Prevention of scopolamine-induced memory deficits by schisandrin B, an antioxidant lignan from *Schisandra chinensis* in mice

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Abstract

The preventive effect of schisandrin B (Sch B), an antioxidant ingredient of *Schisandra chinensis*, was studied on scopolamine-induced dementia in mouse. Scopolamine developed oxidative stress in the brain with the decreased levels of antioxidant enzymes and increased nitrite level. At the same time, a significant impairment of learning and memory occurred when evaluated by passive avoidance task (PAT) and Morris water maze (MWM) with concomitant increase of acetylcholinesterase (AChE) activity and decreased acetylcholine levels. Pre-treatment by Sch B (10, 25, 50 mg/kg) effectively prevented scopolamine-induced oxidative stress and improved behavioural tasks. Further, the scopolamine-induced increase in AChE activity was significantly suppressed and the level of acetylcholine was maintained as normal by Sch B treatment. These results suggest that Sch B have protective function against cerebral functional defects such as dementia not only by antioxidant prevention but also exerting its potent cognitive-enhancing activity through modulation of acetylcholine level.

Keywords: Schisandrin b, oxidative stress, antioxidant, acetylcholinesterase

Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by irreversible, progressive loss of memory followed by complete dementia [1]. Numerous pre-clinical and clinical trials are undergoing for the medications of the dementia associated with AD; however, there is no promised modality to cure the AD so far developed. Studies of AD patients revealed the depleted levels of neurotransmitter acetylcholine (ACh) in the brain. Thus, maintaining the ACh level in the brain is crucial to ameliorate the conditions of AD patients [2]. Cholinergic drugs have been reported to increase the regional cerebral blood flow and thus the level of ACh in the brain regions affected by AD [2]. On the other hand, one approach to increase the ACh level is blocking the activity of acetylcholinesterase (AChE), the enzyme degrading ACh, and thus several cholinesterase inhibitors (AChEI) have been developed and clinically applied for the treatment of AD patients, those are tacrine (THA), donepezil hydrochloride, galantamine hydrobromide and rivastigmine tartrate [3]. However, AChEI presents some limitations because of their short half-lives and excessive side-effects caused by the activation of peripheral cholinergic systems, as well as hepatotoxicity that is the most frequent and critical side-effect of these drugs [4–7]. Therefore, alternate treating modality is prospected for AD. Traditional herbal medicine or natural products thus attracts attention as an alternative or complementary approach for AD treatment because of their basic antioxidant and multiple targeting properties [8]. Several studies have indeed shown the neuroprotective and/or cognition-enhancing properties of natural products and their components using different animal models [9–15]. Although a complex cellular process associates with AD pathogenesis, the
oxidative stress is a basic pathology and plays a critical role in AD development [16]. El-Sherbiny et al. [17] reported that memory impairment in a scopolamine-induced animal model is also associated with altered status of brain oxidative stress. Strong evidence supporting the involvement of oxidative stress in degenerative changes within the forebrain cholinergic system has been suggested. These findings led to the notion that compounds with antioxidant potential might be beneficial for preserving brain function.

In the present study we focused on the neuroprotective effect of schisandrin B (Sch B; Figure 1), a major lignan isolated from fructus Schisandra chinensis (FS), which exert high antioxidant potential both in vitro and in vivo [18,19]. FS is a traditional Chinese herb commonly used to treat cough, mouth dryness, spontaneous sweating, dysentery and insomnia. It is clinically used for the treatment of hepatitis and myocardial disorders and possesses strong antioxidant activities [20–22]. It is also a major component herb of many traditional herbal medicines including Shengmai San (SMS). We previously showed SMS effectively prevents cerebral oxidative damage in the rat and also in the neuronal cell model [23,24]. Neuroprotective function of FS and the lignans of FS have also been published [18,19]. Therefore, Sch B is an attractive target ingredient to study the mechanism of neuroprotection by these brain targeting herbs or prescriptions. The other lignans from FS, such as gomisin A and schizandrin, were reported to ameliorate scopolamine-induced learning and memory impairments in mice [25,26] and it was also reported that Sch B protects the PC12 cells against beta amyloid and homocysteine-induced neurotoxicity [27]; however, no in vivo studies have been conducted on the anti-amnestic effects of Sch B.

Hence, the present study was undertaken to assess the preventive effects of Sch B on scopolamine-induced memory deficits in a mice model and demonstrated that Sch B improved tissue antioxidant potential and prevented learning and memory dysfunctions induced by scopolamine through manipulating ACh level by its inhibitory action on AChE.

