Effect of Chaenomeles sinensis Extract on Choline Acetyltransferase Activity and Trimethyltin-Induced Learning and Memory Impairment in Mice

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Alzheimer’s disease (AD) is a progressive neurodegenerative disease of multifactorial causes.5) AD is characterized by a strong deficiency in acetylcholine (ACh) and choline acetyltransferase (ChAT) activity observed mainly in cholinergic neurons in the basal brain, hippocampus, and cerebral cortex.2,3) The early and most consistently reproduced finding is a profound reduction in the ChAT activity in the neocortex, which correlates positively with the severity of dementia.4) Reduced choline uptake, ACh release, and the loss of cholinergic neurons from the basal forebrain region further indicate a selective presynaptic cholinergic deficit in the hippocampus and neocortex of individuals with AD.5) These observations suggest that ChAT may play an important role in facilitation of learning and memory. Consequently, a decline in cholinergic neurotransmission may play a role in AD.6,7)

In this present work, we screened traditional Korean plants for the presence of ChAT activators and selected the ethanol extract of Chaenomeles sinensis Koehne showed the highest ChAT-activating effect in vitro in an assay that used human neuroblastoma cells and [14C]acetyl-CoA. The active compound was speculated to be stearic acid methyl ester (SAME). In an in vivo experiment, C. sinensis extract and SAME improved trimethyltin (TMT)-induced deficits in learning and memory in mice as assessed by a Y-maze behavioral test and a passive avoidance test. The C. sinensis extract might attenuate the TMT-induced brain disorder. This study suggests that SAME from C. sinensis might be useful in the treatment of Alzheimer’s disease.

Key words Chaenomeles sinensis; learning; memory; cognitive impairment; stearic acid methyl ester; choline acetyltransferase

Note

The aim of this study was to search for a novel choline acetyltransferase (ChAT) activator from plants traditionally grown in Korea. An ethanol extract from Chaenomeles sinensis Koehne showed the highest ChAT-activating effect in vitro in an assay that used human neuroblastoma cells and [14C]acetyl-CoA. The active compound was speculated to be stearic acid methyl ester (SAME). In an in vivo experiment, C. sinensis extract and SAME improved trimethyltin (TMT)-induced deficits in learning and memory in mice as assessed by a Y-maze behavioral test and a passive avoidance test. The C. sinensis extract might attenuate the TMT-induced brain disorder. This study suggests that SAME from C. sinensis might be useful in the treatment of Alzheimer’s disease.

Chemicals

Fetal bovine serum, trypsin–ethylenediamine-tetraacetic acid (EDTA), and penicillin–streptomycin were purchased from Gibco-BRL (Gaithersburg, MD, U.S.A.). Acetyl coenzyme A [acetyl-1-14C] was purchased from NEN (Boston, MA, U.S.A.) and absolute ethyl alcohol was from Hayman (U.K.). Aquasol was purchased from Packard (CT, U.S.A.); eserine hemisulfide, choline chloride, EDTA, sodium tetraphenylborate, and toluene were purchased from Sigma Co. (St. Louis, MO, U.S.A.). Silica gel and analytical thin-layer chromatography (TLC) plates were purchased from Merck Co. (Darmstadt, Germany) and C₁₈ µ-Bondapak column was from Waters Co. (Miliford, MA, U.S.A.).

Cell Line and Medium

MC-IXC cells (a human neuroblastoma cell line) were obtained from American Type Culture Collection (ATCC, CRL number: 2270). Cells were cultured and maintained according to previous methods.11)

Choline Acetyltransferase Preparation and Choline Acetyltransferase (ChAT) Assay

When the culture was 85–90% confluent, the medium was removed and the cells were rinsed with phosphate buffer saline. To add trypsin–EDTA, cell detachment with new medium mixtures were centrifuged for 3 min at 750g. The resulting mixtures were homogenized using a homogenizer (Glas; Col, Terre Haute, IN, U.S.A.) in homogenizing buffer [20mM Tris–HCl (pH 7.5), 150mM NaCl, 10mM MgCl₂, and 0.5% Triton X-100] and cen-

Experimental

Plant Materials

Chaenomeles sinensis and other plants (Table 1) were purchased from Kyungdong Market (an oriental drug store) in Seoul, Korea.

