

Neuroprotective Effect of Ginsenoside Rh1 on Scopolamine-Induced Cognitive Dysfunctions

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Abstract

Background: Ginsenoside Rh1, a 20(S)-protopanaxatriol with a single sugar moiety from ginseng, has benefits on the central nervous system and recently Rh1 had been reported to be effective for the Alzheimer's disease (AD).

Objective: The present study was conducted to investigate the neuroprotective effects of Ginsenoside Rh1 on scopolamine-induced cognitive dysfunctions and to elucidate its underlying mechanisms of action.

Methods: Rh1 (20 and 40 $\mu\text{mol/kg}$) was administered intraperitoneally to mice for 28 days, and scopolamine (0.75 mg/kg) was injected intraperitoneally for 10 days to induce memory deficit. Memory-related behaviors were evaluated using object location recognition (OLR) experiment, novel object recognition (NOR) test, Morris water maze (MWM) test and passive avoidance (PA) task. Cholinergic system function and oxidative stress activities were measured in the hippocampus. The expression levels of CREB, Egr-1, c-Fos and c-Jun were also measured in the hippocampus.

Results: Rh1 administration could improve the cognitive performance of scopolamine-treated mice in the object location recognition, the novel object recognition, the Morris water maze and the passive avoidance tests. And Rh1 significantly enhanced cholinergic system function and suppressed oxidative stress level in the hippocampus of scopolamine-treated mice, as indicated by decreasing acetylcholinesterase (AChE) activity, elevating choline acetyltransferase (ChAT) activity and acetylcholine (ACh) level, increasing superoxide dismutase (SOD) activity and lowering the level of malondialdehyde (MDA) in the hippocampus of scopolamine-treated mice. Moreover, for the first time, Rh1 was found to significantly up-regulate the expression levels of CREB, early growth response-1 (Egr-1), c-Fos and c-Jun in the hippocampus of scopolamine-treated mice.

Conclusion: These results demonstrated that Ginsenoside Rh1 might exert a significant neuroprotective effect on cognitive dysfunctions induced by scopolamine, driven in part by the modulation of cholinergic activity, oxidative stress level and upregulation of CREB, Egr-1, c-Fos and c-Jun expression in the hippocampus.

Keywords:

Ginsenoside Rh1, Scopolamine, Cognitive dysfunction, Cholinergic system function, Oxidative stress

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Introduction

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases characterized as progressive impairment of cognition [1]. Although the exact mechanism of AD remains unclear until now, abundant studies indicated that cholinergic dysfunction and oxidative stress were important in the procession of causing AD [2]. Cholinergic neurons have a noteworthy role in cognitive function, and their loss from the hippocampus is a distinguishing feature of AD [3]. Besides, elevated acetylcholinesterase (AChE) activity in the brain of AD patients has been shown to be associated with increased oxidative stress resulting from mitochondria dysfunction and this in turn accelerates memory loss [4,5]. Scopolamine (Scop), a non-selective muscarinic cholinergic receptor antagonist, causes disturbances in cholinergic system as well as in associated neurochemical cascades resulting in cognitive decline [6]. In addition, recent researchers reported that scopolamine also influences the brain oxidative status which is imbalanced in AD [7]. Therefore, scopolamine-induced dementia model is an appropriate dementia model to reveal AD-related cognitive impairment profile for planning therapeutic strategies against cognitive dysfunctions [8].

Ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae), has been used in traditional oriental medicine for human health in Asian countries, and its neuroprotective effect on different neurologic diseases has been studied [9-12]. Ginsenosides (ginseng saponins) have been considered as the primary active ingredients and mainly are responsible for most of the activities of ginseng [13]. Ginsenoside Rh1 is an important protopanaxatriol ginsenoside that has been reported to be the main hydrolysis product reaching the systemic circulation after oral ingestion of ginseng [14]. Rh1 has attracted increasing attention due to its powerful pharmacological efficacy. It was reported that Rh1 possessed many biological activities including anti-allergic and anti-inflammatory activities [14,15], anti-obesity effect [16] anti-oxidant activity [17]. Its benefits are mainly on central nervous system by showing effective in memory-improving effect of normal mice and scopolamine-treated mice, increasing hippocampal excitability in the dentate gyrus of anesthetized rats and enhancing cell survival in the dentate gyrus of mice [18,19]. Recently, our finding showed that Rh1 exhibited cognition-

