Protective Effect of Arabinoxylan against Scopolamine-Induced Learning and Memory Impairment

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Abstract

The purpose of this study is to investigate the memory enhancing effect and underlying molecular mechanism of arabinoxylan (AX), a major component of dietary fiber in wheat against scopolamine (SCO)-induced amnesia in Sprague-Dawley (SD) rats. Diverse behavior tests including Y-maze, Morris water maze, and passive avoidance tests were performed to measure cognitive functions. SCO significantly decreased the spontaneous alterations in Y-maze test and step-through latency in passive avoidance test, whereas increased time spent to find the hidden platform in Morris water maze test compared with the sham control group. In contrast, oral administration of AX (25 mg/kg and 50 mg/kg) effectively reversed the SCO-induced cognitive impairments in SD rats. Furthermore, AX treatment up-regulated the expression of brain-derived neurotrophic factor (BDNF) in the cortex and hippocampus via promoting activation of cAMP response element binding protein (CREB). Therefore, our findings suggest that AX can improve SCO-induced learning and memory impairment possibly through activation of CREB and up-regulation of BDNF levels, thereby exhibiting a cognition-enhancing potential.

Key Words: Arabinoxylan, Brain-derived neurotrophic factor, cAMP response element binding protein, Learning and memory, Scopolamine

INTRODUCTION

Characteristic neuropathological features of Alzheimer's disease (AD), one of the most common forms of dementia, are accumulation of senile plaques, formation of neurofibrillary tangles, induction of oxidative stress and/or inflammatory responses, and disturbance in neurotransmission (Karran *et al.*, 2011). Particularly, decreased cholinergic function is responsible for the progressive loss of memory in AD and closely correlated with the severity of cognitive impairment (Craig *et al.*, 2011). Conversely, the restoration of cholinergic function by cholinergic agonists or prolongation of the acetylcholine availability into the synaptic cleft by acetylcholinesterase (AChE) inhibitors has been regarded as a representative therapeutic target for the treatment of AD symptoms (Brenner *et al.*, 2008). Therefore, in the present study, we have utilized scopolamine (SCO), a muscarinic acetylcholine receptor (mA-ChR) antagonist to induce memory impairment in Sprague-Dawley (SD) rats as a typical screening model system for anti-amnesic drugs.

Because of the limitation of current medications approved for the management of AD such as relatively low efficacy, severe adverse effects for the long-term use, and ineffectiveness in the late stage of AD, extensive researches have been carried out to search novel and safe phytochemicals with neuroprotective properties. In this regard, an array of natural products from dietary foods with high potency and low side effects has been investigated for the prevention and/or treatment of AD and one promising new candidate of interest in this study is arabinoxylan (AX).

AX is the major constituent of wheat non-starch polysaccharides (NSP) accounting for approximately 2.5% of flour and 18% of bran and acts as dietary fiber in wheat (Gebruers *et al.*, 2008; Ward *et al.*, 2008). AX of wheat is composed of

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xylose and arabinose serving as backbone and substituent, respectively. The relative ratio of arabinose to xylose (A/X) is approximately 0.5 for total AX in flour (Gebruers *et al.*, 2008; Ward *et al.*, 2008). Previously, we have shown that hot water extract of milled wheat and total dietary fiber prepared from it improved memory impairment caused by SCO in SD rats (Jang *et al.*, 2010). In addition, we also have reported that hot water extract of milled wheat restored memory deficit in a rat model of chronic hypoperfusion selected for mimicking vascular dementia in human (Han *et al.*, 2010).

As an extension of the previous studies, the purpose of the present work is to investigate whether AX, an active constituent of wheat, has a novel cognition-enhancing activity against the SCO-induced amnesia in SD rats and exhibits a preventive and/or therapeutic potential for the management of AD. In this study, basic investigations on the action of AX were extended to the molecular levels by examining alterations in the phosphorylation of cAMP response element binding (CREB) protein and expression of brain-derived neurotrophic factor (BDNF).

