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Improvement of scopolamine-induced memory impairment by *Z*-ajoene in the water maze in mice

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Abstract

Z-ajoene, a major compound containing sulfur in oil-macerated garlic products, exhibited inhibitory effects against scopolamine-induced memory impairment in mice using the Morris water maze test. The effects of *Z*-ajoene were observed dose-dependently (0.25–25 mg/kg). At the highest dosage, the memory performance of mice was improved compared to normal mice. The acetylcholinesterase (AChE) activity in the brain was reduced by administration of *Z*-ajoene dose-dependently. However, alliin and diallyl disulfide, organosulfur compounds from garlic, did not improve memory performance nor AChE inhibitory effect. These results suggest that *Z*-ajoene may act on the cholinergic system and on memory impairment caused by excess activity of AChE.

Keywords: *Z*-ajoene; Garlic; Acetylcholinesterase (AChE); Morris water maze; Scopolamine

1. Introduction

It has been demonstrated that there is a specific deficiency in acetylcholine (ACh), cholinacetyltransferase, and acetylcholinesterase (AChE) in autopsy material from patients with Alzheimer's disease (AD; Davies and Maloney, 1976). ACh is an important neurotransmitter related to memory and learning. AChE modulates ACh to proper levels by degradation; accordingly, excessive AChE activity leads to constant ACh deficiency, memory, and cognitive impairments. One way of improving learning and memory is increasing ACh levels. A number of AChE inhibitors have been tested and several effective compounds are available as medicines. The severity of dementia is correlated with neuropathologic indicators of cholinergic loss (Perry et al., 1978). AD is an illness characterized by loss of memory and mental function. In the disease, the most remarkable neurotransmitter change is a decrease in ACh concentration in the brain. Treatment of this disease relies on the "Precursor loading method" (Bartus et al., 1982). This is based on the idea that increasing choline in the brain will enhance the synthesis of ACh.

Garlic has been used as medicinal plant since ancient times, and its major effects on cardiovascular diseases are well known. Aged garlic extract (AGE), prepared by aging chopped garlic in aqueous alcohol for 2 years, has been reported to improve memory in mice (Nishiyama et al., 1997). The active compounds were speculated to be compounds containing *S*-allyl groups but were not identified. Oil-maceration is one method for processing garlic. Ajoene [(*Z*,*E*)-4, 5, 9-trithiadodeca-1, 6, 11-triene-9-oxide](Block et al., 1984; Lawson et al., 1991) is one of the major compounds in oil-macerated garlic products, and has antithrombic, antibiotic, and antitumor effects. *Z*-ajoene contains *S*-allyl groups in its structure, so we tested the inhibitory effects of *Z*-ajoene on scopolamine-induced memory impairment in mice using the water maze test (Morris, 1984) and on AChE activities in the brains of mice. Scopolamine-induced memory impairment was inhibited by *Z*-ajoene in mice, apparently by inhibition of AChE activity.

2. Method

2.1. Animals

Male ddY mice, 8 weeks old (37–40 g), were obtained from Japan SLC (Shizuoka, Japan), housed individually in

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controlled-temperature environment (25 ± 1 °C) with a 12-h light/dark cycle. Food and water were provided ad libitum for 1 week of adaptation. The animal experiments were done according to institutional guidelines of Committee on Animal Research at Nagoya Seiraku.

2.2. Drugs

Z-ajoene, which separated from oil-macerated garlic extract had a purity of 99.2% (0.2% *E*-ajoene), was used in the series of experiments. Preparation and the check of purity were done by the method described previously (Block et al., 1984, 1986; Lawson et al., 1991). Authentic *Z*-ajoene was separated by a high-performance liquid chromatography (HPLC) system.

Scopolamine hydrobromide, a muscarinic antagonist, was obtained from Sigma (St. Louis, MO) and is known to cause memory impairment by increasing AChE activity; therefore, it was used to study dementia. Tetrahydroaminoacridine (tacrine) was obtained from Sigma and was found to improve experimental and clinical memory impairments (Jackson and Soliman, 1996). L-alliin and diallyl disulfide were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Tokyo Kasei Kogyo, respectively.