**Materials and methods**

**Animals**

The experiments were carried out on male Balb c mice (12–14 weeks old, weighing 25–30 g). The mice were housed in stainless steel cages and kept under controlled conditions at 22 ± 3°C, 55 ± 5% relative humidity and a 12 h light/dark cycle throughout the experiment. Food in the form of dry pellets and water were available ad libitum. The animal experiments were performed according to internationally followed ethical standards and with approval from Niigata University of Pharmacy and Applied Life Sciences.

**Sample preparation and treatment**

Sch B was isolated from the petroleum extract of FS as described by Ip et al. [28]. The purity was greater than 95%, as assessed by HPLC. In the Sch B treatment groups, mice were orally administered with Sch B (dissolved/suspended in olive oil) by intubation at a daily dose of 10, 25 or 50 mg/kg for 7 days prior to scopolamine treatment following behavioural and biochemical assessments. Control animals received the vehicle (olive oil) only. To evaluate the pharmacological efficacy of Sch B, THA was used at the same time as a reference (positive control). THA (10 mg/kg p.o. for 7 days) and scopolamine (1 mg/kg i.p.) were dissolved in saline before treatment.

**Drugs and chemicals**

THA, physostigmine, scopolamine, acetylthiocholine iodide (AChI) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used in the study were of analytical grade. Solutions of the drug and chemicals were freshly prepared before use.

**Passive avoidance task (PAT)**

Training for and testing of passive avoidance performance were carried out in two identical light and dark square boxes. On day 7, the mice were initially placed in the light chamber and 10 s later the door between the compartments was opened. When mice entered the dark compartment, the door was closed and an electrical foot shock (0.5 mA) for a period of 3 s was delivered through stainless steel rods (one training trial). Six mice were used per treatment. The last shots of Sch B or THA were given at 1 h before the training trial. Scopolamine (1 mg/kg i.p) was administered...
30 min after Sch B or THA treatment. Twenty-four hours after the training trial, the mice were again placed in the light compartment. The escape latency to enter the dark compartment was measured. If the mice did not enter the dark compartment within 300 s, the experiment was stopped. For investigation of the effect of Sch B on memory in unimpaired animals, Sch B alone was administered 1 h before the acquisition trial without treatment of scopolamine. The intensity of electrical foot shock was set at 0.25 mA instead of 0.5 mA to avoid a ceiling effect in unimpaired animals [24].

Morris water maze test (MWM)

A spatial memory test was performed using the MWM. The MWM is a circular pool (50 cm diameter and 40 cm height) with a featureless inner surface. The circular pool was made opaque by the addition of milk powder. The pool was divided into four quadrants of equal area. A white platform (5 cm diameter and 25 cm height) was centred in one of the four quadrants of the pool and submerged 1 cm below the water surface so that it was invisible at water level. The day prior to the experiment was dedicated to swim training for 60 s in the absence of the platform. In the following days, the mice were given single trial sessions each day for 4 consecutive days. During each trial, the time taken to swim to the platform (escape latency) was recorded. Once the mouse located the platform, it was permitted to remain on it for 10 s and then removed from the pool [29]. One day after the last trial session, mice were subjected to a probe trial session in which the platform was removed from the pool; mice were allowed to swim for 120 s to search for it. A record was kept of the swimming time in the pool quadrant where the platform had been previously placed. Six mice were used per treatment. Mice were treated with Sch B or THA 1 h before the training trial. Scopolamine (1 mg/kg i.p) was administered 30 min after Sch B or THA treatment. All mice were tested for spatial memory 30 min after the administration of scopolamine.

Tissue preparation

The mice were sacrificed by cervical dislocation following the behavioural study. The whole intact brain was carefully removed and placed in an ice-chilled petri dish for cleaning. The cerebellum was rapidly removed and the remaining brain was weighed, washed with isotonic saline and homogenized (10% w/v) in ice-cold sodium phosphate buffer (30 mM, pH 7.0) at 9500 rpm three times with intervals of a few seconds between the runs.

One-half of the volume of this homogenate was separated and used for assay of AChE and antioxidant activity as a salt-soluble (SS) fraction. For preparing a detergent-soluble (DS) fraction for AChE assay, 1% Triton X-100 (1% w/v 30 mM, sodium phosphate buffer, pH 7.0) was added slowly to the SS fraction while stirring on ice and then the volume was adjusted to make the final 10% homogenate. All homogenates were centrifuged at 100 000 g at 4°C for 60 min. The supernatant was stored at 4°C and used as a DS fraction [30]. The protein concentrations were determined by the Bradford assay with bovine serum albumin (BSA) as a standard (0.05–1.00 mg/ml).