MATERIALS AND METHODS

Drugs and reagents

Wheat AX was purchased from Megazyme (Wicklow, Ireland). SCO and anti-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-BDNF and anti-phospho-CREB (p-CREB) antibodies were the products of SantaCruz (Santa Cruz, CA, USA) and Cell Signaling (Boston, MA, USA), respectively.

Experimental animals

Male SD rats weighing 250-300 g (8-week old) were supplied from the Dae Han Bio-Link. Co., Ltd. (Eumseong-gun, Chungcheongbuk-do, South Korea) and maintained under controlled temperature ($21 \pm 2^\circ$C) and humidity (45-60%) with a regular 12 h light - 12 h dark cycle (light period starting at 7 a.m.). The rats freely accessed to standard rodent chow and fresh tap water. The experimental procedure was conducted in compliance with institutional guidelines of NIH and Daegu Haany University for the Care and Use of Laboratory Animals.

Drug administration

The rats were divided into four groups (6-7 animals per group): sham control group, SCO model group, and SCO+ AX (25 mg/kg and 50 mg/kg) treatment groups. The rats were orally administered with normal saline or AX, 1 h before starting the behavior tests. SCO (0.75 mg/kg, 300 μl, *i.p.*) was injected 30 min prior to the behavior tests, whereas the sham control group was injected with same volume of normal saline.

Y-maze test

Three arms of the Y-maze (41 cm long, 25 cm high, and 10 cm wide) were positioned at an equal angle. Each rat was placed at the end of one arm and allowed to navigate freely for an 8-min session. In each trial, the spontaneous alternations were recorded using Ethovision System (Ethovision system, Noldus, Wageningen, Netherlands). Spontaneous alteration behavior was defined as the entry into all three arms on consecutive choices in overlapping triplet sets (e.g. ABC, BCA, CAB). The percentage (%) of spontaneous alternation behav-

ior was calculated by dividing the total number of alternations by the total number of arm entries subtracting 2 and then multiplying by 100: % alternation=[(number of alternations)/(total number of arm entries-2)]×100.

Morris water maze test

The Morris water maze test was conducted in accordance with the protocol previously reported (Jang *et al.*, 2010).

Step-through passive avoidance test

The passive avoidance test apparatus (GEMINI™, San Diego, CA, USA) was consisted of two compartments (26×28×16 cm), illuminated and non-illuminated chambers, equipped with an electrifiable grid floor. The two chambers were connected by an automatically operated guillotine door. During acquisition trial, rats were initially placed in the illuminated chamber and after 30 sec of familiarization, the light was on and the door between the two compartments was opened. When the rats entered the non-illuminated chamber, the guillotine door was automatically closed and an electrical foot shock (0.5 mA) was delivered for 5 sec through the stainless steel rods. The time taken to enter the non-illuminated chamber was recorded as the step-through latency. A retention trial was performed 24 h after training trial. The rats were placed again in the light chamber and the time to enter the dark chamber after door opening was measured without electric foot shock. If the rat did not enter the dark chamber within 300 sec, the step-through latency was regarded as 300 sec.

Reverse transcription-polymerase chain reaction: RT-PCR

After conducting behavior test, rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and sacrificed by decapitation to obtain cortical tissues. Total RNA was extracted from the rat cortex with TRI reagent (Molecular Research center, OH, USA) and then reverse transcribed for 60 min at 42°C using M-MLV reverse transcriptase (Promega, WI, USA). Amplification of cDNA was conducted by polymerase chain reaction (PCR) using synthetic primers specific to choline acetyltransferase (ChAT), M_1 type mAChR (M_1 mAChR), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as follows: ChAT, 5′-AGGGTGAT-CTGTTCACTCAG-3′ (sence), 5′-TCTTGTTGCCTGTCATCA-TA-3′ (antisence); M_1 mAChR, 5′-CAGAAGTGGTGATCAAGA-TGCC-3′ (sence), 5′-GAGCTTTTGGGAGGCTGCTT-3′ (antisence); GAPDH, 5′-GCCAAGGTCATCCATGACAAC-3′ (sense), 5′-AGT-GTAGCCCAGGATGCCCTT-3′ (antisense). The PCR products were analyzed by 1.5% agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer and examined under UV light using a gel documentation system (Geldoc 2000, Bio-Rad, Philadelphia, PA, USA) after staining with ethidium bromide (EtBr).