In this study, scopolamine was subcutaneously injected at a dose of 2 mg/kg of body weight, and drugs were orally administrated at a dose of 0.25–25 mg/kg. Scopolamine was dissolved in physiological saline. Tacrine and L-alliin were administrated by dissolving; *Z*-ajoene and diallyl disulfide were administrated by suspending in a 0.5% carboxymethylcellulose (CMC) solution. The control animals were injected with saline and 0.5% CMC.

2.3. Morris water maze

Water maze testing was performed according to the method (Morris, 1984), performed in a circular pool (diameter: 90 cm, height: 25 cm), filled to a depth of 12 cm of water (19 ± 1 °C). The white platform (10 cm^2) was submerged approximately 1 cm below the surface of the water, and milk was then added to the water to make it opaque. The mice could escape from the water onto the platform. The time was measured from start to escape from the water onto the platform.

2.4. Escape acquisition in mice

The mice were assigned to five groups (see Experiments 1 and 2) for the swimming test. All the mice first learned the location of submerged platform for two consecutive days, and were sorted out the uniform ability of mice, in which they were released from the same starting point in the maze in all sessions and given a maximum of 120 s to the platform. When the mouse reached the platform, it was allowed to remain on it for 20 s. If the mouse failed to find the platform within 120 s, it was removed from the water and placed on the platform for 20 s. The location of the platform remained the same for all tests. Latency was recorded for each trial (data not shown).

Six escape trials were given to all the mice per day for five consecutive days. All latency for the given day was calculated by averaging the six trials. For escape acquisition test, scopolamine (2 mg/kg of body weight) was given by subcutaneous injection 30 min prior to the experiments. *Z*-ajoene and drugs were administrated orally 15 min prior to the experiment.

2.5. Spatial-working memory in mice

After the six trials escape acquisition test, spatial-working memory test was carried out. It was confirmed whether the mice remember the position of the platform. They were released in the water maze, from which the platform was removed for 60 s. Stay time at platform area was recorded. One spatial-working memory trial was given all the mice per day for five consecutive days.

2.6. AChE activity assay

All the mice were given scopolamine (2 mg/kg of body weight) by subcutaneous injection 15 min before *Z*-ajoene and drugs were administrated orally. They were killed by cervical dislocation, 15 min after *Z*-ajoene and drugs administration and their brains were rapidly removed. The brain was homogenized by approximately 1.5 g of the brain per 5 ml of buffer [10 mM ethylenediaminetetraacetate (EDTA)/80 mM tris–hydroxymethyl aminomethane (Tris–HCl), pH 8.0/0.5% Triton X-100] in a homogenizer, centrifuged twice at 10,000 × *g* for 15 min at 4 °C to remove the residue and the supernatant used for the experiment (brain AChE solution).

AChE activity assays were performed by the method of Ellman et al. (1961) with some improvement. The method formulated as follows, substrate solution was prepared by 6.5 mg of acetylthiocholine chloride in 1.0 ml distilled water. A solution of 0.01 M dithiobisnitrobenzoic acid (DTNB) was dissolved in 0.1 M phosphate (sodium) buffer, pH 8.0. Calibration curve of AChE was prepared with standard AChE (Wako Pure Chemical Industries), 5.0 mg of it was dissolved in 1.0 ml of Tris–HCl, pH 8.0 (containing 0.1% albumin), and this solution was diluted 1:20 with Tris–HCl, pH 8.0 (containing 0.1% albumin).

The blank and sample for such a run consists of 2.65 ml of 0.1 M phosphate (sodium) buffer, pH 8.0, 0.2 ml of substrate solution, and 0.05 ml of 0.01 M DTNB solution. After this mixture solution warmed at 25 °C, vehicle treated as blank (nonenzyme) was added to 0.1 ml Tris–HCl, pH 8.0 (containing 0.1% albumin), and sample was added to 0.1 ml brain AChE solution, respectively. After mixing, the rate in absorbency was directly read. The KINETIC MODE of Shimadzu spectrophotometer (UV-160 type) was used.