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The plates were visualized under visible and ultraviolet light. The extracts were applied on the plate in a development chamber and dried (TLC). The extract acetylcholine, a cocktail of toluene–aqueous (250 : 1; 3 parts of acetone ethanol). Radioactivity was measured with a Beckman scintillation counter (Fullerton, U.S.A.). ChAT activity was calculated from the conversion of [14C]acetyl coenzyme A to [14C]acetylcholine.12)

### Table 1. Activation of ChAT by Extracts from Traditional Korean Plants

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mimosa (leaf)</td>
<td>Albizia julibrissin DURAZZ</td>
<td>22.9</td>
</tr>
<tr>
<td>Chinese skullcap (root)</td>
<td>Scutellaria baicalensis</td>
<td>19.3</td>
</tr>
<tr>
<td>Chinese quince (fruit)</td>
<td>Chaenomeles sinensis KOEHNE</td>
<td>24.3</td>
</tr>
<tr>
<td>Japanese sweetflag (rhizome)</td>
<td>Acorus gramineus</td>
<td>22.5</td>
</tr>
<tr>
<td>Channelled waterplantain (rhizome)</td>
<td>Alisma canaliculatum</td>
<td>−7.4</td>
</tr>
<tr>
<td>Cinnamon (bark)</td>
<td>Cinnamomum loureiri</td>
<td>0</td>
</tr>
<tr>
<td>Prickly water lily (fruit)</td>
<td>Euryale ferox</td>
<td>19.3</td>
</tr>
<tr>
<td>Waxgourd (fruit)</td>
<td>Benincasa cerafera SAVI</td>
<td>10.3</td>
</tr>
<tr>
<td>Chrysanthemum (flower)</td>
<td>Chrysanthemum morifolium</td>
<td>18.5</td>
</tr>
<tr>
<td>Moutan cortex radics (petal)</td>
<td>Paeonia suffruticosa ANDR.</td>
<td>−11.0</td>
</tr>
<tr>
<td>Ginger (rhizome)</td>
<td>Zingiber officinale</td>
<td>19.7</td>
</tr>
<tr>
<td>Pomegranate (fruit)</td>
<td>Punica granatum</td>
<td>0.4</td>
</tr>
<tr>
<td>Epimedium koreanum (leaf)</td>
<td>Epimedium koreanum NAKAI</td>
<td>−9.6</td>
</tr>
</tbody>
</table>

Samples were ground until the powder could be passed through a fine screen (about 1 mm). Evaporated ethanol extracts of plant materials were used as samples for screening. Samples were dissolved (1 mg/mL) in distilled water containing 5% (v/v) DMSO.11)

Activity (%) = ([sample reaction – enzyme reaction] / enzyme reaction) × 100

trifuged for 10 min at 10000×g. The supernatants were used for determination of ChAT activity.

The mixture (crude enzyme, 200 µL; sample, 100 µL; 50 mM K-phosphate buffer, 82 µL; 0.1 mM eserine, 4 µL; 8 mM chloride, 4 µL; and 25 µL coenzyme A [acetyl-1-14C], 10 µL) was incubated for 1 h at 37°C. The reaction was stopped by the addition of 5 mg/mL sodium tetraphenylborate (1 mL). To extract acetylcholine, a cocktail of toluene–aqueous (250 : 1; 2 mL) was added and the mixture was centrifuged for 10 min at 10000×g. To determine ChAT activity, 1 mL of the organic layer was transferred to a scintillation vial (Greiner Labortechnik, Germany) containing 1 mL of another cocktail solution [7 parts of toluene–aqueous (250 : 1) and 3 parts of absolute ethanol]. Radioactivity was measured with a Beckman scintillation counter (Fullerton, U.S.A.). ChAT activity was calculated from the conversion of [14C]acetyl coenzyme A to [14C]acetylcholine.12)

### Isolation of ChAT Activation from the C. sinensis Extract

Dried C. sinensis (fruits) was ground into powder. The powder was dissolved in ethanol by shaking at room temperature for 24 h. The extract was filtered through a Whatman filter paper No. 41 (Whatman International Ltd., U.K.) and dried in a rotary evaporator at 40°C. The sample (10.82 g) was dissolved in chloroform. Silica-gel was separated by a stepwise chloroform–ethanol gradient 100 : 0, 90 : 10, 80 : 20, 70 : 30, 60 : 40, 50 : 50, 40 : 60, 30 : 70, 20 : 80, 10 : 90, 0 : 100 (v/v, repeated 3 times) at a flow rate of 3.3 mL/min (silica-gel column chromatography). The bed volume of solvent was 650 mL. Thirty three fractions were evaporated and tested for ChAT activation. The fraction (the third fraction of the 40:60 fraction; 70 mg) was evaporated at 40°C, dissolved in ethanol and repeatedly 0.5 µL aliquots were spotted on the plate in a development chamber and dried (TLC). The plates were visualized under visible and ultraviolet light (254 and 360 nm) and the Rf values were evaluated. Analysis was conducted with a Waters apparatus using a reverse phase C18 µ-Bondapak column (3.9×300 mm) at a flow rate of 1 mL/min and detection wavelength range of 200–800 nm; detection was performed at 210 nm. Samples were dissolved in ethanol at a concentration of 10 mg/mL; the injection volume was 20 µL. The mass spectrometer (JMS AX505WA; JEOL, Japan) was operated in the electron ionization (EI) mode. Samples (0.5 mg) were analyzed in MeOH (electron ionization mass spectrometry; EI-MS). 13C-/1H-NMR (600 MHz) was carried out using an Avance-600 spectrometer (Bruker, Germany). Samples (2 mg) were analyzed in MeOH (nuclear magnetic resonance spectroscopy; NMR).