Immunoblotting

After completing behavior test, rats were sacrificed and the brains were dissected on an ice-cold operating table. The cortex and hippocampus were taken from the brain and homogenated thoroughly with RIPA buffer using automatic homogenizer (Precellys 24, Bertin Technologies, Montigny le Bretonneux, France). The homogenates were centrifuged at 14,000 *g* for 15 min on 4°C. The concentration of isolated protein was determined by BCA protein assay kit (Pierce Biotechnology, IL, USA). Immunoblotting was performed according to the procedures previously described (Lee *et al.*, 2011a).

Immunohistochemistry

Rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and perfused with phosphate buffered saline (PBS) followed by 4% cold paraformaldehyde in PBS. Brain tissues were post-fixed in the same fixative for additional 2 h at room temperature and then sequentially immersed in 10%, 20%, and then 30% sucrose solution. Frozen tissue blocks were made with OTC compound (Tissue Tek, Torrance, CA, USA) and coronal brain sections (30 μm thick) were serially taken using a cryostat (Leica, Wetzlar, Germany). Immunohistochemical staining was conducted following the procedures previously reported with some modifications (Lee *et al.*, 2011b). Correspondent slides with adhered sections were washed with 1% Triton X-100 in PBS for 10 min and then with PBS for 5 min twice. Endogenous peroxidase was inactivated by incubating the tissue sections with 1% H_2O_2 in PBS for 30 min. After washing with PBS for three times, the slides were blocked in 3% normal horse serum containing 0.1% Triton X-100 for 1 h at room temperature and incubated with primary anti-p-CREB antibody (1:200) prepared in PBS containing 0.1% bovine serum for 24 h at 4°C. After washing again three times with PBS, biotinylated secondary antibody was added for 2 h, followed by incubation with avidin-biotin peroxidase complex (Santa Cruz) for 1 h at room temperature. Then slides were developed by using DAB as substrate, dehydrated with a series of ethanol solution, and mounted with 50% (v/v) glycerol. The stained images were examined under a bright field microscope (Leica).

Statistical analysis

The data were expressed as mean values ± standard error of the mean (S.E.M.). Statistical analysis was performed by one-way ANOVA followed by the Tukey's test for multiple comparisons using SPSS software (SPSS 12.0 KO for windows). Differences were considered to be significant when *p* value was less than 0.05.
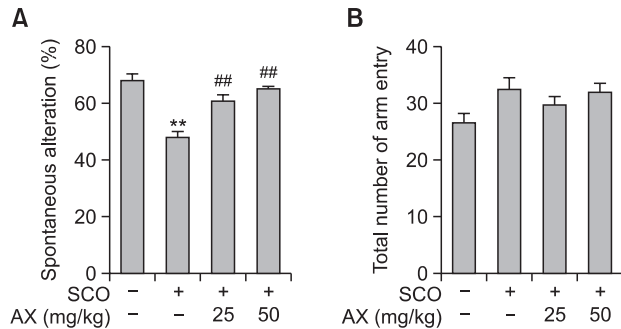
RESULTS

Effect of AX on SCO-induced memory impairments

Immediate working memory performance was assessed by recording spontaneous alteration behavior in Y-maze test. In this test, SCO (0.75 mg/kg, *i.p.*) significantly decreased the percentage of spontaneous alteration (Fig. 1A). Treatment of SD rats with AX (25 or 50 mg/kg) effectively increased % spontaneous alteration and improved memory impairment caused by SCO in a dose-related manner (Fig. 1A). However, under the same experimental condition, the total number of arm entries was similar in each experimental group (Fig. 1B).

