Therapeutic potential of *Gastrodia elata* Blume for the treatment of Alzheimer's disease☆

Guang-Biao Huang[1], Tong Zhao[1], Sushma Shrestha Muna[1], Hong-Mei Jin[1], Jong-Il Park[1], Kyu-Sik Jo[2], Bo-Hee Lee[2], Soo-Wan Chae[3], Sun-Young Kim[3], Soo-Hyun Park[3], Eun-Ock Park[3], Eun-Kyung Choi[3], Young-Chul Chung[1, 4]

1 *Department of Psychiatry, Chonbuk National University Medical School, Jeonju 561-756, Republic of Korea*
2 *Muju Chunma Native Local Industrial Center, Muju 568-844, Republic of Korea*
3 *Clinical Trial Center for Functional Foods, Chonbuk National University Hospital, Jeonju 561-756, Republic of Korea*
4 *Department of Pharmacology, Chonbuk National University Medical School, Jeonju 561-756, Republic of Korea*
5 *Research Institute of Clinical Medicine of Chonbuk National University-Biomedical Research Institute of Chonbuk National University Hospital, Jeonju 561-756, Republic of Korea*

Abstract

Several studies have demonstrated that the Chinese herb *Gastrodia elata* Blume can protect against amyloid beta-peptide (Aβ)-induced cell death. To investigate the possible therapeutic effects of *Gastrodia elata* Blume on Alzheimer's disease, we established a rat model of Alzheimer's disease by injecting $Aβ_{25-35}$ into bilateral hippocampi. These rats were intragastrically administered 500 or 1 000 mg/kg *Gastrodia elata* Blume per day for 52 consecutive days. Morris water maze tests showed that *Gastrodia elata* Blume treatment significantly improved the spatial memory of Alzheimer's disease rats. Congo red staining revealed that *Gastrodia elata* Blume significantly reduced the number of amyloid deposits in the hippocampus of these rats. Western blot analysis showed that choline acetyltransferase expression in the medial septum and hippocampus was significantly increased by the treatment of *Gastrodia elata* Blume, while Ellman method showed significant decrease in the activity of acetylcholinesterase in all three regions (prefrontal cortex, medial septum and hippocampus). These findings suggest that long-term administration of *Gastrodia elata* Blume has therapeutic potential for Alzheimer's disease.

Key Words

neural regeneration; neuroprotective effect; Traditional Chinese medicine; *Gastrodia elata* Blume; Alzheimer's disease; Morris water maze test; choline acetyltransferase; acetylcholinesterase; grants-supported paper; neuroregeneration

Research Highlights

(1) Intrahippocampal injection of amyloid beta-peptide (25–35) ($Aβ_{25-35}$) impaired spatial reference memory in rats.
(2) $Aβ_{25-35}$ had detrimental effects on choline acetyltransferase expression and acetylcholinesterase activity in the brain.
(3) Long-term administration of *Gastrodia elata* Blume partially reversed these effects in Alzheimer's disease rats.
(4) *Gastrodia elata* Blume is a potential drug candidate for the treatment of Alzheimer's disease.

Guang-Biao Huang☆, M.D., Researcher.

Guang-Biao Huang and Tong Zhao contributed equally to this paper.

Corresponding author: Young-Chul Chung, M.D., Professor, Department of Psychiatry, Chonbuk National University Medical School, Keumam Dong 634-18, Jeonju 561-712, Republic of Korea, chungyc@jbnu.ac.kr.

INTRODUCTION

Gastrodia elata Blume, a saprophytic, perennial herb in the Orchidaceae family, is native to several Asian countries, including China, Korea and Japan. The dry tuber of *Gastrodia elata* Blume (*Gastrodiae rhizome*; "*Tianma*" in Chinese) has been used for centuries in traditional Chinese medicine to treat dizziness, paralysis and epilepsy, and is listed in the Chinese Pharmacopoeia[1]. Recently, *Tianma* and its main bioactive component, gastrodin, have been shown to have diverse effects, including cognition enhancing[2-4], neuroprotective[5-6], antidepressant[7] and anti-inflammatory[8] effects.

Hsieh *et al* [9] reported that a single administration of gastrodin and *p*-hydroxybenzyl alcohol, an aglycone of gastrodin, prolonged cycloheximide- and apomorphine-induced step-through latency in a passive avoidance task, but did not prolong scopolamine-induced step-through latency in the same task. Their conclusion was that gastrodin and *p*-hydroxybenzyl alcohol can facilitate the consolidation and retrieval of memories, but not the acquisition of memories. However, the administration of gastrodin or *p*-hydroxybenzyl alcohol for 1 week significantly prolonged the shortened step-through latency induced by scopolamine in the passive avoidance task[2, 10]. In addition, *Gastrodia elata* Blume improves d-galactose-[11] and aluminum chloride-induced[4] impairment in the passive avoidance test. Interestingly, *Gastrodia elata* Blume has also been reported to protect against amyloid β-peptide (Aβ)-induced cell death, suggesting that *Gastrodia elata* Blume has therapeutic potential for Alzheimer's disease[3, 12]. The strong hydroxyl radical scavenging activity of *Gastrodia elata* Blume has been suggested to underlie the neuroprotective effect of the herb[3]. However, the mechanisms by which *Gastrodia elata* Blume improves memory remain poorly understood.

Alzheimer's disease is a multifaceted neurodegenerative disorder of the central nervous system characterized by progressive cognitive dysfunction. A major pathologic hallmark of Alzheimer's disease is the presence of numerous senile plaques, neurofibrillary tangles in brain regions, and cholinergic dysfunction. The degree of cognitive dysfunction in Alzheimer's disease patients significantly correlates with a decline in choline acetyltransferase activity and the loss of cholinergic neurons[13-14]. Interestingly, it has been shown that the G4 globular form of acetylcholinesterase is selectively decreased in the brain of Alzheimer's disease patients, whereas levels of the monomeric forms of the protein are increased in the vicinity of amyloid plaques[15]. Direct injection of Aβ$_{25-35}$ into the hippocampus has been used successfully to create animal models of Alzheimer's disease[16-18], although some studies have yielded conflicting results[19-20]. The potential benefits of *Gastrodia elata* Blume for the treatment of Alzheimer's disease have never been investigated *in vivo*.

