble Aβ peptides was traumatically reduced by the repeated intake of the REUO, but did not shown by the repeated intake of the AIE. The brain is particularly susceptible to oxidative stress because of its high oxygen consumption, high polyunsaturated fatty acid content and low antioxidant defences [23].

Numerous studies have demonstrated that generation of reactive oxygen species [24] and inhibition of choline uptake [18] plays a crucial role in the pathogenesis of AD. In Tg2576 mice, the REUO could improve memory deficits and decrease the levels of total Aβ proteins in the mouse brains. In the discussion, further purification to isolate one or more bioactive components from the red ginseng and further analysis on their structure and effects should be carried out for a better understanding of its pharmacologic mechanisms. Although the action mechanisms of plant extract that have been used medicinally and traditionally need to be investigated. Furthermore, it is thought that the outer fraction of the ultra filtrated red ginseng extracts, especially non-saponin fractions of red ginseng, may be considered as cognition-enhancing food supplements that protect cholinergic dysfunction, oxidative stress, and Aβ production

Table 1: The contents of ginsenosides in ginseng extract and fractions

Ginsenoside (mg/g)	RE	REUI	REUO	REUO-30
PPT type				
Rg_1	3.52	2.22	4.49	N. D.
Re	3.49	2.71	4.13	N. D.
Rf	1.64	1.81	0.90	N. D.
F_1	0.29	2.02	N. D.	N. D.
Subtotal	8.93	8.75	9.52	
PPD type				
Rb_1	6.21	35.31	1.03	N. D.
Rc	5.04	26.91	0.19	N. D.
Rb_2	2.60	16.67	N. D.	N. D.
Rd	0.68	7.38	N. D.	N. D.
S-Rg_3	0.35	1.67	N. D.	N. D.
R-Rg_3	0.22	4.11	N. D.	N. D.
Rk_1	0.19	2.45	N. D.	N. D.
Rg_5	0.34	3.68	N. D.	N. D.
Subtotal	15.64	98.19	1.22	
Total	24.57	106.94	10.74	
PPD/PPT	1.75	11.22	0.13	
Rb_1/Rg_1	1.77	15.90	0.23	

RE, Red ginseng extract with 80 % ethanol; REUI, the inner fraction of red ginseng extract concentrated up to 20 % of extraction volume by ultrafiltration system with Hollow Fiber cartridge (pore size; 3 kDa); REUO, the filtrated outer fraction of red ginseng extract prepared by ultrafiltration system; REUO-30, the eluting fraction with 30 % ethanol of REUO fractioned by Diaion HP-20 column chromatography using H_2O, 30 % EtOH, 50 % EtOH and 70 % EtOH in a sequential elution process; PPT, protopanaxatriol; PPD, protopanaxadiol; N. D., not detected.

CONCLUSION

We conclude that the outer fraction of the ultra filtrated red ginseng extract, including the majority of its hydrophobic part, may be more effective for memory improvement, will be strongly considered as a novel candidate for the memory-enhancing ingredients against cholinergic dysfunctions and cognitive impairments of neuro-degenerative diseases including Alzheimer's disease. However, we remained further purification and identification of smaller unknown component with memory improvement effect of Korean red ginseng.

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CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest

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