In another experiment, Morris water-maze was conducted to compare the spatial memory. The rats in the vehicle-treated sham control group readily learned and memorized the location of submerged hidden platform during four consecutive training days, which was reached within 30 sec by the last day
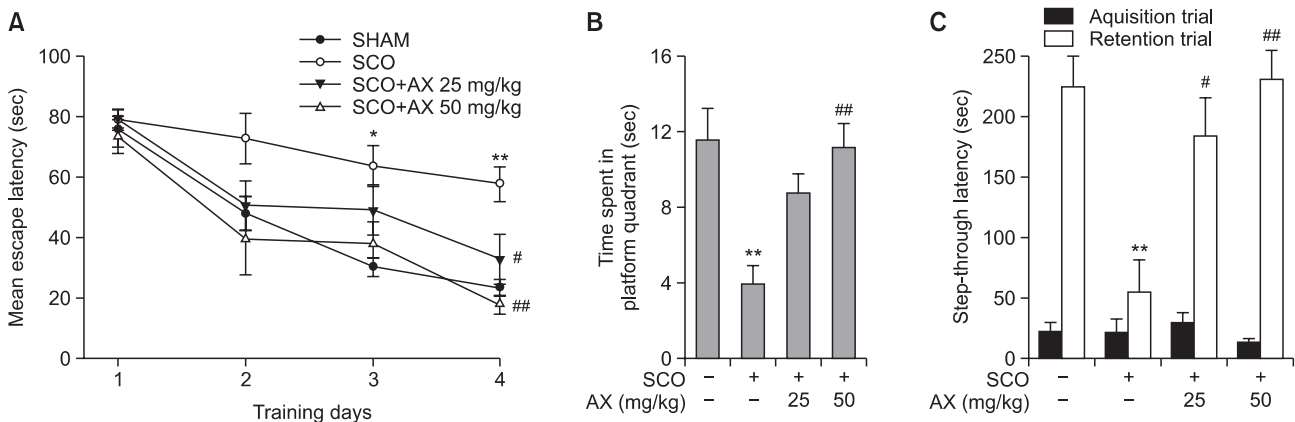

Fig. 1. Memory enhancing effect of AX in Y-maze test. (A) Effect of AX on the SCO-decreased spontaneous alternation was monitored by Y-maze test. (B) Total number of arm entries during 8-min session of Y-maze test. Data are presented as mean ± S.E.M. (n=7). Significant difference between groups: **p<0.01, vehicle-treated control vs. SCO alone group; ##p<0.01, SCO alone group vs. AX-treated groups in combination with SCO.

Fig. 2. Memory promoting activity of AX in Morris water-maze and passive avoidance tests. (A) Effect of AX on the SCO-induced spatial memory impairment which was represented by mean escape latency during training trials in Morris water-maze test. (B) Comparison of time spent in the platform quadrant during probe test in Morris water-maze test. (C) Protective effect of AX on the learning and memory deficit caused by SCO in the passive avoidance test. Data are presented as mean ± S.E.M. (n=6). Significant difference between groups: *p<0.05 and **p<0.01, vehicle-treated control vs. SCO alone group; #p<0.05 and ##p<0.01, SCO alone group vs. AX-treated groups in combination with SCO.
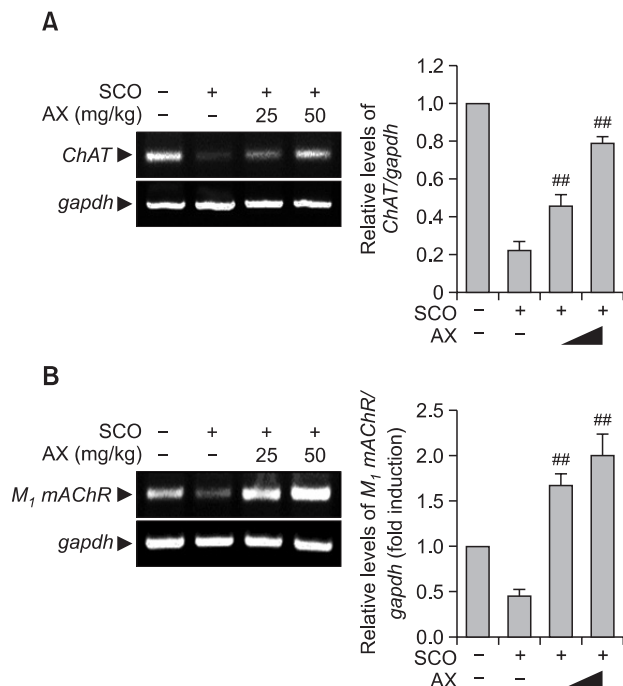
A