Enzymatic inhibitory activity rates were observed during the first 0–5 min of the reaction at 25 °C. The slope is the change in absorbency at 1 min at 405 nm.

2.7. Experiment 1

The effects of a single administration of *Z*-ajoene on water maze performance were assessed in this experiment. As described above, a new group of mice was divided into five groups: a nonscopolamine group (N-SCP), a scopolamine-treated group (SCP), 0.25 mg/kg *Z*-ajoene-administrated scopolamine-treated group (0.25A-SCP), 0.5 mg/kg *Z*-ajoene-administrated scopolamine-treated group (0.5A-SCP), and 25 mg/kg *Z*-ajoene-administrated scopolamine-treated group (25A-SCP). Escape acquisition tests were given 30 min after scopolamine subcutaneous injection. *Z*-ajoene (0.25–25 mg/kg) was administrated orally 15 min prior to the experiment.

2.8. Experiment 2

This study was designed to make a comparative study of *Z*-ajoene, other garlic constituents (L-alliin and diallyl disulfide), and tetrahydroaminoacridine (tacrine) on water maze performance experiment. Tacrine has been reported to have beneficial effects on memory and learning in experimental animals (Jackson and Soliman, 1996). As described above, a new group of mice was divided into five groups: an N-SCP, an SCP, 25 mg/kg L-alliin-administrated scopolamine-treated group (AL-SCP), 25 mg/kg diallyl disulfide-administrated scopolamine-treated group (DADS-SCP), and 0.5 mg/kg tacrine-administrated scopolamine-treated group (THA-SCP). Escape acquisition tests were given 30 min after scopolamine subcutaneous injection. Drugs were administrated orally 15 min prior to the experiment.

2.9. Protein assay

Protein assay was similar to that described previously (Bradford, 1976). The protein standards were made by dissolving bovine serum albumin (Wako Pure Chemical Industries) in distilled water. Optical density was measured at 595 nm in a spectrophotometer. The values were plotted against protein standard concentrations and regression analysis was performed to determine protein concentrations for the samples. Using the protein values, AChE activity was then calculated according to the levels of protein in the tissue samples.

2.10. Statistics

Each data value is presented as the mean ± S.D. The treatment effects, in the case of the AChE activity and escape latency, were analyzed by two-way ANOVA, and the differences between means were tested by Duncan's multiple-range test (Duncan, 1995) when the F value was significant. Other data were analyzed by one-way ANOVA. Student's t test and Aspin–Welch's test were used to evaluate significant differences between means with either the same or different variance, respectively (Snedecor et al., 1990). Differences of $P < .01$ are considered to be significant.

3. Results

3.1. Experiment 1

N-SCP reduced the escape latency from 23 (first learning average time) to 17 s at the first day and remained constant after the experiment (Fig. 1). The SCP showed an increase in escape latency by means of memory impairment.

Z-ajoene-treated (0.25, 0.5, and 25 mg/kg) mice groups recovered from memory impairment dose-dependently. The escape latency at 0.5 mg/kg was comparable to N-SCP. Furthermore, at 25 mg/kg, *Z*-ajoene showed a higher performance of escape latency than that of N-SCP (Fig. 1).

The platform was removed to examine the spatial-working memory of mice. We measured the time in which the mice wandered the place where there was a platform. Fig. 2 shows the result of the spatial probe trials. The oral administration of *Z*-ajoene prior to every training test increased swimming time in a dose-dependent manner in the pool where the platform was placed during the training trials. To elucidate the mechanism for recovery of memory impairment by *Z*-ajoene, the brain AChE activity was measured the day after the 5-day escape acquisition test. Table 1 shows the brain AChE activity effects on scopolamine-induced memory deficits in mice. Mice injected with scopolamine (2 mg/kg) showed increased errors of entering nonexits and prolonged the path to find the platform. The mice received *Z*-