The aim of this study was to investigate the effects of *Gastrodia elata* Blume on spatial memory in a rat Alzheimer's disease model using the Morris water maze test. To explore the mechanisms behind these putative effects, we examined Alzheimer's disease model rats treated with cellulose or *Gastrodia elata* Blume for choline acetyltransferase expression and acetylcholinesterase activity in several brain regions.

RESULTS

Quantitative analysis of experimental animals

A total of 43 rats were initially included in this study, and divided into four groups. Some rats (Alzheimer's disease model rats) received bilateral injections of Aβ$_{25-35}$ into the hippocampus ($n = 31$) and were then treated daily with oral administration of either 0.5% cellulose (Aβ$_{25-35}$/cellulose group, $n = 9$) (control), 500 mg/kg *Gastrodia elata* Blume (Aβ$_{25-35}$/GEB$_{500}$ group, $n = 11$) or 1 000 mg/kg *Gastrodia elata* Blume (Aβ$_{25-35}$/GEB$_{1000}$ group, $n = 11$) for 52 days (GEB: *Gastrodia elata* Blume). The vehicle group ($n = 12$) consisted of vehicle-injected rats treated daily with oral administration of 0.5% cellulose for 52 days. All 43 rats were included in the final analysis.

Effects of *Gastrodia elata* Blume on spatial memory in Alzheimer's disease rats

Typical swim paths are shown in Figure 1, and the water maze data latency and distance data are shown in Figure 2. The acquisition day had a significant effect on latency and on the distance traveled to the hidden platform [latency: $F_{(3, 32)} = 85.04$, $P < 0.000\ 1$; distance: $F_{(3, 33)} = 101.43$, $P < 0.000\ 1$], indicating that all of the rats readily learned the task. There was also a main effect of treatment on latency and on distance traveled to the hidden platform [latency: $F_{(3, 32)} = 4.21$, $P = 0.013$; distance: $F_{(3, 33)} = 3.27$, $P = 0.033$]. No significant interactions between acquisition day and treatment were found for latency [$F_{(3, 32)} = 1.01$, $P > 0.05$] or distance traveled [$F_{(3, 33)} = 0.45$, $P > 0.05$]. *Post hoc* analysis for

the main effect of treatment during acquisition revealed a significant difference in the escape latencies of the control and $A\beta_{25-35}/GEB_{500}$ groups ($P = 0.001\ 42$) and revealed a trend towards a difference in escape latencies between the control and $A\beta_{25-35}/cellulose$ and between the control and $A\beta_{25-35}/GEB_{1000}$ groups. It also revealed a significant difference in the distances traveled by the control and $A\beta_{25-35}/cellulose$ groups as well as in the distances traveled by the control and $A\beta_{25-35}/GEB_{500}$ groups ($P < 0.05$).
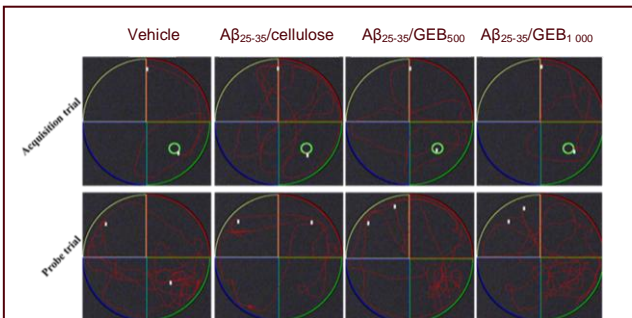

Figure 1 Spatial probe swim paths in the Morris water maze.

Swimming paths recorded during the acquisition and probe trials of representative Alzheimer's disease model rats treated with 0.5% cellulose and *Gastrodia elata* Blume (GEB; 500 and 1 000 mg/kg).
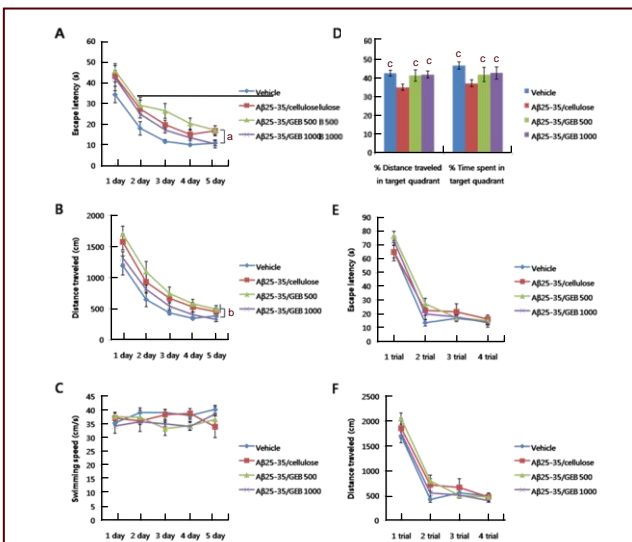

Figure 2 Effects of *Gastrodia elata* Blume (GEB) on amyloid beta-peptide (25–35) ($A\beta_{25-35}$)-induced spatial cognitive deficits in the water maze test in rats (A, B and C, and D are acquisition trials and probe trials of the reference memory test, respectively, and E and F are working memory tests).

Data are expressed as means ± SEM (each group n = 6–10 rats) and were analyzed by repeated measures analysis of variance or one-way analysis of variance, followed by Fisher's *post hoc* test. [a]$P < 0.05$, $A\beta_{25-35}/GEB_{500}$ group, *vs.* vehicle group for days 1–5; [b]$P < 0.05$, $A\beta_{25-35}/cellulose$ and $A\beta_{25-35}/GEB_{500}$ groups, *vs.* vehicle group; [c]$P < 0.05$, *vs.* $A\beta_{25-35}/cellulose$ group.