enhancing effect on sleep-deprivation mice by reducing oxidative stress in cortex and hippocampus [20]. Although previous study showed that Rh1 has a protective effect in scopolamine-induced mice and improve its cognitive performance in step-through passive avoidance test [18], researches about cognitive improving effect of Rh1 on AD is still limited and the *in vivo* study of possible mechanism is insufficient. Therefore, the present study firstly used object location recognition (OLR), novel object recognition (NOR), Morris water maze (MWM) and passive avoidance (PA) tests to evaluate the cognitive effect of Rh1 in scopolamine-induced mice and then analyzed the changes of cholinergic system function, oxidative stress biomarkers and memory-related molecules to explore the mechanism of Rh1.

Materials and Methods

Chemicals and reagents

Rh1 (purify >98% by HPLC) was purchased from Chengdu Herbpurify Co., Ltd (Chengdu, Sichuan, China). Scopolamine (Scop) was purchased from Sigma-Aldrich Inc. (Saint Louis, USA). Donepezil hydrochloride (Aricept), the positive control used in this study was purchased from Eisai Inc. (Ibaraki, Japan). Rh1, Donepezil (DNP) and Scop were dissolved in normal saline solution for the animal experiment. The commercial kits for ChAT, AChE, Ach, superoxide dismutase (SOD) and malondialdehyde (MDA) were obtained from Jiancheng Biological Technology Co., Ltd (Nanjing, Jiangsu, China). Primary antibodies for CREB, Egr-1, c-Fos, c-Jun and β -actin were obtained from Cell Signaling Technology (Cell Signaling, Danvers, MA, USA).

Animals

Sixty male ICR mice (weighing 20-22 g) with animal quality certificate number SCXK 2012-0001, were purchased from the Beijing Vital River Laboratories and housed at a temperature controlled (23 ± 2)°C condition with humidity of $50\% \pm 10\%$ and a 12 h light/dark cycle. The mice were given free access to water and diet. All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and performed under the approval and supervision of the Academy of Experimental Animal Center of the Institute of Medicinal Plant Development.

■ Experimental design

After habituation for 7 days, the mice were randomly divided into five groups (n=12 per group): normal untreated control, Scop (0.75 mg/kg)/vehicle-treated, Scop (0.75 mg/kg)/Rh1 (20 μ mol/kg)-treated, Scop (0.75 mg/kg)/Rh1 (40 μ mol/kg)-treated and Scop (0.75 mg/kg)/DNP (1.6 mg/kg)-treated. The control group was treated with saline at the equivalent volume of physiological saline. The Scop group was treated with scopolamine (0.75 mg/kg). The Rh1 min group was treated with scopolamine (0.75 mg/kg) and Rh1 (20 μ mol/kg). The Rh1 max group was treated with scopolamine (0.75 mg/kg) and Rh1 (40 μ mol/kg). Dose of Rh1 was determined based on our previous report [20]. The DNP group was treated with scopolamine (0.75 mg/kg) and donepezil (1.6 mg/kg). The scopolamine was injected intraperitoneally to mice for 10 consecutive days, while Rh1 and DNP were administered intraperitoneally 30 min before Scop injection for 28 consecutive days. The experiment procedure was illustrated in **Figure 1**.

■ Open field (OF) test

After the pretreatment, the open field test was first conducted as described in a previous study [21] to assess the effect of Rh1 on the locomotor activity for eliminating its interference in cognitive function. The apparatus consists of four metal tanks (30 cm \times 28 cm \times 35 cm) with a 120 Lux light source on the ceiling and a video

camera fixed at the top of each tank. 30 min after drug administration, each animal was placed into the center of the tank and allowed to explore freely for 3 min. The total distances and average speeding in the following 10 min were recorded automatically as the index of locomotor activity.