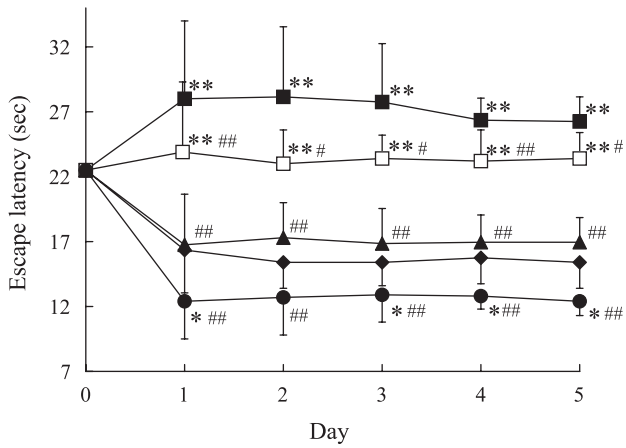

Fig. 1. Improvement by *Z*-ajoene on water maze performance for scopolamine-induced memory impairment in mice. (♦)—N-SCP, (■)—SCP, (□)—0.25A-SCP, (▲)—0.5A-SCP, (●)—25A-SCP. Each value is expressed as the mean ± S.D. Significance: $*P < .05$, $**P < .01$ vs. N-SCP (blank). $^{\#}P < .05$, $^{\#\#}P < .01$ vs. SCP (control).

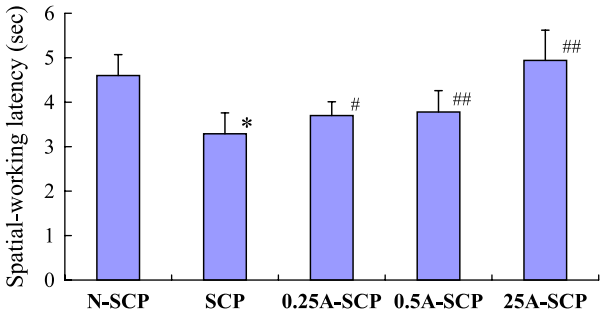

Fig. 2. The effect of *Z*-ajoene on spatial-working latencies in the Morris water maze in mice. N-SCP: a nonscopolamine group, SCP: a scopolamine-treated group, 0.25A-SCP: 0.25 mg/kg *Z*-ajoene-administrated scopolamine-treated group, 0.5A-SCP: 0.5 mg/kg *Z*-ajoene-administrated scopolamine-treated group, 25A-SCP: 25 mg/kg *Z*-ajoene-administrated scopolamine-treated group. Each value is expressed as the mean ± S.D. Significance: **P*<.05 vs. N-SCP (blank). #*P*<.05, ##*P*<.01 vs. SCP (control).

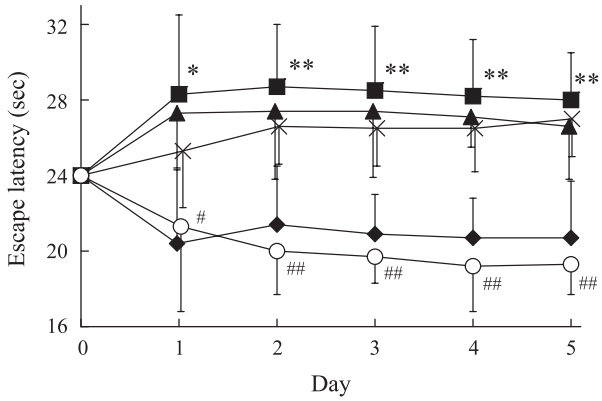

Fig. 3. Escape latency of scopolamine-induced memory impairment in mice using water maze. (◆)—N-SCP, (■)—SCP, (×)—AL-SCP, (▲)—DADS-SCP, (○)—THA-SCP. Each value is expressed as the mean ± S.D. Significance: * *P* < .05, ** *P* < .01 vs. N-SCP (blank). # *P* < .05, ## *P* < .01 vs. SCP (control).