In the probe trial, both the time spent [$F_{(3, 37)} = 2.79$, $P = 0.54$] and the distance traveled [$F_{(3, 37)} = 3.29$, $P = 0.031$] in the target quadrant varied significantly among the four groups. Fisher's *post hoc* comparisons demonstrated significant differences in time spent and distance traveled in the target quadrant between the $A\beta_{25-35}/cellulose$ group and the control ($P = 0.018$ and $P = 0.012$, respectively), $A\beta_{25-35}/GEB_{500}$ ($P = 0.033$ and $P = 0.008$, respectively) and $A\beta_{25-35}/GEB_{1000}$ ($P = 0.017$ and $P = 0.029$, respectively) groups. In the working memory test, all animals exhibited an improvement in performance with repeated trials in terms of latency and distance traveled to the hidden platform [latency: $F_{(3, 35)} = 307.41$, $P < 0.001$; distance: $F_{(3, 32)} = 285.63$, $P < 0.001$]. However, no significant main effects of treatment on latency [$F_{(3, 35)} = 0.33$, $P > 0.05$] or distance traveled [$F_{(3, 32)} = 1.93$, $P > 0.05$] were observed, nor were any significant interactions between trial number and treatment for latency [$F_{(3, 35)} = 1.93$, $P > 0.05$] or distance traveled [$F_{(3, 32)} = 2.94$, $P > 0.05$] observed.

Effects of *Gastrodia elata* Blume on amyloid deposits in Alzheimer's disease rats

Congo red staining showed that the CA1 neurons in control rats were arranged in an orderly fashion, with distinct edges and clear nuclei, without Congo red deposits. However, the CA1 neurons in $A\beta_{25-35}/cellulose$ group rats were shrunken, with an irregular morphology, and were surrounded by Congo red staining. In comparison, stained amyloid deposits in the hippocampus were scarce in the $A\beta_{25-35}/GEB_{500}$ and $A\beta_{25-35}/GEB_{1000}$ groups (Figure 3). Few or no stained amyloid deposits were seen in the prefrontal cortex or medial septum (data not shown).
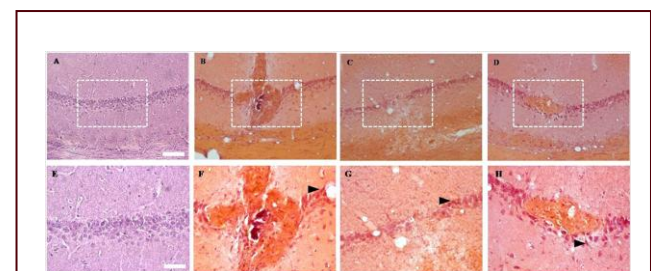

Figure 3 Congo red staining in the hippocampal CA1 region in amyloid beta-peptide (25–35) ($A\beta_{25-35}$)-induced Alzheimer's disease rats treated with *Gastrodia elata* Blume (GEB).

(A, E) Vehicle group; (B, F) $A\beta_{25-35}/cellulose$ group; (C, G) $A\beta_{25-35}/GEB_{500}$ group; (D, H) $A\beta_{25-35}/GEB_{1000}$ group. Dotted boxes indicate the regions shown in the second row. Arrowheads point to Congo red-stained cells (shrunken neurons with intense staining in the nucleus). Scale bar: 100 μm for the top row, 50 μm for the bottom row.

Effects of *Gastrodia elata* Blume on choline acetyltransferase expression

Significant differences in choline acetyltransferase expression in the medial septum [$F_{(3, 33)} = 4.71$, $P = 0.008$] and hippocampus [$F_{(3, 34)} = 3.53$, $P = 0.025$] were found among the four groups. Fisher's *post hoc* analysis revealed significant differences in medial septum expression between the Aβ_{25-35}/cellulose and Aβ_{25-35}/GEB$_{500}$ and Aβ_{25-35}/GEB$_{1000}$ groups ($P < 0.001$ for both comparisons), as well as significant differences in hippocampal expression between the Aβ_{25-35}/cellulose and control, Aβ_{25-35}/GEB$_{500}$ and Aβ_{25-35}/GEB$_{1000}$ groups ($P < 0.05$ for all three comparisons). However, no significant difference in choline acetyltransferase expression in the prefrontal cortex was found among the four groups [$F_{(3, 34)} = 0.32$, $P > 0.05$] (Figure 4).
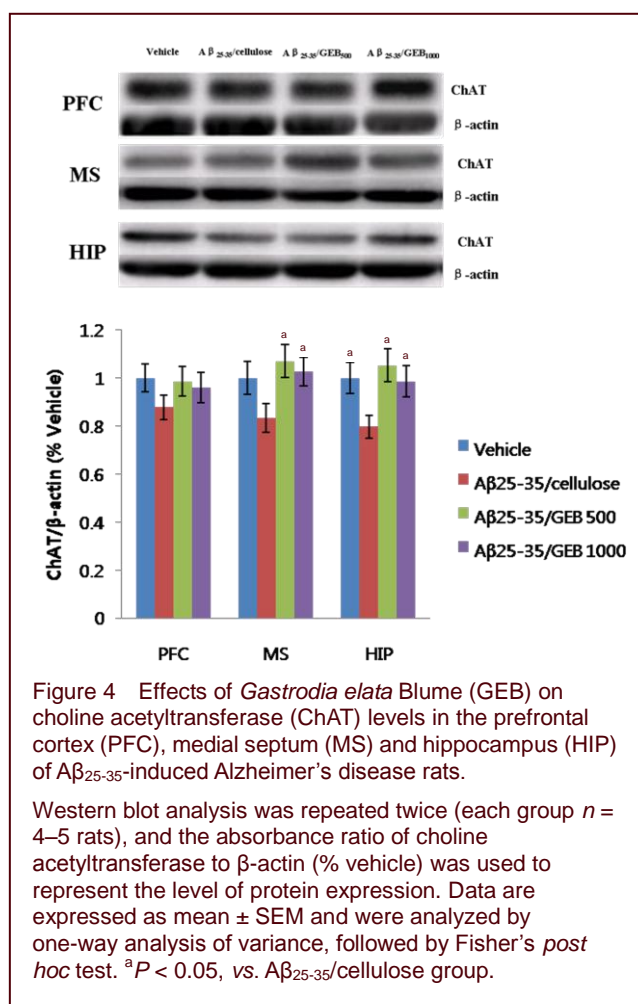

Figure 4   Effects of *Gastrodia elata* Blume (GEB) on choline acetyltransferase (ChAT) levels in the prefrontal cortex (PFC), medial septum (MS) and hippocampus (HIP) of Aβ_{25-35}-induced Alzheimer's disease rats.

Western blot analysis was repeated twice (each group $n = $ 4–5 rats), and the absorbance ratio of choline acetyltransferase to β-actin (% vehicle) was used to represent the level of protein expression. Data are expressed as mean ± SEM and were analyzed by one-way analysis of variance, followed by Fisher's *post hoc* test. [a]$P < 0.05$, *vs.* Aβ_{25-35}/cellulose group.