■ Object location recognition (OLR) experiment

The object location recognition (OLR) test was performed as previously described [22]. The apparatus was a rectangular box (40 cm \times 50 cm \times 50 cm), made of a black polyester plastic material, camera mounted on the top of the chamber to record animals' exploratory behavior. This experiment process could be divided into 3 stages: habituation, familiar and test phase. In the habituation phase, mice were allowed to freely explore the arena for 10 mins without any objects in order to reduce animals' fear of new environment and it tested for 3 consecutive days. During the familiar phase, the animals had free access to two of the identical objects for 5 min, after which they were returned to their cage. The total exploration time they were touching and sniffing the objects was recorded. After a waiting period of 30 min, the test phase was conducted: the animals were placed back into the same box, in which one of the original objects placed in the same position (familiar), whereas the other one was removed to a new location (novelty). Locations of the objects were counterbalanced among groups. To control for

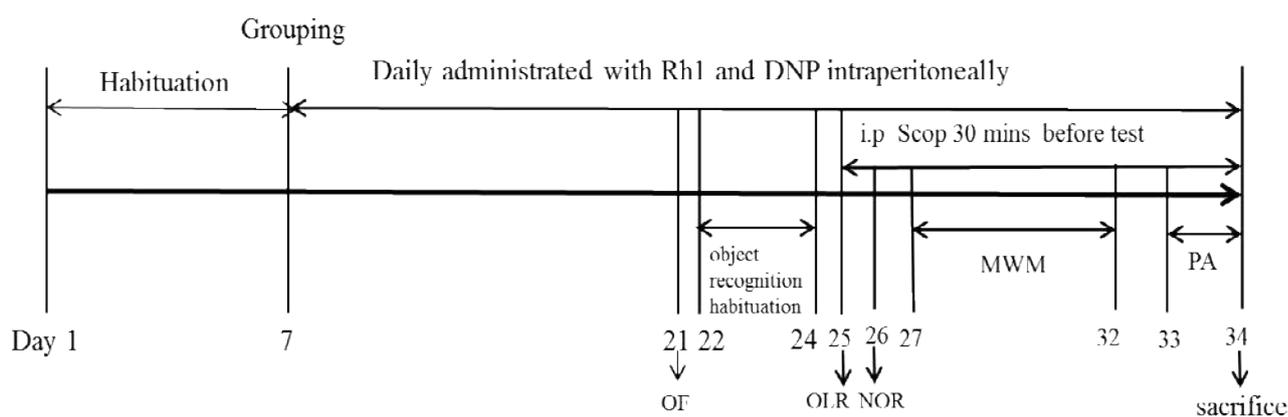


Figure 1: The experimental procedure. After habituation for 7 days, the mice were randomly divided into five groups (n=12 per group): normal untreated control group, Scop (0.75 mg/kg)/vehicle-treated group, Scop (0.75 mg/kg)/Rh1 (20 μ mol/kg)-treated group, Scop (0.75 mg/kg)/Rh1 (40 μ mol/kg)-treated group and Scop (0.75 mg/kg)/DNP (1.6 mg/kg)-treated group. Mice received pretreatment with Rh1 and DNP for 19 days before inducing amnesia by Scop. The Scop was injected intraperitoneally to mice for 10 consecutive days, while Rh1 and DNP were administered intraperitoneally 30 min before Scop injection for 28 consecutive days. After pretreatment, the open field (OF) test was firstly used to evaluate the effect of Rh1 on the locomotor activity without Scop administration. Thereafter, memory impairment was induced by intraperitoneal administration of Scop (0.75 mg/kg) once daily and object location recognition (OLR) experiment, novel object recognition (NOR) test, Morris water maze (MWM) task and passive avoidance (PA) test were conducted 30 min after Scop administration. Following the behavioral tests, animals were sacrificed and their hippocampus was isolated for further biochemical analyses.

possible odor cues, the objects and the floor of the arena were cleaned with 70% ethanol at the end of each trial to eliminate possible scent/trail markers. Recognition memory was evaluated using a discrimination index (DI) calculated for each animal using the formula: $DI = (TN - TF) / (TN + TF)$ (TN=exploration time of “novel” object; TF=exploration time of “familiar” object).