AChE inhibitory effects of Sch B

AChE activity was determined according to the colourimetric assay of Ellman et al. [31], as previously described. Briefly, 25 μl of 15 mM AChI, 75 μl of 3 mM, DTNB and 75 μl of 50 mM Tris-HCl, pH 8.0, containing 0.1% BSA, were taken into 96-well plates, and the absorbance was measured at 405 nm after 5 min of incubation at room temperature. Then, 25 μl of each sample (SS and DS fractions) was added to above the reaction mixture and the absorbance was measured again after 5 min of incubation at room temperature. Duplicate determinations were carried out for each sample in one grouped three equivalent samples for one data. In order to determine the in vitro inhibitory effects of Sch B, it was initially dissolved in dimethyl sulphoxide and further diluted to various concentrations with ethanol immediately before use. Aliquots of diluted Sch B (1.5 ml) were then mixed with 2.6 ml of buffered Ellman’s reagent (75 mM) and 100 μl of buffered Ellman’s reagent (DTNB 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. Readings were taken at 30 s intervals for 5 min. The concentrations of Sch B required to inhibit AChE activity by 50% were calculated using the dose response curves. THA was used as a positive control (1 nM–100 μM) [24,31].

Estimation of nitrite

The accumulation of nitrite, an indicator of the production of nitric oxide, was determined using Greiss reagent as described by Green et al. [32]. The supernatant was added with equal volume of Greiss reagent and incubated for 10 min at room temperature in the dark and absorbance was determined at 540 nm (Shimazu model UV/Vis spectrophotometer). The concentration of nitrite in the supernatant was determined from the standard curve prepared for sodium nitrite and expressed as μM/mg protein.
Assay of ACh

ACh was determined by the method of Hestrin [33]. Briefly, 10% brain homogenate in cold saline was prepared (10 000 rpm, 10 s twice with 30 s interval) on ice. The aliquots (0.8 ml) of brain homogenate were mixed with 1.4 ml distilled water, 0.2 ml of 1.5 mM physostigmine and 0.8 ml of 1.84 M trichloroacetic acid blending adequately. After centrifugation, 1 ml of each supernatant was added to 1 ml of basic hydroxylamine. The mixture was incubated for 15 min at 25°C and then 0.5 ml of 4 M HCl and 0.5 ml of 0.37 M FeCl3 were added. Absorbance were read at 540 nm and calibrated with standard (ACh 0.2 μM/ml)

Antioxidant assay

The SS fraction of brain homogenate was used for a different antioxidant enzyme assay. The quantitative measurement of malondialdehyde (MDA), end product of lipid peroxidation, in brain homogenate was performed according to the method of Ohkawa et al. [34]. Reduced glutathione (GSH) was determined by the Ellman [35] method, which is based on the development of a yellow colour due to the reaction of DTNB with compounds containing sulphhydryl groups. The glutathione peroxidase (GPx) activity was determined according to the method described by Lankin et al. [36] using tert-butyl hydroperoxide as a substrate. The superoxide dismutase activity (SOD) was determined using the water-soluble tetrazolium method [37].

Statistical analysis

The results are given as mean ± SEM. The data obtained was analysed by one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. Differences were considered significant at the 5% level.

Results

Effects of Sch B on memory impairment induced by scopolamine: Assessed by PAT

The effect of Sch B on the scopolamine-induced memory deficit was evaluated using PAT. During acquisition trials, latency times were not different among the experimental groups of mice by the treatment with Sch B. For retention trials, the ANOVA followed by Tukey’s test revealed significant differences for the latency time in the Sch B treatment group. The step through latency was shortened in scopolamine-treated mice compared to that of normal mice (Figure 2A) but the shortened step through latency induced by scopolamine was significantly restored by the Sch B (10, 25 and 50 mg/kg) treatment at all doses. In mice treated with Sch B at a dose of 25 mg/kg, the step through latency was increased to 75.4% of normal control mice and was comparable to the value (76.9%) attained by the reference drug THA. Furthermore, the step through latencies of the groups treated with Sch B (25, 50 mg/kg) alone also significantly (p < 0.05) increased more than those in the normal control group (Figure 2B).

Effects of Sch B on memory impairment induced by scopolamine: Evaluated by MWM test

The effect of Sch B on spatial learning was evaluated using the MWM task. As shown in Figure 3A, the scopolamine-treated group exhibited longer escape latencies throughout training compared with the
control group. Sch B (25, 50 mg/kg) significantly shortened the increased escape latency by scopolamine. THA also significantly reduced the escape latencies compared with those in the scopolamine-treated group. On the day following the final day of training trial sessions, swimming times within the target quadrant in the scopolamine-treated group were significantly ($p < 0.01$) shorter than those in the control group (Figure 3B). However, the swimming time within the platform quadrant shortened by scopolamine treatment was significantly increased by Sch B treatment at the dose of 25 and 50 mg/kg and THA treatment. In mice treated with Sch B at a dose of 25 mg/kg, the swimming time in the target quadrant was increased to 68% of normal control mice and was comparable to that (69.8%) of reference drug THA.