Effects of *Gastrodia elata* Blume on acetylcholinesterase activity

Acetylcholinesterase activity in the prefrontal cortex, medial septum and hippocampus differed significantly among the four groups [$F_{(3, 29)} = 8.37$, $F_{(3, 30)} = 7.9$ and $F_{(3, 32)} = 23.85$, respectively; $P < 0.001$ for all]. Fisher's *post hoc* analysis showed significantly higher acetylcholinesterase activity in the prefrontal cortex and hippocampus in the Aβ_{25-35}/cellulose group compared with the control, Aβ_{25-35}/GEB$_{500}$ and Aβ_{25-35}/GEB$_{1000}$ groups ($P < 0.001$ for all). In addition, acetylcholinesterase activity in the medial septum was significantly higher in the Aβ_{25-35}/cellulose group than in the control or Aβ_{25-35}/GEB$_{500}$ groups ($P < 0.05$ for both) (Figure 5).
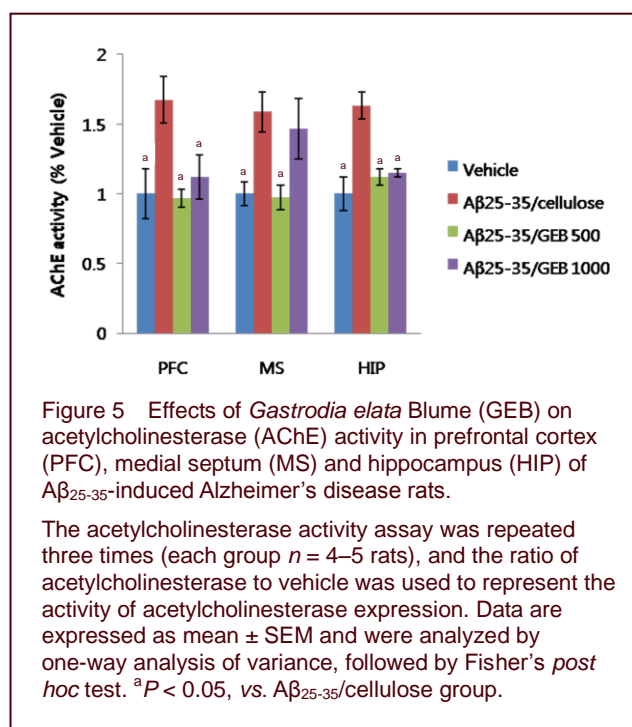

Figure 5   Effects of *Gastrodia elata* Blume (GEB) on acetylcholinesterase (AChE) activity in prefrontal cortex (PFC), medial septum (MS) and hippocampus (HIP) of Aβ_{25-35}-induced Alzheimer's disease rats.

The acetylcholinesterase activity assay was repeated three times (each group $n = 4$–5 rats), and the ratio of acetylcholinesterase to vehicle was used to represent the activity of acetylcholinesterase expression. Data are expressed as mean ± SEM and were analyzed by one-way analysis of variance, followed by Fisher's *post hoc* test. [a]$P < 0.05$, *vs.* Aβ_{25-35}/cellulose group.

DISCUSSION

To produce the rat model of Alzheimer's disease, we used bilateral hippocampal injections of Aβ_{25-35}. Significant differences between the control (vehicle-injected, cellulose-treated non-Alzheimer's disease rats) and the Aβ_{25-35}/cellulose groups were observed in the probe trial and in the distance traveled in the acquisition trial. In a preliminary experiment, significant differences in both escape latency and distance traveled were demonstrated between the control and Aβ_{25-35}/cellulose groups in the probe and acquisition trials (data not shown). These findings suggest that bilateral hippocampal injection of Aβ_{25-35} produces a valid rat model of Alzheimer's disease. The validity of this model is supported by our findings of a significant decrease in choline acetyltransferase expression and a significant increase in acetylcholinesterase activity in the Aβ_{25-35}/cellulose group relative to the control group, and by our observation of Congo red staining, revealing amyloid deposition in the hippocampus of the

$A\beta_{25-35}$/cellulose rats. Interestingly, Congo red deposition was not detected in the prefrontal cortex of the $A\beta_{25-35}$/cellulose group, suggesting that intracerebroventricular injection of $A\beta$ might result in broader deposition of peptide in areas such as the prefrontal cortex[21].

In the Morris water maze test, daily gastric gavage of *Gastrodia elata* Blume at 500 or 1 000 mg/kg enhanced cognition in the probe trial, but not in the working memory test. These findings suggest that *Gastrodia elata* Blume improves spatial reference memory, which is long-term, but not working memory, which is short-term. However, *Gastrodia elata* Blume had no apparent effect in our acquisition (learning) trials. These findings are similar to those of Hsieh *et al* [9], who reported that gastrodin and *p*-hydroxybenzyl alcohol facilitate memory consolidation and retrieval, but not acquisition. It should be noted that they evaluated memory function using a passive avoidance task and administered gastrodin and *p*-hydroxybenzyl alcohol only once. Our finding that *Gastrodia elata* Blume has a beneficial effect on long-term memory is consistent with a recent report[22] that gastrodin supplementation effectively relieves the impairment of long-term potentiation in the hippocampus of rats exposed to lead.

While our Alzheimer's disease ($A\beta_{25-35}$-injected) rats displayed a significant decrease in choline acetyltransferase levels in the hippocampus, *Gastrodia elata* Blume treatment restored choline acetyltransferase to levels comparable to those in non-Alzheimer's disease (vehicle-injected) rats. These results suggest that *Gastrodia elata* Blume protects against the reduction in choline acetyltransferase expression induced by amyloid deposits. The observed lack of an effect of $A\beta_{25-35}$ injection on choline acetyltransferase levels in the prefrontal cortex may be explained by the fact that little or no amyloid deposition occurred in the prefrontal cortex, as indicated by the lack of Congo red staining.