B

Fig. 3. Effect of AX on the mRNA expression of acetylcholine-related proteins The mRNA expression of enzymes involved in the synthesis of acetylcholine (A) and M_1 type mAChR (B) was analyzed by RT-PCR (n=3 per lane). mRNA levels of *gapdh* were determined to confirm equal amount of mRNA loading. Quantitative values for the relative mRNA expression were calculated from three independent experiments and represented in the right panels (n=9). Significant difference between groups : ##p<0.01, SCO alone group vs. AX-treated groups in combination with SCO.

of the training period (Fig. 2A). Conversely, the performance in the SCO alone-injected group was significantly impaired. However, repeated daily administration of AX (25 or 50 mg/kg) reduced mean escape latencies starting from the second day and particularly 50 mg/kg of AX-administered group exhibited almost similar mean escape latency to the vehicle-treated sham control group during four consecutive training days (Fig. 2A). Immediately after the last training session on day 4, the platform was removed and probe test was conducted for 30 s. The time spent in the target quadrant to search for the removed platform was monitored. The sham control and AX-treated groups spent more time in the probe quadrant compared with SCO alone-treated group (Fig. 2B) suggesting the retention of learning and memory by oral administration of AX.

To confirm the effect of AX on the SCO-induced learning and memory impairments, passive avoidance test was performed. In the acquisition trial, there was no significant difference in step-through latency time among experimental groups (Fig. 2C). However, during retention trial, the step-through latency was dramatically decreased in the SCO-injected rats and SCO-reduced step-through latency was effectively restored by oral administration of increasing doses of AX (25 and 50 mg/kg) (Fig. 2C).

Effect of AX on the expression of enzymes and receptors related with acetylcholine

To verify the effect of AX on the acetylcholine metabolism,

the mRNA levels of enzymes and receptors involved in the synthesis as well as pharmacological action of acetylcholine were monitored by RT-PCR. In the cortex, SCO decreased the mRNA expression of ChAT which plays a key role in the formation of acetylcholine (Fig. 3A). SCO-induced a decrease in the ChAT mRNA levels was restored by AX (25 or 50 mg/kg) in a dose-dependent manner (Fig. 3A). However, under the same experimental condition the mRNA expression of AChE, acetylcholine decomposing enzyme, was not much affected by SCO or AX treatment (data not shown). In another experiment, SCO injection also suppressed mRNA expression of M_1 type of muscarinic acetylcholine receptor (M_1 mAChR), which was significantly up-regulated by oral administration of AX (25 or 50 mg/kg) dose-dependently (Fig. 3B).

Effect of AX on the expression of BDNF and activation of CREB

To further elucidate the molecular mechanisms underlying the memory enhancing effect of AX, we have examined the expression of BDNF in the cortical and hippocampal brain tissues. Treatment of SD rats with AX (25 or 50 mg/kg) dose-dependently increased the protein levels of BDNF which was decreased by SCO in the cortex and hippocampus as well (Fig. 4A). To further determine an upstream regulator for the up-regulation of BDNF by AX, the possible involvement of CREB was investigated. Proteins from cortical or hippocampal tissue lysates were analyzed by Western blot analysis with p-CREB specific antibody. SCO-decreased phosphorylation of CREB was marked restored by oral administration of AX with maximal induction at the dose of 50 mg/kg (Fig. 4B). We also verified activation of p-CREB by AX (50 mg/kg) in the cortex as well as hippocampus (Fig. 4C) using immunohistochemistry. As a result, the phosphorylation of CREB was evident after AX treatment in accordance with the Western blot data of BDNF.

