Ameliorating effect of a herbal medicinal prescription, Kyung-Ok-Ko, on scopolamine-induced memory impairment in mice

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Abstract

Kyung-Ok-Ko (KOK) which is widely used as a tonic in traditional Korean herbal medicine, and contains six main ingredients, such as, Ginseng Radix, Rehmanniae Radix, Hoelen, Honey, Lycium Fructus, and Aquilaria Lignum. In the present study, we assessed the effect of KOK on the learning and memory impairments induced by scopolamine in mice. The ameliorating effect of KOK was investigated using passive avoidance, Y-maze, and Morris water maze tasks. Drug-induced amnesia was introduced by administering scopolamine (1 mg/kg, i.p.). KOK (1 or 2 g/kg, p.o., single treatment 30 min before scopolamine) significantly prevented scopolamine-induced cognitive impairments in the passive avoidance task and the Y-maze task (p < 0.05), and improved escape latency in the Morris water maze task at 1 g/kg (p < 0.05). Moreover, KOK was also found to inhibit acetylcholinesterase activity in a dose-dependent manner in vitro (IC_{50} value; 162 μg/ml), and to inhibit it ex-vivo. These results suggest KOK may be a useful treatment for cognitive impairment, and that its beneficial effects are mediated, in part, by enhancing the cholinergic neurotransmitter system.

Key words Kyung-Ok-Ko, memory, passive avoidance task, Y-maze task, Morris water maze task, acetylcholinesterase.

Introduction

The central cholinergic neurotransmitter system is known to play an important role in learning and memory.1) Moreover, cholinergic neurons in the central nervous system are degenerated in Alzheimer's disease (AD) and degrees of cholinergic neurons degeneration correlate well with cognitive functional loss.2,4) The cholinergic receptor agonists (muscarinic and nicotinic) and enhancers of the endogenous acetylcholine level (synthesis promoters and inhibitors of acetylcholine metabolizing enzyme) have been examined as potential treatments for senile dementia of the Alzheimer's type. Of the various approaches attempted, the inhibition of acetylcholinesterase (AChE) was found to be most successful.5) Galantamine and huperzine A, which were also isolated from plant extracts, have been used to treat the early symptoms of AD. We considered that if empirically used herbal prescription has an inhibitory effect on the AChE, it would be useful for treating senile dementia.
The traditional Korean prescription, Kyung-Ok-Ko (KOK), which contains 6 main ingredients, i.e., Ginseng Radix, Rehmanniae Radix, Hoelen, Honey, Lycium Fructus and Aquilarie Lignum. KOK has been used empirically as a tonic for the elderly in Korea, which suggests that it may ameliorate some age-related symptoms, such as amnesia or dementia. However, the memory enhancing effects of KOK have not been previously examined. Available evidences suggests that the activities of neurotransmitter systems decline with age, and of these the cholinergic neurotransmitter system is the most specific system. The relation between age and decreases in cholinergic neurotransmitter activity is generally accepted, and treatments for senile dementia are based on the enhancing the cholinergic neurotransmitter system as mentioned above. These findings suggest a possibility that KOK may also be useful for enhancement of cholinergic signaling in aged people. If KOK enhances the cholinergic signaling, it can relieve aged person from senile dementia.

In the present study, we investigated the effects of KOK on memory impairment induced by scopolamine in mice using a passive avoidance task, a Y-maze task, and the Morris water maze task in mice. We also investigated in vitro and ex-vivo whether the memory ameliorating effects of KOK are associated with its inhibition of AChE.

Materials and Methods

Animals: ICR Male mice, weighing 25-30 g, were purchased from the Orient Co., Ltd, a branch of Charles River Laboratories (Seoul). Animals were housed 5 per cage, allowed access to water and food ad libitum, and maintained in a constant temperature (23 ± 1 °C) and humidity (60 ± 10%) environment under a 12-h light/dark cycle (light on 07:30 - 19:30 h). Animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea.

Materials: Tacrine (9-amino-1, 2, 3, 4-tetrahydroacridine hydrochloride), (-) scopolamine hydrobromide, acetylthiocholine iodide and DTNB (5, 5'-dithiobis [2-nitrobenzoic acid]) were purchased from the Sigma Chemical Co. (USA). KOK is composed of 6 kinds of crude herbal materials; Ginseng Radix, Rehmanniae Radix, Hoelen, Honey, Lycium Fructus, Aquilarie Lignum (Table 1). KOK (Lot No., OV30) was donated by Kwang Dong Pharmaceutical Co. (Pyongtaek, Korea) as a sticky extract. All other materials were obtained from normal commercial sources and were of the highest grade available.