**Animals** ICR mice (male, 5 weeks old) were purchased from Daehan Bio (Gyeonggido, Korea). Mice were housed (eight per cage) at 24±1°C and humidity of 55% with a 12 h light–dark cycle for 3 weeks. During this time, the mice had free access to feed and water. C. sinensis extract was mixed with water and supplied to mice instead of drinking water at 400, 800, or 1200 mg/kg of body weight per day. After C. sinensis extract in vivo testing, stearic acid methyl ester (SAME) was dissolved in water and supplied to mice at 5, 10, 20, or 40 mg/kg of body weight per day. Trimethylin (TMT; Sigma Co.) was dissolved in 0.85% (w/v) NaCl solution and injected intraperitoneally (i.p.). The injection was 2.5 mg/kg. All experimental procedures were performed according to the guidelines established by the Animal Care and Use Committee of Korea University.

**In Vivo Behavior Tests** Y-Maze and passive avoidance test were performed according to previous methods.13) The Y-maze test was performed 2 d after TMT injection. The passive avoidance test was performed 4 d after TMT injection.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Reduction Assay** Cell viability was measured by the MTT reduction assay. MC-IXC cells were plated at a density of 10^5–10^6 cells in a 96-well plate and preincubated with various concentrations of SAME for 48 h. After incubation with TMT (300 µmol) cell viability was determined on the basis of the amount of MTT formazan crystals.14)

**Statistical Analysis** Results were expressed as the mean±standard deviation (S.D.). The data of in vivo tests were
analyzed using Duncan’s multiple range tests in Statistical Analysis System (SAS). The statistical significance of differences among groups was calculated by one way ANOVA.

**Results**

**Screening of Extracts from Traditional Korean Plants for Their Ability to Activate ChAT** Among extracts from 13 species of edible and herbal plants tested, the extract from *C. sinensis* demonstrated the highest ability to activate ChAT in MC-IXC cells (Table 1). Therefore, this extract was selected for further analysis.

**Isolation of ChAT Activator from the *C. sinensis* Extract**

The ethanol extract of *C. sinensis* was solvent-partitioned with hexane, chloroform, and ethyl acetate, respectively. The first fraction of chloroform exhibited the highest activity (data not shown). The first chloroform fraction was subjected to silica-gel chromatography. The ability to activate the enzyme was calculated for each fraction relative to the positive control values. Fraction 21st (chloroform–ethanol, 40 : 60, v/v) had the highest ability to activate ChAT (data not shown). Fraction 21st from silica-gel chromatography was evaporated and separated by TLC. The range of the *Rf* values was 0.37–0.77.

The third fraction (*Rf* value of 0.77) showed the highest activity (106.64%, data not shown) and was selected for further purification. Isolation fractions were evaporated and tested for ChAT activation. Purification of the ChAT activator from *C. sinensis* extract by HPLC, EI-MS and NMR. A peak was observed, showing the molecular weight of the active component to be 284.48 Da (calculated for C\textsubscript{18}H\textsubscript{36}O\textsubscript{2}: 284.48) and called a stearic acid (SAME) (data not shown).

**In Vivo Behavior Tests (the *C. sinensis* and SAME)**

Spontaneous alternation behavior was investigated using the Y-maze test (Table 2). TMT-injected mice exhibited significantly impaired spatial working memory (a 18% decrease in alternation behavior in comparison with that of the control group). Treatment of TMT-injected mice with the ethanol extract of *C. sinensis* and SAME increased spontaneous alternation behavior in comparison with that of TMT-injected mice. In contrast, the number of arm entries did not change among any of the experiment groups. In the passive avoidance test, TMT-injected mice exhibited a significant reduction (a 263s decrease) in the step-through latency in comparison with that of the control group (Table 2). Treatment of TMT-injected mice with the ethanol extract of *C. sinensis* and SAME at-
tenuated the TMT-induced impairment, with the maximal effect observed at 1200 mg/kg. This may indicate that SAME treatment improved learning ability and memory.