■ Novel object recognition (NOR) test

The novel object recognition (NOR) test was performed as previously described [23], with slight modifications. The familiar neither phase of NOR was performed in the same way as that of OLR. During the test phase, one of the objects used during the familiar phase was replaced with a novel object and the other remained unchanged (familiar). Each mouse was allowed 5 min of free exploration. Also, the total exploration time in familiar phase and the DI in test phase were calculated.

■ Morris Water maze (MWM) task

Morris water maze task (MWM) was carried out to evaluate the long-term, spatial memory [24]. The water maze was consisted of circular, black pool measuring 1.0 m in diameter × 0.38 m in height, and filled with opaque water (black ink) at the temperature of $(23 \pm 1)^\circ\text{C}$ to a depth of 25 cm. A video camera monitored the behavior of the mice in the pool. The test room contained several permanent extra cues such as lights, a picture on the wall, etc. For the acquisition test, mice were repeatedly placed into the tank and must learn to locate a hidden platform (6 cm in diameter) beneath in water. Acquisition test consisted of five consecutive daily trails in which mice were placed (facing the wall) three times into each of four quadrants in turn (except for target quadrant), and the order was unchanged each day. Before each trail, mice were placed on the hidden platform for 10 s. If mice did not locate the safe platform within 90 s, the mice were gently guided to the platform, allowing mice to stay the platform 10 s. The escape latency and the percent of time spent in the target quadrant were recorded. On the day following completion acquisition testing, animals were probed in a 90 s “retention” trail in which the platform was removed (probe test). The mice were placed in the pool side opposite to the target (platform) quadrant. The virtual-platform crossing numbers and the percent of time spent in the target quadrant were recorded and used as the indexes of memory retention.

■ Passive Avoidance (PA) task

The passive avoidance (PA) test was performed according to a previous study [25]. The apparatus in trough-shape consisted of a white illuminated chamber and a dark chamber (17 cm × 13.5 cm × 25 cm, respectively). In training trial, following 180 s adaptations, each mouse was put into the light chamber to explore with the door opened for 300 s. When it entered the dark chamber, a 0.5 mA electric foot shock (5 s) was delivered. 24 h later, the consolidation trial was performed in the same way as training. The latency into the dark chamber and time in the dark chamber was recorded in 300 s test session.

■ Brain Sample Preparation

After the last behavioral tests, all mice were anesthetized and decapitated quickly. The hippocampus were dissected from the whole brain on ice, separated from the midsagittal plane and immediately snap-frozen and stored at -80°C until further analysis.

■ Analysis of AChE, ChAT, Ach, SOD and MDA in the hippocampus

The hippocampus were weighed and homogenized in 9 volumes (1:9, w/v) ice-cold 0.9% normal saline. After centrifugation at 3000 rpm for 10 min at 4°C , the supernatant was collected and was further diluted with appropriate buffer solution of the determination of the relevant biochemical index. AChE, ChAT and Ach in the hippocampus were tested on automatic enzyme standard instrument, using commercial kits according to the manufacturer’s instructions. The levels of AChE, ChAT and Ach were normalized and expressed as U/mg protein, U/g protein, U/mg protein, respectively. The SOD activity and MDA level in the hippocampus were measured using commercially available assay kits according to the protocols and expressed as U/mg protein and nmol/mg protein, respectively.

■ Western blotting analysis

Mouse hippocampus was homogenized on ice in CellLytic™ MT mammalian tissue lysis reagent (Sigma, C3228) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma, P3840). The homogenate was centrifuged at $13,523 \times g$ for 10 min at 4°C . Protein concentration in the supernatant was quantified by BCA assay. Protein samples (30 µg/ sample)

were electrophoresed on a 10% SDS-PAGE and transferred onto PVDF membrane (Millipore, USA). The membrane was thus blocked with 5% non-fat dried milk at room temperature for 1 h followed by incubation with respective primary antibodies against cAMP responsive element-binding (CREB), Egr-1, c-Fos, c-Jun and β -actin (Cell Signaling Technology) at 4°C overnight. After thoroughly washed with PBST (PBS with 0.1% Tween 20), the membrane was incubated with HRP-conjugated secondary antibodies at room temperature for another 1 h. The protein bands were visualized with ECL prime kit (GE Healthcare, NA, UK).