<table>
<thead>
<tr>
<th>Table 1. Composition of Kyung-Ok-Ko</th>
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<tr>
<td>KOK</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Juice of Rehmannia Radix</td>
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<tr>
<td>Powder of Ginseng Radix</td>
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<tr>
<td>Powder of Hoelen</td>
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<tr>
<td>Powder of Lycium Fructus</td>
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<td>Powder of Aquilarie Lignum</td>
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<td>Honey</td>
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<td>Simple syrup</td>
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Passive avoidance task: Passive avoidance tasks were carried out as described elsewhere. For acquisition trial, mice were initially placed in the illuminated compartment and the door between the two compartments was opened 10 s later. When mice entered the dark compartment, the door automatically closed and an electrical foot shock (0.5 mA) of 3 s durations was delivered through the stainless steel rods. One hour before the acquisition trial, mice were administered KOK (0.25, 0.5, 1, and 2 g/kg, p.o.) or tacrine (10 mg/kg, p.o.) as a positive control. Memory impairment was induced by scopolamine treatment (1 mg/kg, i.p.) 30 min after the administration of KOK, tacrine, or 10% Tween 80 solution. Control animals were administered 10% Tween 80 solution only. Twenty-four hours after acquisition trial, the mice were again placed in the illuminated compartment for the retention trials. The time taken for a mouse to enter the dark compartment after door opening was measured as latency times in both acquisition and retention trials. If a mouse did not enter the dark compartment within 300 s, it was assumed that the mouse had remembered the single training trial.

Y-maze task: Y-maze is used as a measure of immediate spatial working memory which is a form of short-term memory. Mice were initially placed within one
arm, and the sequence (i.e., ABCCAB, etc) and number of arm entries were recorded manually for each mouse over an 8 min period. An actual alternation was defined as entries into all three arms on consecutive choices (ABC, CAB, or BCA but not BAB). One hour before this test, mice were treated with KOK at various dosages (0.25, 0.5, 1, and 2 g/kg, p.o.) or tacrine (10 mg/kg, p.o.) as a positive control, and 30 min later memory impairment was induced by administration of scopolamine (1 mg/kg, i.p.). Control group animals received 10% Tween 80 solution instead of KOK. Maze arms were thoroughly cleaned between tests to remove residual odors. The percentage of alternation was determined by dividing the total number of alternations by the total number of choices minus 2 multiplied by 100 as shown in the following equation: % Alternation = [(Number of alternations) / (Total arm entries - 2)] × 100. The number of arm entries serves as an indicator of locomotor activity.

Morris water maze task: Morris water maze tasks were carried out as described elsewhere. The first experimental day was dedicated to swimming training for 60 s in the absence of the platform. During the four subsequent days the mice were given four trials per day with the platform in place. When a mouse located the platform, it was permitted to remain on it for 10 s and was then placed in a holding cage for 30 sec. until the start of the next trial. If the mouse did not locate the platform within 60 s, it was placed on the platform for 10 s. The animal was taken to its home cage and was allowed to dry up under an infrared lamp after each trial. During each trial, the time taken to find the hidden platform (latency) was recorded using a video camera-based Ethovision System (Nodulus, Wageningen, The Netherlands). One day after the last training trials, mice were subjected to a probe trial session in which the platform was removed from the pool, allowing the mice to swim for 60 s to search for it. A record was kept of the swimming time in the pool quadrant where the platform had previously been placed. KOK (1 g/kg, p.o.) or tacrine (10 mg/kg, p.o.) as a positive control was given 1 h before the first trial at every consecutive day. Memory impairment was induced in mice with scopolamine (1 mg/kg, i.p.) at 30 min before the first trial at every consecutive day. Control group received 10% Tween 80 solution only.