**ChAT Activity is Stimulated by SAME**  The activation of ChAT by SAME was assessed. SAME increased ChAT activity in a concentration-dependent manner (0.001 μM, 102.1%; 0.01 μM, 110.0%; 0.1 μM, 125.2%). The activation capacity of SAME is better than that obtained using 393.3 mM of daidzein (Fig. 1A).

**MTT Reduction Test** To measure the protective effect of the various concentration of SAME against TMT-induced MC-IXC cell death, cell viability was evaluated by MTT assay. Compared with the control, concentration and cell protection ability of the SAME (0.001–0.1 μM) were proportional (Fig. 1B).

**Discussion**
This experiment is to find the ChAT activator from the ethanol extract of *C. sinensis*. Trimethyltin is a potent neurotoxin, particularly for the hippocampus and results in extreme behavioral and cognitive deficits in both humans and experimental animals. In this study, dietary supplementation with *C. sinensis* extract improved cognitive impairment in TMT-injected mice. Remarkably, the group administered 1200 mg/kg of the extract had significantly improved learning and memory in comparison with the TMT-only group.

To find a novel biochemical and physiological ChAT activator, we screened various traditional Korean plants. We purified an active compound from *C. sinensis* by using solvent partitioning, silica-gel column chromatography, TLC, and HPLC, and analyzed it by EI-MS and 13C-/1H-NMR to predict its chemical structure. The main activator was identified as SAME. Daidzein, used in this study as a positive control, is a major isoflavonoid in dietary soybean and is thought to play an important role in cancer prevention. Furthermore, it is a plant derived estrogen-like compound that shows protective effect on memory in cognition tests in mice. We found that, similar to daidzein, SAME is able to ameliorate TMT-induced impairment of memory and cognition in an *in vivo* test. Dietary supplementation with SAME significantly improved the percentage of spatial alternation behavior in TMT-injected mice in a dose-dependent manner. These results suggest that SAME improves acquisition of short-term memory in TMT-treated mice. To investigate the cell viability of MC-IXC cell induced by TMT, results of processing by the SAME concentration, SAME concentration and cell protection effect were proportional. This result indicate that SAME has the ability to protect MC-IXC cells from neurotoxicity. However, it has not previously been reported to mitigate dementia. SAME is a long-chain fatty acid consisting of 18 carbon atoms without double bonds, which is present in fairly constant proportion in beef, pork, lamb, and veal (approximately 9–12% of the total fatty acid content), with lower proportions found in poultry (approximately 6–7% of the total fatty acid content). In addition, SAME has a beneficial effect on clotting factors and may result in a less thrombogenic state.

The brain is rich in diverse fatty acids (saturated, mono-unsaturated, and polyunsaturated), with the complex lipids composed of fatty acids with chain lengths ranging from less than 16 to more than 24 carbons. SAME is one of the most common fatty acids in brain phospholipids; it originates in the circulation and is sequestered from blood by the brain along with precursor fatty acids. Several fatty acids can function as natural ligands of peroxisome proliferator-activated receptors (PPARs) to regulate lipid homeostasis. PPAR-α and PPAR-γ can significantly protect the brain from ischemic or oxidative insult. SAME can function as an endogenous PPAR-α ligand. Furthermore, as a natural ligand of PPAR, SAME may protect from dementia after an ischemic insult. Vascular-ischemic dementia or vascular cognitive impairment (VCI) is caused by or related to various types of cerebrovascular lesions and ischemic brain damage. Patients with VCI develop dementia according to standard diagnostic criteria. Vascular disorders are important in chronic neurodegeneration in AD. A neuroprotective effect of SAME on neurotoxicity induced by oxygen and glucose deprivation, NaN₃, or H₂O₂ in rat cortical and hippocampal slices and cell viability was reported. SAME also increased extracellular glutamate uptake; this property undoubtedly would help its neuroprotective effect against glutamate toxicity. In the present study, we found that the neuroprotective effect of SAME was similar to that of daidzein. Therefore, SAME may effectively protect against some central nervous system injuries. In patients with AD, the levels of Ach in cholinergic neurons in the basal forebrain are reduced, which is coupled with profound neuronal degeneration. However, the molecular and cellular mechanisms by which SAME exerts neuroprotection at cholinergic or other sites are still unclear.

In conclusion, SAME is an effective activator of ChAT and it might protect against TMT-induced memory and cognition deficit in vivo. These results demonstrate that SAME isolated from *C. sinensis* may be useful for the prevention of neurodegenerative diseases such as AD.

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**Conflict of Interest** The authors declare no conflict of interest.

**References**