DISCUSSION

In this study, we have investigated the neuroprotective effects of AX against SCO-induced cognitive dysfunction in SD rats. First, to induce learning and memory impairment, we have intraperitonically injected SCO in SD rats and conducted a series of behavior tests. In the Y-maze test, SCO-treated rats showed decreased spontaneous alterations compared with that of vehicle-treated animals, which was improved by AX pretreatment. In Morris water-maze task, SCO-treated rats took longer time to find platform than the rats in sham control group and AX-treated group in combination with SCO easily found the location of hidden platform. In the passive avoidance test, SCO treatment reduced step-through latency, which was effectively restored by oral daily administration of AX.

As a molecular mechanism underlying the memory enhancing effect of AX, we showed that AX treatment increased phosphorylation of CREB, an activated form of CREB, and stimulated the expression of BDNF, ChAT and M_1 mAChR genes. It has been reported that BDNF, ChAT and mAChR genes have CRE, the binding site of CREB (Zhang *et al.*, 2005) implying that phosphorylation of CREB can stimulate the transcription of aforementioned genes. Indeed, phosphorylation of CREB ameliorated stresses generated by diverse pathophysiological conditions such as AD (Jin *et al.*, 2009), ischemia (Kim *et al.*, 2009), aging (Asanuma *et al.*, 1996; Hu *et al.*, 2010) in as-
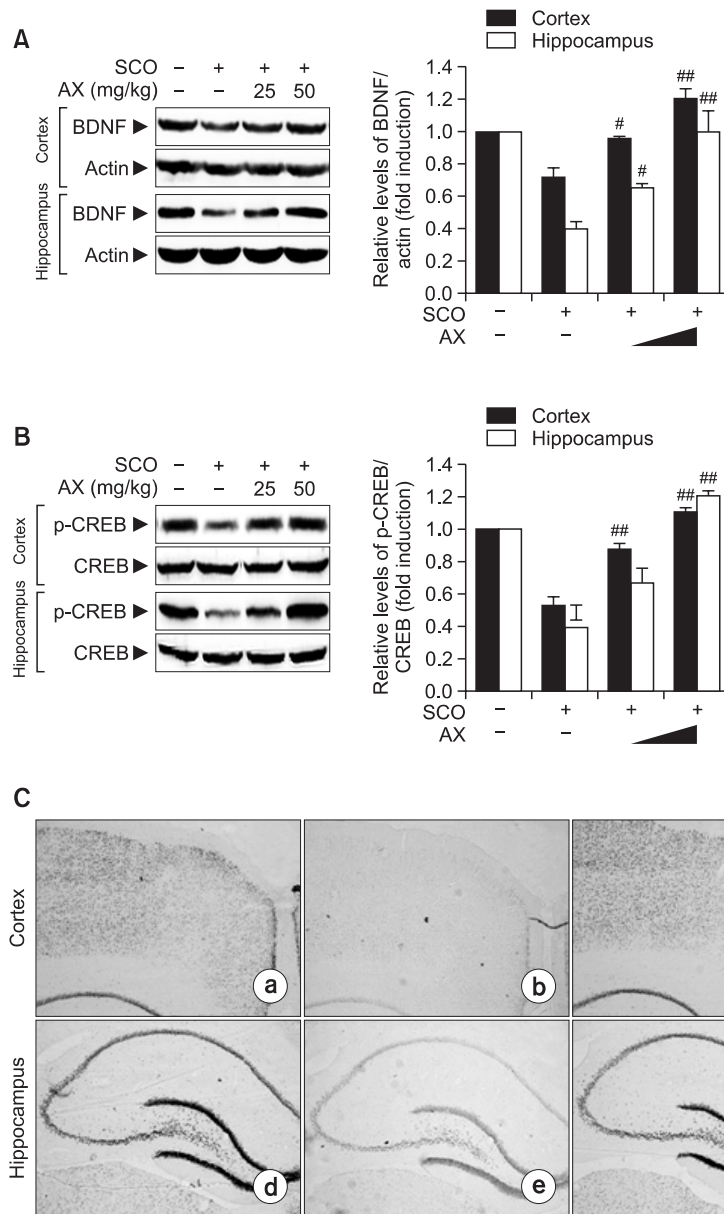
Fig. 4. Effect of AX on the protein levels of BDNF and activation of CREB The protein expression of BDNF (A) and p-CREB (B) was examined by Western blot analysis (n=3 per lane). Proteins from tissue lysates were analyzed by using BDNF or p-CREB specific antibody. Actin expression levels were monitored to ensure equal amount of protein loading. Relative protein expression calculated from three independent experiments was represented in the right panel (n=9). Significant difference between groups : [#]$p<0.05$ and [##]$p<0.01$, SCO alone group vs. AX-treated groups in combination with SCO. (C) Activation of CREB was verified by immunohistochemistry utilizing anti-p-CREB antibody. a and d : sham control, b and e : SCO alone (0.75 mg/kg) group, c and f : SCO (0.75 mg/kg)+AX (50 mg/kg).