**AChE inhibitory effects of Sch B**

Since AChE inhibitors are known to antagonize scopolamine-induced amnesia, the effect of Sch B on AChE activities in salt and detergent-soluble brain homogenates were evaluated (Figures 4A and B). There was a significant increase in AChE activity in both SS and DS ($p < 0.01$) fractions of the scopolamine-treated group. Sch B (25 mg/kg ($p < 0.01$), 50 mg/kg ($p < 0.05$)) treatment significantly inhibited the AChE activity in both SS and DS fractions of brain homogenate at the concentration higher than 25 mg/Kg. The inhibitory activity of Sch B was almost the same as that given by a reference drug THA that also inhibited the AChE activity in both SS and DS ($p < 0.01$) fractions of brain homogenate.

In order to know whether the inhibitory activity of Sch B on AChE is direct or indirect, the AChE activity was measured by adding elevated concentrations of Sch B directly into the brain homogenate. The 50% inhibitory concentration (IC$_{50}$) of Sch B was $\sim 667 \mu$M. The value was far larger than that (120 nM) determined simultaneously.

**Effect of Sch B on ACh level**

As Figure 4C shows, scopolamine induced a significant decrease of ACh level ($p < 0.01$) in the brain homogenate and the results were in consistant with a previous report by Saito et al. [38]. At the same time, Sch B significantly prevented the decrease (25 mg/kg, 50 mg/kg ($p < 0.01$)) and maintained the ACh level as normal control. THA as a reference drug also significantly ($p < 0.01$) inhibited the decrease of ACh level.
Figure 4. The effect of Sch B (10, 25, 50 mg/kg) administration for 7 days on AChE activity in SS fraction (A) and DS fraction (B) on ACh levels (C) and nitrite levels (D) of brain homogenate in scopolamine-induced memory deficit mice. Data represents mean ± SEM (n = 6). **p < 0.01 statistically different from control group. ##p < 0.01, #p < 0.05 statistically different from scopolamine-treated group.

Effect of Sch B on nitrite level

The nitrite level was increased in the scopolamine-treated group, but the extent was not statistically significant compared to that in the control group. In the present study, we observed the nitrite inhibitory effect of Sch B in the brain homogenate in a dose-dependent (25 mg/kg (p < 0.05), 50 mg/kg (p < 0.01)) manner, as shown in Figure 4D. Similarly, Guo et al. [39] reported the nitrite inhibitory effect of Sch B in vitro and in vivo systems.

Antioxidant effects of Sch B on scopolamine-induced oxidative stress

To further elucidate the biochemical mechanism of the anti-amnesic activity of Sch B in mice, effects of Sch B against oxidative stress were examined in the brain tissue. The treatment of mice with scopolamine resulted in a significant increase in MDA levels (Figure 5A) (p < 0.01) and significant decreases both in glutathione (Figure 5B) (p < 0.01) and SOD (Figure 5D) (p < 0.01) levels. The activity of GPx was also reduced, but the difference was not statistically significant (Figure 5C). The treatment of amnesic mice with Sch B retained the GSH level (10 mg/kg (p < 0.05), 25 mg/kg (p < 0.01), 50 mg/kg (p < 0.01)) and the activities of GPx (25 mg/kg, 50 mg/kg (p < 0.05)) and SOD (25 mg/kg (p < 0.05), 50 mg/kg (p < 0.01)) to normal control levels. Sch B also significantly decreased the MDA (10 mg/kg (p < 0.05), 25 mg/kg (p < 0.05), 50 mg/kg (p < 0.01)) levels compared with those of the scopolamine-treated group, as shown in Figure 5.

Discussion

In the present study, we examined the neuroprotective effects of Sch B by biochemical and behavioural evaluations using a pharmacologically-induced amnesia by scopolamine in mice. Scopolamine, a muscarinic antagonist that induces central cholinergic blockade, produces a reversible and well-described impairment in both (i) maintaining attention; and (ii) processing of information and the acquisition of new knowledge both in rodents and in humans. The cognitive deterioration caused by scopolamine resembles the memory disturbances observed in AD [40,41]. In this study, mice were given scopolamine to induce memory impairment at a dose of 1 mg/kg; this dose has been reported to have no effect on acquisition latency or swimming ability [10]. Under this condition, scopolamine significantly shortens the step-through latency.
in the PAT task, which is a useful tool for the estimation of standard learning and memory deficits, and is thus used as an indicator of short-term and long-term memory [42]. In the Sch B treated group, the scopolamine-induced shortening of the step-through latency was successfully prevented at every dose of Sch B examined. Sch B at the dose of 25 mg/kg recovered the memory level to 75.4% and thus the activity was comparable to THA (76.9%).