ajoene at doses of 0.25, 0.5, and 25 mg/kg 30 min before the water maze tasks. The mice were then divided into five groups: an N-SCP, an SCP, *Z*-ajoene 0.25 mg/kg-injected scopolamine-treated group (0.25A-SCP), *Z*-ajoene 0.5 mg/kg-injected scopolamine-treated group (0.5A-SCP), and *Z*-ajoene 25 mg/kg-injected scopolamine-treated group (25A-SCP). While scopolamine administration resulted in significant increase in the AChE activity (1.34-fold of N-SCP level) in the brain, AChE activity was reduced dose-dependently by the *Z*-ajoene administration. In the group administrated with highest dosage (25A-SCP), the brain AChE activity was 64.9% that of SCP (Table 1). These results show that administration of *Z*-ajoene suppressed the increase of AChE activity by scopolamine administration.

3.2. Experiment 2

Fig. 3 shows the results of escape latency of other garlic constituents alliin and diallyl disulfide, and tacrine as an AChE inhibitor. N-SCP-injected mice group reduced the escape latency from 24 to 20 s, which remained constant. The tacrine-administrated group showed higher performance of escape latency than those of alliin and diallyl disulfide.

The effects of alliin (25 mg/kg), diallyl disulfide (25 mg/kg), and tacrine (0.5 mg/kg) on spatial-working memory performance in the Morris water maze are shown in Fig. 4. A tacrine-administrated scopolamine-treated group (THA-SCP) showed a significantly longer effect than the SCP ($P < .01$). However, there was no significant difference between AL-SCP- and DADS-SCP-treated groups and SCP group during the 5 days of testing. Thus, the administration of THA-SCP group significantly improved SCP group spatial-working memory impairments.

The results of brain AChE activity are shown in Table 2. In N-SCP mice, the AChE activity in the brain was 418.8 ± 74.6 pmol/min/mg protein. Alliin and diallyl disulfide administration resulted in a significant increase in the

Table 1
Effects of *Z*-ajoene in the brain AChE activity

Groups[a]	*n*	AChE activity (pmol/min/mg protein)
N-SCP	6	389.1[b] ± 52.9
SCP	6	522.9 ± 59.3 *
0.25A-SCP	8	463.7 ± 42.7 **
0.5A-SCP	8	436.4 ± 76.1***
25A-SCP	8	339.2 ± 36.3***

[a] Group name: a nonscopolamine group (N-SCP), a scopolamine-treated group; (SCP), 0.25 mg/kg *Z*-ajoene-administrated scopolamine-treated group (0.25A-SCP), 0.5 mg/kg 2-ajoene-administrated scopolamine-treated group (0.5A-SCP), and 25 mg/kg *Z*-ajoene-administrated scopolamine-treated group (25A-SCP). *n*: represents the number of mice.

[b] Values are means ± S.D.

* *P* < .01 vs. N-SCP (blank).

** *P* < .05 vs. SCP (control).

*** *P* < .01 vs. SCP (control).

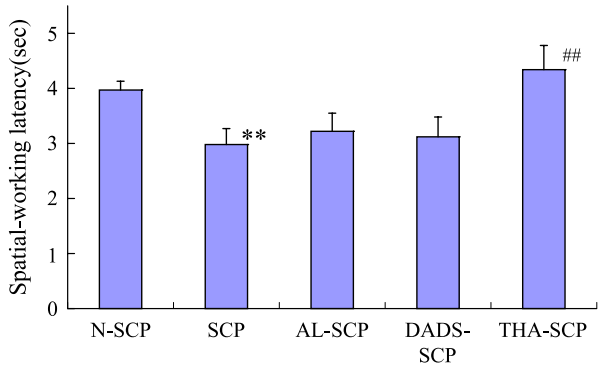

Fig. 4. The effect of *Z*-ajoene on spatial-working latencies in the Morris water maze in mice. N-SCP: a nonscopolamine group, SCP: a scopolamine-treated group, AL-SCP: 25 mg/kg alliin-administrated scopolamine-treated group, DADS-SCP: 25 mg/kg diallyl disulfide-administrated scopolamine-treated group, THA-SCP: 0.5 mg/kg tacrine-administrated scopolamine-treated group. Each value is expressed as the mean ± S.D. Significance: ** *P* < .01 vs. N-SCP (blank). ## *P* < .01 vs. SCP (control).