AChE activity assay: AChE activity assays were carried out using an acetylthiocholine iodide substrate based colorimetric method, as described by Ellman et al. Whole brains of male ICR mice (25-30 g) were homogenized in a glass Teflon homogenizer (Eyela, Japan) containing 10 volumes of homogenization buffer (12.5 mM sodium phosphate buffer with 400 mM NaCl, pH 7.0), and then centrifuged at 1,000 x g for 10 min at 4°C. The supernatant solution obtained was used as source of enzyme for the assay. KOK was initially dissolved in 0.02% dimethyl sulfoxide (DMSO) and diluted to various concentrations in Buffer A (100 mM sodium phosphate buffer, pH 8.0) immediately before use. An aliquot of diluted KOK solution in Buffer A (1.5 ml) was then mixed with 2.6 ml of Buffer A, 20 μl of acetylthiocholine iodide solution (75 mM) and 100 μl of buffered Ellman’s reagent (10 mM 5,5'-dithio-bis[2-nitrobenzoic acid] and 15 mM sodium bicarbonate) and reacted at room temperature for 30 min. Absorbance was measured at 410 nm immediately after adding the enzyme source (400 μl) to the reaction mixtures (OPTIZEN 2120UV, Mecasys Co. Ltd., Korea). Readings were taken at 30 s intervals for 5 min. The concentration of KOK required to inhibit acetylcholinesterase activity by 50% (IC50) was calculated using an enzyme inhibition dose response curve. Tacrine was used as a positive control.

For ex-vivo testing, the most effective doses as determined by the passive avoidance task were chosen. Mice were administered vehicle, KOK (1 g/kg, p.o.), or tacrine (10 mg/kg, p.o.). Animals were decapitated 1 h after each administration, and brains were removed to assay AChE activity. AChE activity was assessed as described above for the in vitro assay. Protein concentrations were determined by the Lowry’s method using bovine serum albumin as a standard.

Statistics: Values are expressed as means ± S.E.M. For the passive avoidance task, data were analyzed using a Kruskal-Wallis non-parametric ANOVA test. If results were significant, treatment groups were compared using the Tukey’s post hoc test. In all the other tests, data were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-
Keuls test for multiple comparisons. Especially, group differences in the escape latency in the Morris water maze training task were analyzed using two-way ANOVA with repeated measures. Statistical significance was set at $p < 0.05$.

Results

**Effect of KOK on passive avoidance task:** During acquisition trials, latency times were not different among the experimental groups [$H(6) = 3.42, p = 0.75$]. For retention trials, the Kruskal-Wallis test revealed significant differences for latency times [$H(6) = 51.64, p < 0.001$]. The step-through latency of scopolamine-treated mice was significantly shorter than that of vehicle-treated control mice (Fig. 1, $p < 0.05$). In tacrine with scopolamine-treated mice (a positive control group), step-through latency was significantly higher than that of the scopolamine-treated group ($p < 0.05$). Moreover, the shorter step-through latencies induced by scopolamine were significantly attenuated by KOK (0.5, 1 or 2 g/kg) versus the scopolamine-treated group ($p < 0.05$).

**Effect of KOK on Y-maze task:** Significant group effects on alternation behavior were observed [$F(6, 63) = 5.33, p < 0.001$] (Fig. 2A). Spontaneous alternation of scopolamine-treated mice was significantly lower than that of vehicle-treated control mice (Fig. 2A, $p < 0.05$), and the lowered spontaneous alternation induced by scopolamine was significantly reversed by KOK (1 and 2 g/kg).

![Figure 1](image1.png)  
**Figure 1** Effect of Kyung-Ok-Ko (KOK) administration on scopolamine-induced memory deficits in the passive avoidance task. At 60 min before acquisition trials, KOK (0.25, 0.5, 1 or 2 g/kg, p.o.), tacrine (THA, 10 mg/kg, p.o.) or vehicle (same volume of 10% Tween 80) solution were administered to mice. Memory impairment was induced by scopolamine treatment (1 mg/kg, i.p.). Acquisition trials were carried out 30 min after the single scopolamine treatment. At 24 h after acquisition trials, the retention trials were carried out for 5 min. Data represent means ± S.E.M. ($n = 10$ per group) (*$p < 0.05$ versus vehicle control group, **$p < 0.05$ versus scopolamine treated group).