Injection of $A\beta_{25-35}$ induced significant increases in acetylcholinesterase activity in the rat prefrontal cortex, medial septum and hippocampus, whereas *Gastrodia elata* Blume treatment of $A\beta_{25-35}$-injected rats diminished acetylcholinesterase to levels comparable to those in control rats in all three areas. These findings suggest that *Gastrodia elata* Blume also protects against the increase in acetylcholinesterase expression induced by amyloid deposits. The deposition of $A\beta$ has been reported to be neurotoxic, resulting in increased acetylcholinesterase levels[23-25], which in turn promotes

aggregation of $A\beta$ peptide and amyloid deposition[26]. It should be noted that, apart from its well-known role in cholinergic transmission, acetylcholinesterase is involved in cell proliferation, differentiation, and responses to various insults, including stress and amyloid formation[27]. Given that minimal or no amyloid deposition was detected in the prefrontal cortex or the medial septum, it is not clear why intrahippocampal injection of $A\beta$ peptide induced significant changes in acetylcholinesterase activity in the prefrontal cortex and medial septum, but not in choline acetyltransferase expression in the same areas. Acetylcholinesterase might be more sensitive than choline acetyltransferase to the neurotoxic effects of amyloid deposition.

This report is the first to demonstrate that hippocampal injection of $A\beta$ peptide induces impairment of spatial memory and has detrimental effects on choline acetyltransferase expression and acetylcholinesterase activity, and that *Gastrodia elata* Blume treatment partially restores spatial memory function through its ability to restore these critical cholinergic enzymes to normal levels. Similar evidence supporting the detrimental effects of $A\beta$ peptide on the cholinergic system comes from reports that the injection of $A\beta$ peptide into rat brain impairs learning as a result of a hypofunction of the septohippocampal cholinergic system caused by a reduction in acetylcholine release mechanisms[28] and as a consequence of cholinergic neuronal degeneration[29]. The detrimental effect of $A\beta$ peptide on cholinergic markers may be related to its ability to self-aggregate and form Ca^{2+}-permeable channels in membranes, resulting in excessive Ca^{2+} influx and the induction of neurotoxic cascades[30-31]. Thus, the protective effect of *Gastrodia elata* Blume on spatial memory function may be related to its ability to counteract the harmful changes in levels of critical cholinergic enzymes induced by $A\beta$ peptide. The mechanisms underlying the neuroprotective effects of the herb have been investigated in several studies, and include the following: suppressing tumor necrosis factor-α-induced vascular inflammation[8], increasing Bcl-2 expression and inhibiting caspase-3 activation[5], antioxidative effect and GABAergic neuromodulation[32], suppressing nitric oxide synthase and microglial activation[6], and increasing blood supply to the vertebral-basilar artery[33]. Taken together, these findings suggest that *Gastrodia elata* Blume preserves choline acetyltransferase and acetylcholinesterase activities from $A\beta$ toxicity through various neuroprotective mechanisms, resulting in partial recovery from spatial memory impairment.

Several methodological considerations should be mentioned. First, in the present study, *Gastrodia elata* Blume was administered through gastric gavage, not through spontaneous oral intake. This method was selected because the *Gastrodia elata* Blume solution had a peculiar odor that might have caused inter-individual variation in intake and because forced feeding might have affected study parameters. In addition, contrary to the manufacturer's instructions, ethopropasine-HCl was not added to the incubation mixture in the QuantiChrom acetylcholinesterase assay. Since ethopropasine-HCl inhibits nonspecific cholinesterase activity, our acetylcholinesterase activity results represent total cholinesterase activity. Even though the physiological role of the nonspecific cholinesterase has not been established, it comprises approximately 10% of the total cholinesterase activity[34]. Therefore, this limitation should be considered in interpreting our acetylcholinesterase activity results. Furthermore, we did not investigate how preservation of choline acetyltransferase expression by *Gastrodia elata* Blume impacts acetylcholine levels. In future studies, other cholinergic markers such as acetylcholine and vesicular acetylcholine transporter, as well as central nervous system inflammatory markers, should be examined to clarify the mechanisms by which *Gastrodia elata* Blume enhances cognitive performance.

In conclusion, the present study demonstrated that (i) intrahippocampal injection of Aβ peptide impairs spatial reference memory and has detrimental effects on choline acetyltransferase expression and acetylcholinesterase activity in the rat brain; and (ii) long-term administration of *Gastrodia elata* Blume partially reverses these effects. These findings suggest that *Gastrodia elata* Blume has potential therapeutic value for Alzheimer's disease.

MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
Experiments were performed from February 2011 to December 2011 at the Research Institute of Clinical Medicine, Chonbuk National University-Biomedical Research Institute, Republic of Korea.
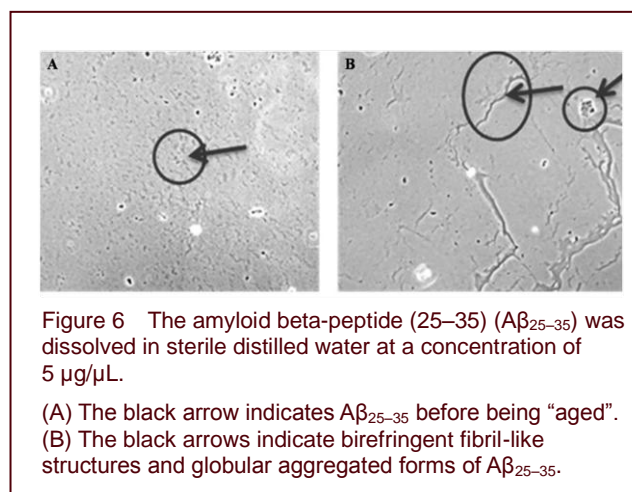
Materials
Male Wistar rats (8 weeks old, 220–250 g) were purchased from Orient Bio Inc. (Seoul, Republic of Korea) and were used throughout the study. The rats were housed in a temperature-controlled room under a 12-hour light-dark cycle (lights on from 7:00 a.m. to 7:00 p.m.) with food and water *ad libitum*. Experiments were carried out between 9:00 a.m. and 4:00 p.m. All possible efforts were made to minimize animal suffering and the number of animals used, in accordance with the Guidelines for Animal Experiments of Chonbuk National University Medical School, Republic of Korea.

Methods
Intrahippocampal injection of Aβ25-35 and administration of Gastrodia elata Blume and cellulose
Aβ25-35 (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile distilled water at a concentration of 5 µg/µL. To obtain the aggregated form of Aβ25-35, the peptide solution was placed in an incubator at 37°C for 96 hours. Light microscopic observation demonstrated the precipitation of insoluble globular aggregates (Figure 6).