■ Statistical Analysis

All results were expressed as mean \pm standard error of the mean (SEM). The data was carried out using one-way analysis of variance (ANOVA). Following significant ANOVAs, multiple post hoc comparisons were performed using the LSD test. The data recorded from the acquisition trails of MWM among the groups over a period of five days were analyzed with repeated measures and a multivariate analysis of variance (ANOVA) process of the general linear model. Statistical significance was set at $p < 0.05$ in all of the evaluations. The analysis was finished using SPSS 19.0 for Windows (Chicago, Illinois, USA).

Results

■ Rh1 did not affect the locomotor activities

Open field (OF) test was used to evaluate the locomotor activity in mice which received pretreatment with Rh1 administration for 14 days. As shown in **Figure 2**, there were no

significant changes in total distance and average speed among all groups. These results indicated that the mice in all groups had no effects on their locomotor activities (motor function) in the OF test and could preclude the influence of locomotor activity change on the evaluation of cognitive function.

■ Rh1 improved the short-term, spatial recognition memory on Scop-treated mice in OLR test

Results of OLR were shown in **Figure 3**. In familiar phase, there were no differences in the total exploration time among all groups (**Figure 3A**), which suggested that there were no differences for animals' ability of exploration. As the **Figure 3B** shown, in test phase, when comparing between groups, the DI of Scop group was significantly decreased compared to control group ($P < 0.01$). However, treatment with Rh1 (40 μ mol/kg) significantly elevated the DI compared with Scop group ($P < 0.05$), showing the short-term, spatial recognition memory-enhancing effect.

■ Rh1 improved the short-term, non-spatial recognition memory on Scop-treated mice in NOR test

Results of NOR were shown in **Figure 4**. The total exploration time during the familiar phase (**Figure 4A**) was demonstrated none significance among all groups, which was similar to the result of OLR and it also suggested that there were no differences for animals' ability of exploration. In test phase, the DI of Scop group was significantly decreased compared to control group ($P < 0.001$). While Rh1 (40 μ mol/kg) administration elevated the DI with significance compared with Scop group ($P < 0.05$) (**Figure 3B**).

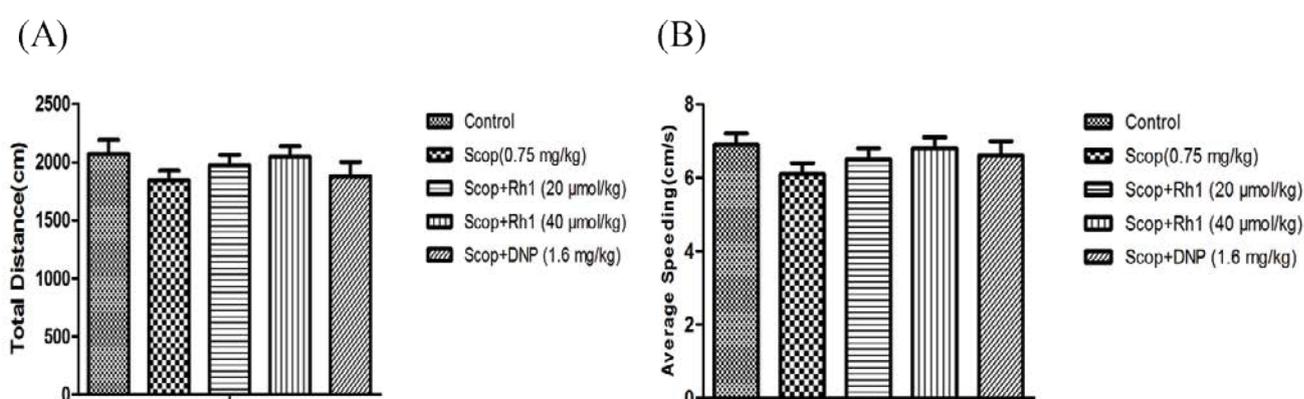


Figure 2: Effect of Rh1 on locomotor activities. (A) Total distance and (B) Average speed. Data are expressed as means \pm SEM (n=12).