![Figure 2](image2.png)  
**Figure 2** Effect of Kyung-Ok-Ko (KOK) administration on scopolamine-induced memory deficits in the Y-maze task. At 60 min before training trials, KOK (0.25, 0.5, 1 or 2 g/kg, p.o.), tacrine (THA, 10 mg/kg, p.o.) or vehicle (same volume of 10% Tween 80) solution was administered to mice. Memory impairment was induced by scopolamine treatment (1 mg/kg, i.p.). The test was carried out 30 min after a single scopolamine treatment. Spontaneous alternation behavior (A) and the number of arm entries (B) were measured during an 8-min session were measured. Data represent means ± S.E.M. ($n = 10$ per group) (*$p < 0.05$ versus vehicle control group, **$p < 0.05$ versus scopolamine-treated group).
Effect of KOK on Morris water maze task: The effect of KOK (1 g/kg, p.o.) on spatial learning was evaluated using the Morris water maze test. As shown in Fig. 3A, the scopolamine-treated group exhibited longer escape latencies throughout the training days than did the control group \( [F(1, 72) = 100.02, p < 0.001] \). KOK (1 g/kg) significantly shortened the escape latencies prolonged by scopolamine treatment \( [F(1, 72) = 55.34, p < 0.001] \). Moreover, tacrine also significantly reduced escape latencies compared with scopolamine-treated group \( [F(1, 72) = 34.82, p < 0.001] \). On the day following the final day of training trials, swimming times within the platform quadrant for the scopolamine treated group was significantly lower than those of the vehicle treated control group animals (Fig. 3B, \( p < 0.05 \)). The shorter swimming time within the platform quadrant induced by scopolamine was significantly reversed by KOK or tacrine (Fig. 3B, \( p < 0.05 \)). However, there were no significant differences in the swimming speed within the platform zone among all groups (Fig. 3C).

**Effect of KOK on acetylcholinesterase activity:** KOK inhibited AChE activity in a concentration-dependent manner with IC_{50} values of 162.07 µg/ml in vitro (Fig. 4A). Ex-vivo testing showed a significant group effects on AChE activity \( [F(2, 12) = 8.93, p = 0.004] \) (Fig. 4B). AChE activity was also significantly inhibited by

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**Figure 3** Effect of Kyung-Ok-Ko (KOK) on the latency time during training trial sessions (A) and on the swimming time (B) and speed (C) during the probe trial session of the Morris water maze task in scopolamine-induced memory deficits mice. At 60 min before the first trial on each training day, KOK (1 g/kg, p.o.), tacrine (THA, 10 mg/kg, p.o.) or vehicle (same volume of 10% tween 80) solution was administered to mice. Memory impairment was induced by scopolamine treatment (1 mg/kg, i.p.). The training trial and the probe trial sessions were performed as described in the Materials and Methods. Data represent means ± S.E.M. (n = 10 per group) (*p < 0.05 versus vehicle control group, #p < 0.05 versus scopolamine-treated group in graph).
KOK treatment as did tacrine in ex-vivo study ($p < 0.05$).

![Inhibition of acetylcholinesterase activity](image)

**Figure 4** In vitro (A) and ex-vivo (B) studies of Kyung-Ok-Ko (KOK) on the acetylcholinesterase activity. Inhibition efficacy was expressed as percent inhibition of enzyme activity compared to the control value (100%). The concentration required for 50% enzyme inhibition (IC50) of KOK was 162.07 $\mu$g/ml. In the ex-vivo study, four different animals were used per each treatment group. Data represent means ± S.E.M. (*$p < 0.05$ versus vehicle control group).

**Discussion**

The main findings of the present study are that KOK prevented the memory deficits induced by scopolamine as assessed in the passive avoidance, Y-maze, and Morris water maze tasks without changing general behavior, and that KOK inhibited AChE activity in vitro and ex-vivo. These results suggest that cholinergic signaling is involved in the ameliorating effect of KOK on cognitive dysfunctions.