Table 2
Effects of alliin and diallyl disulfide in the brain AChE activity

Groups[a]	*n*	AChE activity (pmol/min/mg protein)
N-SCP	6	418.8[b] ± 74.6
SCP	6	542.3 ± 97.9 *
AL-SCP	8	506.8 ± 130.4 * *
DADS-SCP	8	515.0 ± 136.0 * *
THA-SCP	8	397.8 ± 69.2* * *

[a] Group name: a nonscopolamine group (N-SCP), a scopolamine-treated group (SCP), 25 mg/kg alliin-administrated scopolamine-treated group (AL-SCP), 25 mg/kg diallyl disulfide-administrated scopolamine-treated group (DADS-SCP), and 0.5 mg/kg tacrine-administrated scopolamine-treated group (THA-SCP). *n*: represents the number of mice.

[b] Values are means ± S.D.

* $P < .05$ vs. N-SCP (blank).

** $P < .05$ vs. SCP (control).

*** $P < .01$ vs. SCP (control).

AChE activity (1.21-fold of N-SCP level) in comparison with the N-SCP. In the THA-SCP, the brain AChE activity was 73% that of SCP (Table 2).

4. Discussion

In this study, the inhibitory effects of memory impairment on scopolamine-treated mice were demonstrated using the Morris water maze test. *Z*-ajoene exhibited inhibitory effects against scopolamine-induced memory impaired mice dose-dependently; that is, the mice showed shorter escape latency. In the group administrated 0.5 mg/kg of *Z*-ajoene, the escape latencies were comparable to those of nonscopolamine-treated mice. Furthermore, the group administrated 25 mg/kg of *Z*-ajoene showed shortest escape latency among all the groups including nontreated mice. This result suggested that *Z*-ajoene improved memory impairment by improving learning or exercise ability of mice. Neither alliin nor diallyl disulfide, at 25 mg/kg, showed significant improvement.

To elucidate the effect of *Z*-ajoene, the brain AChE activity was measured. The brain AChE activity of normal mice was increased 1.34-fold by scopolamine administration; consequently, escape latency was elongated. *Z*-ajoene administration decreased AChE activity dose-dependently, and the maximum dosage of 25 mg/kg *Z*-ajoene reduced the activity to 65% of scopolamine-treated mice. The brain AChE activity was directly related to the length of escape latency; that is, higher AChE activity caused longer escape latency.

From these results, improved memory by *Z*-ajoene is presumed to be caused by its inhibitory effect on AChE activity. Alliin and diallyl disulfide also slightly inhibited AChE in the brain. These results showed the relationship between escape latency of water maze tests and the brain AChE activity. Tacrine, an AChE inhibitor, significantly decreased AChE activity to 73% of scopolamine-treated mice. This effect was superior to the administration of 0.5 mg/kg *Z*-ajoene but inferior to 25 mg/kg *Z*-ajoene.

Nishiyama et al. (1997) reported improved memory by AGE in mice. Their results were obtained with whole AGE, not individual constituents. The active compounds were speculated to the compounds containing *S*-allyl groups, with *S*-allyl cysteine (SAC) as a key candidate. SAC is the deoxygenated form of alliin and has an *S*-allyl group. In our study, alliin and diallyl disulfide showed the improvement of AChE activity, so that the structures, *S*-allyl or *S*-allyl sulfoxide was important for the activity. Because *Z*-ajoene contains both *S*-allyl and *S*-allyl sulfoxide groups, it showed stronger activity than alliin or diallyl disulfide.

In conclusion, scopolamine-induced memory impairment in mice was inhibited by *Z*-ajoene. The effect of *Z*-ajoene appears to be due to inhibition of AChE activity. Although our results obtained from animal experiment are preliminary, from the view of preventive medicine, the results demonstrate the importance of future studies of *Z*-ajoene in dementia.

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