Figure 6   The amyloid beta-peptide (25–35) (Aβ25–35) was dissolved in sterile distilled water at a concentration of 5 µg/µL.

(A) The black arrow indicates Aβ25–35 before being "aged".
(B) The black arrows indicate birefringent fibril-like structures and globular aggregated forms of Aβ25–35.

The rats were anesthetized with intraperitoneal injection of a 4:1 mixture of ketamine (Yuhan, Seoul, Korea) and xylazine (Bayer, Seoul, Korea) and placed in a David Kopf stereotaxic instrument (IWOO Scientific Corporation, Seoul, Korea) with the incisor bar set 3.3 mm below the interaural line. Holes were drilled in the skull, and 2 µL of Aβ25-35 (10 µg) or vehicle was bilaterally infused into the hippocampus (CA1 region) through a stainless-steel 25-gauge cannula (Small Parts Inc., Seattle, WA, USA) connected *via* polyethylene tubing to a 10 µL Hamilton microsyringe. The coordinates used were –3.6 mm anteroposterior from the bregma, +2.0 mm bilateral from the midline, and –2.7 mm ventral from the skull according to the stereotaxic atlas[35]. The correct placement of the cannula was verified in preliminary experiments by the injection of India ink. The skull holes were filled with bone wax (Lukens Bone Wax; Angiotech Inc, Vancouver, BC,

Canada) to prevent outflow, and the skin was sutured. One day after the injection of $A\beta_{25-35}$ or vehicle, rats received their first dose of powdered *Gastrodia elata* Blume (Muju Tianma Native Local Industrial Center, Korea) dissolved in water (500 or 1 000 mg/2 mL per kg body weight) or 0.5% cellulose (2 mL/kg) *via* gastric gavage. Dosing was continued daily throughout the experimental period of 52 days (6 weeks plus 10 days for the Morris water maze test).

Gastrodia elata Blume preparation

The rhizome of *Gastrodia elata* Blume (*Tianma*) was collected from Muju, Korea and was provided by Muju Tianma Native Local Industrial Center. Whole dried tubers of *Gastrodia elata* Blume were hammered into smaller pieces and ground to a fine powder. *Gastrodia elata* Blume was dissolved in deionized water (1.25 or 2.5 g powder in 5 mL deionized water) to yield stock solutions of 250 and 500 mg/mL for use in experiments.

Morris water maze test

Morris water maze testing was initiated after 6 weeks of daily *Gastrodia elata* Blume or cellulose dosing, which continued throughout the 10-day water maze experiment. The water maze consisted of a circular pool (diameter: 180 cm; height: 60 cm) with a black (painted) interior. A small (diameter: 10 cm), black (painted), non-slippery, Plexiglas platform was immersed 1 cm below the water surface so that it was invisible in the black tank. The water temperature was maintained at 25 ± 2°C with a Thermo Heater (Warmtone, Shenzhen, Guangdong, China). Three extra-maze cues (a black, white, or black/white-striped circle, square or triangle, each 60 cm × 80 cm) were hung on the surrounding black curtain in a dimly lit (40 lx at water level) and soundproof test room. A digital camera mounted above the water maze captured five images per second and transmitted them to a personal computer running Smart software (Panlab, Barcelona, Spain) for calculation of the escape latency, distance traveled and average swim speed for each trial. Four points, equally spaced along the circumference of the water maze, were arbitrarily designated north (N), east (E), south (S) and west (W) to create NE, SE, NW and SW quadrants of equal size.

Visible platform training

An initial visual-training day was used to familiarize each animal with the water maze and allow between-treatment comparison of possible differences in visual discrimination and motor control. This training was composed of three trials using a 10-cm-diameter visible cued platform positioned in the center of the

water maze. For each trial, the animal was positioned against the maze wall at a randomly chosen start position (N, E, S or W) and released into the water maze. The rat was allowed a maximum of 90 seconds to locate the escape platform. Animals failing to locate the platform were guided to the platform by the experimenter. The animals were then allowed to spend 15 seconds on the platform and 45 seconds in the home cage before commencement of the next trial. The inter-trial interval was therefore approximately 1 minute. All animals acquired the swimming response and were able to climb onto the escape platform by the end of the third trial.

Reference memory acquisition

After the initial training day, each animal was tested for reference memory using the submerged invisible platform with a visual cue placed in a fixed position (SW). Each animal participated in five trials per day for 5 consecutive days using a procedure similar to that used for the training task. When an animal failed to locate the platform within the 90-second time limit, an escape latency of 90 seconds was assigned. Between trials, the water was strained of feces and gently swirled to diffuse odor cues. A different starting position was used for each trial performed on the same day, and the sequence of starting positions was varied from day to day. Acquisition of reference memory was indicated by a gradual reduction in escape latency and the distance traveled as the training progressed.

The day after the acquisition training period ended (the 6th day), a probe test was performed, in which the platform was removed and the animals were allowed to search for it for 90 seconds. The time spent and distance traveled in the target quadrant (*i.e.*, where the platform was located during the acquisition training sessions) were computed (SMART software, Panlab, Barcelona, Spain).

Working memory performance

Working memory was tested after the reference memory experiment using new extra-maze cues placed in a novel spatial arrangement. The animals were tested over 4 consecutive days, with four trials per day, using a procedure similar to that used for the visual task. The submerged platform was placed at a new location on each test day, but remained unchanged between the four trials on a given day. The start positions were counterbalanced across days and trials in a given day. The escape latency and distance traveled values for each trial number (*i.e.*, trials 1–4) over the 4 days were averaged, and these average values were used as a measure of the working

memory performance for that trial number.

Preparation of brain tissue

After behavioral tests were completed, rats were killed by decapitation under ether anesthesia. The brains were quickly removed and frozen in liquid nitrogen. From the appropriate coronal sections in a pre-cooled cryostat (Richard-Allan Scientific, Kalamazoo, MI, USA), the prefrontal cortex, medial septum, and CA1 and dentate gyrus regions of the hippocampus were excised bilaterally using a 2.0-mm Harris Uni-Core micropunch (Electron Microscopy Sciences, Hatfield, PA, USA). The approximate bregma levels from which the punches were acquired were 3.70–2.20 mm and 0.70–0.20 mm for the prefrontal cortex and medial septum, respectively, and –3.60 to –4.30 mm for the hippocampus. Tissues were stored at –80°C before use.