On the other hand, MWM evaluates spatial memory and detects changes in the central cholinergic system [43]. In the present study, scopolamine increased the escape latency as compared to normal in the MWM trial. At the probe trial session, Sch B (25 and 50 mg/kg) significantly increased the swimming time within the target quadrant. At the dose of 25 mg/kg of Sch B, the swimming in the target quadrant was 68% and was comparable to that of THA, 69.8%. Collectively, from these behavioural studies, it was revealed that Sch B improves the long-term memory in amnesic mouse models induced by scopolamine treatment.

The cholinergic neurotransmission system in the basal forebrain plays an important role in learning and memory and thus maintaining ACh level is critical for the brain function. One of the targets for treating AD is the AChE inhibitors that increase the availability of ACh in central cholinergic synapses [43,44]. It is well documented that the AChE occurs in different molecular isoforms having differential localizations in neuronal cells [45]. Two major isoforms are globular monomer (G1) and globular tetramer (G4) of the same monomer sub-unit. The G1 isoform is reported to present in the cytoplasm of neuronal cells, whereas the G4 isoform is predominantly membrane-bound [46]. Results showed that the AChE (G1 and G4 isoforms) levels in both SS and DS fractions were significantly increased compared to normal control after scopolamine treatment, but Sch B treatment reduced the level in both SS and DS fractions. Further, we studied the direct inhibitory action of Sch B on AChE activity in vitro; however, the IC_{50} values obtained were > 500 μM, which was far larger than the value of THA (120 nM). Therefore, the inhibitory effect of Sch B may involve other mechanisms than its direct inhibition of the enzyme. Further we analyzed the ACh levels in the brain homogenate of memory deficits mice. We observed that ACh levels were significantly reduced in scopolamine-treated mice, but treatment with Sch B increased the reduced ACh level as did THA. Altogether, our data suggest that the ameliorating effects of Sch B on memory
deficit might have resulted from the modulation of ACh level through an inhibition of AChE enzyme by certain mechanisms other than direct interactions with enzymes. Since Sch B is metabolized in the cells, the metabolites could be one of the candidates inhibiting AChE activity.

Another beneficial property of Sch B found in the present study is the potential antioxidant activity. It is well accepted that oxidative stress is at the forefront of AD research. Evidence indicates that a long ‘dormant period’ of gradual oxidative damage accumulation precedes and actually leads to the seemingly sudden appearance of clinical and pathological AD symptoms, including cognitive decline [47]. Several reports have emphasized the potential therapeutic role of antioxidant agents in AD treatment [48]. Memory impairment induced by acute administration of scopolamine in rats is associated with the altered level of GSH in the brain [17]. In our experimental conditions, scopolamine treatment resulted in a significant increase of TBARS, an important marker for lipid peroxidation, in the brain and a reduction in both GSH and SOD activities. Although the activity of GPx was not changed by the administration of scopolamine, Sch B elevated the activity of GPx to a level higher than that found in normal control mice. The most remarkable effect of Sch B is the increased activity of SOD. Treatment with Sch B not only preserved the reduced SOD activity induced by scopolamine but also elevated to a level comparable to that of normal control mice. Most recently, SOD mimics have come to the forefront of anti-oxidative therapeutics of neurodegenerative disease [49]. In addition to its antioxidant and anti-cholinesterase properties, the anti-inflammatory function of Sch B may play an important role in preserving brain function. The anti-inflammatory effect of Sch B was observed in vitro and in vivo [39], but its role in the dementia model is unclear. In order to clarify the role of Sch B in the inflammatory cascade, we are extending the present study using an amyloid beta-induced dementia model in rats.

In conclusion, the present study revealed that Sch B effectively prevents neuronal defects induced by scopolamine. The underlying mechanism should be associated with enhancing cholinergic signalling through the inhibition of AChE and preventing the oxidative stress. Since the oxidative stress is a basic pathology of degenerative brain diseases, the natural antioxidant molecules carrying additional functions such as AChE inhibition should be more promising for the treatment of AD. Hence, the present findings suggest that Sch B will provide complementary advantage for the treatment of neurodegenerative diseases such as AD.

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Declaration of Interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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