It has been demonstrated that several traditional herbal prescriptions have memory enhancing properties. For example, DX-9386, a Chinese traditional medicine, which consists of Ginseng Radix, Polygala Radix, Acorus Radix and Hoelen has been reported to have anti-aging and memory ameliorating effects. Ninjin-yoeto (Ren-Shen-Yang-Rong-Tang) provides another example. Egashira et al. reported that ninjinyoeto may ameliorate memory dysfunction by enhancing the cholinergic system, and suggested that Polygala Radix might be involved in its action. Recently, they also concluded that tenuifoliside B, a main ingredient of Polygala Radix, is responsible for its cognitive improving properties. Moreover, kamiuntanto which contains Polygala Radix and 11 other herbs and is used to treat psychoneurological diseases, was found to improved memory in the passive avoidance test. Polygala Radix is presumed to be the main active ingredient in these prescriptions. Park et al., also reported that BT-11, an extract formulation based on Polygala Radix, has some protective effect against the cognitive impairments of AD and other neurodegenerative diseases related to central cholinergic dysfunction. Furthermore, when Polygala Radix was removed from kamiuntanto, the effects of choline acetyltransferase activity were not pronounced. Ginseng Radix and Hoelen, which are ingredients of KOK, are also present in the DX-9386. If Polygala Radix is the only ingredient responsible for cognitive enhancement as was previously suggested, the memory enhancing effects of KOK would be expected to be equivocal or absent. However, in the present study, we observed that step through latency, which was reduced by scopolamine treatment, recovered to approximately 47% (0.5 g/kg), 76% (1 g/kg), and 60% (2 g/kg) of that of the vehicle treated controls, by administering KOK. In terms of short-term spatial memory, KOK also reversed scopolamine-induced decreases in spontaneous alternation in the Y-maze test. Furthermore, KOK also improved hippocampal-dependent spatial learning ability as determined by the Morris water maze task. Collectively, these behavioral findings suggest that KOK contains a memory enhancing ingredient which ameliorates memory dysfunctions in amnesic mouse.
models induced by scopolamine treatment. Since these effects could not be explained by reported ingredients as discussed above, the effective ingredient may be novel. It is an interesting to determine the effective ingredient(s) other than reported one.

To confirm the mode of action of KOK, we assessed its inhibitory activity on AChE using mouse brain homogenates, as it is well known that the antiamnestic effects of tacrine and donepezil are due to AChE inhibition in brain. This in vitro study showed that KOK inhibited AChE activity in a concentration-dependent manner with an IC$_{50}$ of 162.07 µg/ml. Moreover, as was expected, our ex-vivo studies also showed that KOK significantly blocked AChE activity 1 h after administration, as did tacrine (Fig. 4B), but the IC$_{50}$ of KOK was much higher than that of tacrine (data not shown). Because its inhibitory effect on the AChE activity was similar to that of tacrine in our ex-vivo study, we believe that the inhibition of AChE activity by KOK is sufficient to enhance memory. Taken together, these behavioral and biochemical investigations suggest that short- and long-term memory improvements by KOK via cholinergic signaling enhancement. However, it has still not determined which ingredient of KOK has the predominant effect.

Several reports have been issued on the usefulness of the ingredients of KOK in cognitive disorders. Hoelen, an ingredient of tokishakuyakusan (TSS), was reported to have an ameliorating effect on scopolamine-induced memory impairment in a radial-arm maze task. However, the authors concluded that this effect was mainly due to the presence of Toki (Angelica acutiloba) not Hoelen. No report has been issued concerning the effect of Hoelen on the cholinergic neurotransmitter system. In the case of Ginseng Radix, many pre-clinical and clinical studies have demonstrated that it has promising therapeutic potential as a cognitive enhancing drug. Various possible mechanisms have been proposed to explain the anti-amnesic effect of Ginseng Radix, i.e., increased acetylcholine release in the hippocampus, increased proliferation and differentiation of neural progenitor cells in dentate gyrus of hippocampus, and increased long-term potentiation of the hippocampal CA3 region. Furthermore, Ginseng Radix was reported to have ameliorating effects on an Aβ-induced memory impairment model.

Nevertheless, no information is available concerning the AChE inhibitory activities of Ginseng Radix. Moreover, Rehmanniae Radix, Lycium Fructus, or Aquilaria Lignum is also the same cases in point of the AChE inhibitory activities. In the present study, we observed KOK inhibits AChE and that the levels of AChE inhibition by KOK were comparable with those of tacrine ex-vivo AChE study. As mentioned above, no published evidence explains the mode of action of KOK in terms of AChE inhibition in terms of its ingredients. Moreover, we also observed that any herbal ingredients of KOK extracted by boiling water or 70% ethanol did not show remarkable inhibitory activities on AChE (data not shown). Accordingly, an active compound might be produced during processing of KOK. We suggest that an activity guided-fractionation method should be utilized to further probe the action mechanisms of the main components of KOK.

In conclusion, the present study demonstrates that KOK has the ability to improve or ameliorate short- and long-term working memory by inhibiting AChE and thus activating cholinergic neurotransmitter system. Although these findings of KOK may not in general provide clinically useful outcomes in patients or in normal humans, the findings of this study suggest that KOK has therapeutic potential for the treatment of age-related senile dementia.

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