Two rats selected randomly from each group for Congo red staining were transcardially perfused under deep anesthesia with ketamine/xylazine (4:1), then with 200 mL of calcium-free Tyrode's solution, and thereafter with 200 mL of ice-cold 4% paraformaldehyde in 0.1 M PBS. Each brain was removed immediately after perfusion, placed in fresh fixative for at least 4 hours, and then transferred sequentially to 20% sucrose in PBS and to 30% sucrose in PBS for 12 and 24 hours, respectively. The brains were embedded with Tissue-Tek® Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetek USA, Inc.) in liquid nitrogen and kept at –80°C until further use. Coronal sections (10-μm thickness) were cut using a cryostat microtome (Richard-Allan Scientific). The sections were collected in PBS in 24-well plates for Congo red staining.

Congo red staining

Congo red staining[36] was used to identify amyloid deposits in the prefrontal cortex, medial septum and CA1 region of the hippocampus. Briefly, brain sections mounted on slides were hydrated in water (5 minutes), stained in 0.5% Congo red reagent (Sigma-Aldrich Inc.) for 15–20 minutes, rinsed in distilled water, quickly differentiated (5–10 dips) in alkaline alcohol solution, rinsed in tap water (1 minute), counterstained with Gill's hematoxylin (30 seconds), rinsed in tap water (2 minutes), dehydrated with 95% and 100% ethanol, cleared in xylene, covered with balsam, and cover-slipped. Under a light microscope, the amyloid deposits were red to brown, whereas the nuclei were blue.

Western blot analysis

Tissue samples (prefrontal cortex, medial septum, CA1

and dentate gyrus regions of the hippocampus) were homogenized in 20 mM ice-cold Tris-HCl (pH 7.4) containing 1% protease and phosphatase inhibitor cocktails (Sigma) using a hand homogenizer with a Teflon pestle (Vintage Thomas, Philadelphia, PA, USA). The homogenates were centrifuged for 15 minutes at 14 000 r/min at 4°C, and the resulting supernatant fractions were used for western blot analyses. Protein concentrations were determined using the Bradford assay[37] with commercial reagents (Bio-Rad Laboratories, Foster City, CA, USA) and spectrophotometric measurement at 595 nm. The protein samples (20 μg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to a hydrophobic polyvinylidene difluoride membrane. The membrane was blocked in 5% (w/v) dried non-fat skim milk in PBS containing 0.1% Tween 20 for 120 minutes at room temperature. The membranes were then incubated overnight at 4°C with a polyclonal rabbit anti-choline acetyltransferase antibody (AB143; diluted 1:10 000; Chemicon International Inc., Temecula, CA, USA) or β-actin antibody (4967; diluted 1:1 000; Cell Signaling Technology, Inc., Danvers, MA, USA) in 5% non-fat milk. The next day, the membranes were washed with PBS containing 0.1% Tween 20, and the primary antibody was detected using a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (PI-1000; Vector; Burlingame, CA, USA) diluted 1:20 000 in PBS (60 minutes, 25°C).

The protein band corresponding to choline acetyltransferase (68 kDa) was detected using an enhanced chemiluminescence immunoblotting detection system (RPN2232; GE Healthcare, UK). The band intensities were evaluated with an LAS-3000plus luminescent image analyzer (Fuji Photo Film Company, Kanagawa, Japan). Band densities were quantified using Multi Gauge version 3.0 (Fujifilm, Tokyo, Japan).

Acetylcholinesterase assay

Tissue samples (prefrontal cortex, medial septum, CA1 and dentate gyrus regions of the hippocampus) were homogenized in 20 volumes of cold 0.1 M phosphate buffer (pH 7.5). The homogenates were centrifuged at 14 000 r/min for 15 minutes at 4°C, and 10 μL aliquots of the supernatant fractions were used in a commercial acetylcholinesterase assay (QuantiChrom acetylcholinesterase Assay Kit; BioAssay Systems, Hayward, CA, USA). This assay was performed based on an improved Ellman method[38] in which the thiocholine produced by the action of

acetylcholinesterase formed a yellow color with 5,5'-dithiobis(2-nitrobenzoic acid). The intensity of the product color, measured at 412 nm, was proportionate to the enzyme activity in the sample.

Statistical analysis

Results are presented as mean ± SEM. The data were analyzed with SPSS 12.0 (IBM, Armonk, NY, USA) using two-way repeated measures analysis of variance or one-way analysis of variance, followed by Fisher's *post hoc* test. In all the cases, differences were considered statistically significant at $P < 0.05$.

REFERENCES

[1] Xu X. Practical Chinese material medica for health care. In: Xu X, ed. The English-Chinese Encyclopedia of Practical Traditional Chinese Medicine. Beijing: Beijing Higher Education Press. 1992.

[2] Hsieh MT, Peng WH, Wu CR, et al. The ameliorating effects of the cognitive-enhancing Chinese herbs on scopolamine-induced amnesia in rats. Phytother Res. 2000;14:375-377.

[3] Kim HJ, Moon KD, Lee DS, et al. Ethyl ether fraction of Gastrodia elata Blume protects amyloid β peptide-induced cell death. J Ethnopharmacol. 2003; 84:95-98.

[4] Shuchang H, Qiao N, Piye N, et al. Protective effects of Gastrodia elata on aluminium-chloride-induced learning impairments and alterations of amino acid neurotransmitter release in adult rats. Restor Neurol Neurosci. 2008;26:467-473.

[5] Yu SS, Zhao J, Zheng WP, et al. Neuroprotective effect of 4-hydroxybenzyl alcohol against transient focal cerebral ischemia via anti-apoptosis in rats. Brain Res. 2010; 1308:167-175.

[6] Hsieh CL, Chen CL, Tang NY, et al. Gastrodia elata BL mediates the suppression of nNOS and microglia activation to protect against neuronal damage in kainic acid-treated rats. Am J Chin Med. 2005;33(4):599-611.

[7] Chen PJ, Hsieh CL, Su KP, et al. The antidepressant effect of Gastrodia elata Bl. on the forced-swimming test in rats. Am J Chin Med. 2008;36:95-106.

[8] Hwang SM, Lee YJ, Kang DG, et al. Anti-inflammatory effect of Gastrodia elata rhizome in human umbilical vein endothelial cells. Am J Chin Med. 2009;37:395-406.