sociation with BDNF (Jin *et al*., 2009; Kim *et al*., 2009), ChAT (Asanuma *et al*., 1996; Jin *et al*., 2009), and mAChR (Jin *et al*., 2009; Hu *et al*., 2010). Particularly, the activation of CREB and increased expression of BDNF are involved in enhancement of long-term memory (Cunha *et al*., 2010; Bitner, 2012). In addition, up-regulated expression of ChAT and mAChR results in an increased production of acetylcholine and amplification of signal transduction with acetylcholine acting as a ligand. Thus, CREB-mediated expression of BDNF, ChAT and mAChR by AX may contribute to the improvement of memory impaired

by SCO.

In accordance with our findings, a wide range of phytochemicals have been reported to have neuroprotective and memory enhancing effects via CREB-BDNF signals. Triterpenoid saponin PGS32 (polygalasaponin XXXII) isolated from the roots of *Polygala tenuifolia* Willd. prevented SCO-induced cognitive impairments possibly through improvement of synaptic transmission, activation of the mitogen-activated protein kinase (MAPK) and CREB, and enhancement of BDNF levels (Xue *et al*., 2009). Kalopanaxsaponins A and B derived

from stem-bark of *Kalopanax pictus* ameliorated SCO-induced memory deficits by inducing BDNF and p-CREB expression and conversely inhibiting AChE activity as well (Joh *et al*., 2011).

To determine which compounds might be responsible for improving memory in rats administered with SCO, we have to follow the passages that the AX undergoes in the gastrointestinal tract. Zhang *et al*. (Zhang *et al*., 2003) demonstrated that approximately 3% of arabinose on AX was released in 3 h of incubation at pH 3. Subsequently the remaining AX entering the small intestine is suitable for the breakdown as it is not a substrate for enzymatic digestion in the small intestine. Finally, the remaining AX entering the large intestine is first degraded into monosaccharides such as arabinose and xylose by bacteria inhabiting in the large intestine, followed by fermentation into short chain fatty acids (SCFA) such as butyrate, propionate and acetate (Broekaert *et al*., 2011). As a result, both the monosaccharides generated in the small and large intestines and SCFA formed in the large intestine are promising candidates that can be absorbed into the blood and reach the brain. Arabinose (Eidelberg *et al*., 1967) and butyrate (Dienel *et al*., 2001) have been reported to penetrate across blood-brain barrier (BBB) and be transported into the brain. However, mechanisms by which arabinose and/or xylose might play in enhancing learning and memory functions have not been elucidated yet, although oral administration of arabinose attenuated brain injury by inhibiting inflammatory responses caused by hypoperfusion in SD rats (Han *et al*., 2010). On the other hand, memory enhancing mechanisms of butyrate are beginning to be elucidated. Butyrate can be metabolized to produce ATP that can be converted to cAMP by adenylyl cyclase. Therefore, consequent increase of cAMP activates protein kinase A (PKA) leading to phosphorylation of CREB (Wang *et al*., 2012). Thus, contribution of AX to enhancing memory can be explained, at least in part, by conversion of AX into arabinose and butyrate.

In summary, we demonstrated that AX improved learning and memory functions in SD rats with SCO-induced amnesia. This cognition enhancing effect of AX was mediated partly through activation of CREB cascade and subsequent expression of BDNF. To translate the novel effect of AX in CNS into clinical applications for the improvement of cognitive functions, the pharmacology and mode of action of its active components should be further investigated.

ACKNOWLEDGMENTS

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