[9] Hsieh MT, Wu CR, Chen CF. Gastrodin and p-hydroxybenzyl alcohol facilitate memory consolidation and retrieval, but not acquisition, on the passive avoidance task in rats. J Ethnopharmacol. 1997;56:45-54.

[10] Wu CR, Hsieh MT, Huang SC, et al. Effects of Gastrodia elata and its active constituents on scopolamine-induced amnesia in rats. Planta Med. 1996;62:317-321.

[11] Kao NN, Yu SZ, Liu RH, et al. Improving effects of Gastrodia elata Bl on D-galactose-induced aging in mice. Zhongcaoyao. 1994;25:521-523.

[12] Kim DS, Kim JY, Han YS. Alzheimer's disease drug discovery from herbs: neuroprotectivity from beta-amyloid (1-42) insult. J Altern Compliment Med. 2007;13(3):333-340.

[13] Perry EK, Tomlinson BE, Blessed G, et al. Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. BMJ. 1978;2:1457-1459.

[14] Wilcock GK, Esiri MM, Bowen DM, et al. Alzheimer's disease: correlation of cortical choline acetyltransferase activity with the severity of dementia and histological abnormalities. J Neurol Sci. 1982;57:407-417.

[15] García-Ayllón MS, Silveyra MX, Sáez-Valero J. Association between acetylcholinesterase and β-amyloid peptide in Alzheimer's cerebrospinal fluid. Chem Biol Interact. 2008;175:209-215.

[16] Deng J, Shen C, Wang YJ, et al. Nicotine exacerbates tau phosphorylation and cognitive impairment induced by amyloid-beta 25–35 in rats. Eur J Pharmacol. 2010;637: 83-88.

[17] Díaz A, De Jesús L, Mendieta L, et al. The amyloid-β 25-35 injection into the CA1 region of the neonatal rat hippocampus impairs the long-term memory because of an increase of nitric oxide. Neurosci Lett. 2010;468:151-155.

[18] Rush DK, Aschmies S, Merriman MC. Intracerebral β-amyloid(25–35) produces tissue damage: is it neurotoxic? Neurobiol Aging. 1992;13:591-594.

[19] Games D, Khan KM, Soriano FG, et al. Lack of Alzheimer pathology after β-amyloid protein injections in rat brain. Neurobiol Aging. 1992;13:569-576.

[20] Stein-Behrens B, Adams K, Yeh M, et al. Failure of β-amyloid protein fragment 25–35 to cause hippocampal damage in the rat. Neurobiol Aging. 1992;13:577-579.

[21] Maurice T, Lockhart BP, Privat A. Amnesia induced in mice by centrally administered β-amyloid peptides involves cholinergic dysfunction. Brain Res. 1996;706: 181-193.

[22] Yong W, Xing TR, Wang S, et al. Protective effects of gastrodin on lead-induced synaptic plasticity deficits in rat hippocampus. Planta Med. 2009;75:1112-1117.

[23] Arendt T, Bruckner MK, Lange M, et al. Changes in acetylcholinesterase and butyrylcholinesterase in Alzheimer's disease resemble embryonic development: a study of molecular forms. Neurochem Int. 1992;21: 381-396.

[24] Fodero LR, Mok SS, Losic D, et al. α7-nicotinic acetylcholine receptors mediate an $A\beta_{1-42}$-induced increase in the level of acetylcholinesterase in primary cortical neurons. J Neurochem. 2004;88:1186-1193.

[25] Sberna G, Saez-Valero J, Beyreuther K, et al. The amyloid β-protein of Alzheimer's disease increases acetylcholinesterase expression by increasing intracellular calcium in embryonal carcinoma P19 cells. J Neurochem. 1997;69:1177-1184.

[26] Inestrosa NC, Alarcon R, Arriagada J, et al. Blood markers in Alzheimer's disease: subnormal acetylcholinesterase and butyrylcholinesterase in lymphocytes and erythrocytes. J Neurol Sci. 1994;122:1-5.

[27] Grisaru D, Sternfeld M, Eldor A, et al. Structural roles of acetylcholinesterase variants in biology and pathology. Eur J Biochem. 1999;264:672-686.

[28] Abe E, Casamenti F, Giovannelli L, et al. Administration of amyloid β-peptides into the medial septum of rats decreases acetylcholine release from hippocampus in vivo. Brain Res. 1994;636:162-164.

[29] Nitta A, Itoh A, Hasegawa T, et al. β-amyloid protein-induced Alzheimer's disease animal model. Neurosci Lett. 1994;170:63-66.

[30] Arispe N, Rojas E, Pollard B. Alzheimer disease amyloid β protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminium. Proc Natl Acad Sci U S A. 1993;90:567-571.

[31] Mattson MP, Barger SW, Cheng B, et al. β-amyloid precursor protein metabolites and loss of neuronal Ca^{2+} homeostasis in Alzheimer's disease. Trends Neurosci.1993;16:409-414.

[32] Ha JH, Lee DU, Lee JT, et al. 4-hydroxybenzaldehyde from Gastrodia elata BL is active in the antioxidation and GABAergic neuromodulation of the rat brain. J Ethnopharmacol. 2000;73:329-333.

[33] Wang ML, Lu XH, Wang FX. Observations of curative effect of Tianxuanqing injection on atherosclerotic vertebrobasilar ischemia. Zhongcaoyao. 2001;32: 823-825.

[34] Lassiter TL, Barone Jr S, Padilla S. Ontogenetic differences in the regional and cellular acetylcholinesterase and butyrylcholinesterase activity in the rat brain. Brain Res. 1998;105:109-123.

[35] Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. 2nd ed. New York: Academic Press. 1986.

[36] Johansson S, Radesäter AC, Cowburn RF, et al. Modelling of amyloid beta-peptide induced lesions using roller-drum incubation of hippocampal slice cultures from neonatal rats. Exp Brain Res. 2006;168:11-24.

[37] Simonian MH, Smith JA. Spectrophotometric and colorimetric determination of protein concentration. Curr Protoc Mol Biol. 2006;Chapter 10:Unit 10.

[38] Ellman GL, Courtney KD, Andres V Jr, et al. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961;7:88-95.

(Reviewed by Lakshmana MK, Patel B, Jin XM, Li YW)
(Edited by Li CH, Song LP)