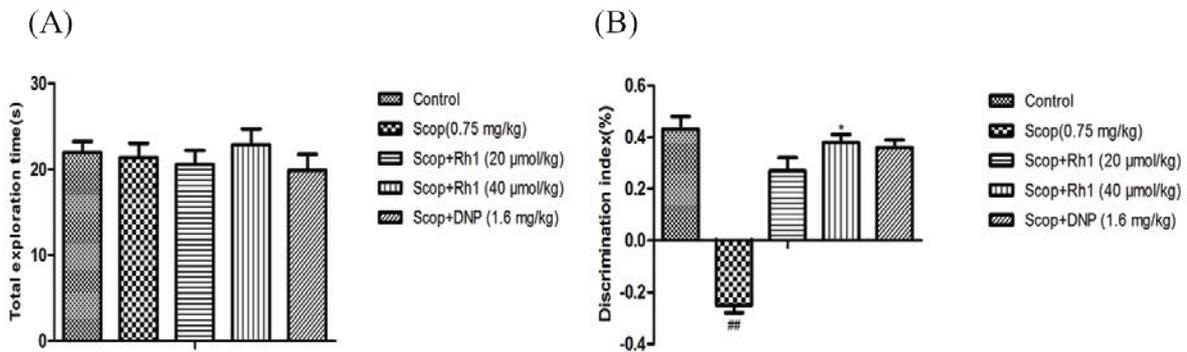


Figure 3: Effect of Rh1 on Scop-induced memory deficit in OLR task. (A) Total exploration time in familiar phase and (B) DI in test phase. Data are expressed as means ± SEM (n=12). Significant differences ##*p*<0.01 compared with the control group; **p*<0.05 compared with the Scop group.

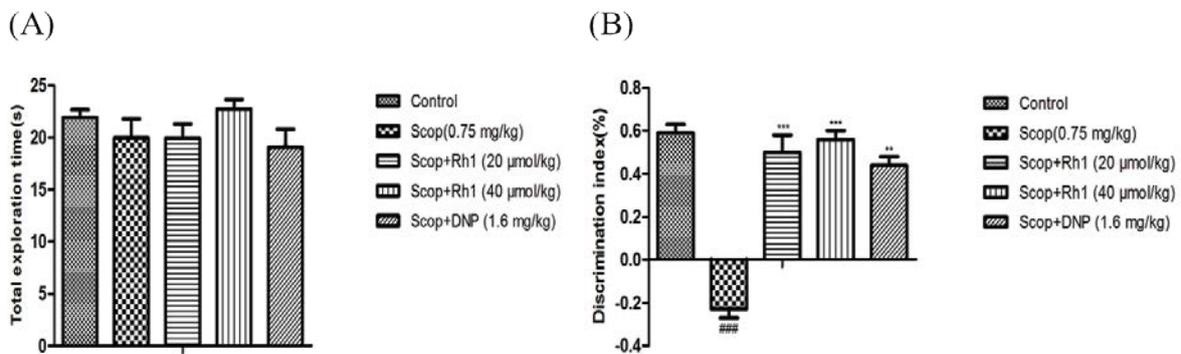


Figure 4: Effect of Rh1 on Scop-induced memory deficit in NOR task. (A) Total exploration time in familiar phase and (B) DI in test phase. Data are expressed as means ± SEM (n=12). Significant differences ###*p*<0.001, compared with the control group; ***p*<0.05, ****p*<0.01 compared with the Scop group.

■ **Rh1 improved the long-term, spatial memory on Scop-treated mice in the MWM task**

In acquisition test, as shown in **Figure 5A**, the escape latency to locate the platform gradually decreased during the acquisition test in all groups except the Scop group. Scop-treated mice exhibited significantly longer escape latency compared to the control group from day 1 to day 5 (*P*<0.01, *P*<0.01, *P*<0.001, respectively), illustrating the memory deficit successfully induced by Scop treatment. Rh1 (20 and 40 μmol/kg) treatments effectively decreased the escape latency on the fourth and fifth day compared to Scop treatment (*P*<0.01 and *P*<0.001, respectively). DNP (1.6 mg/kg) also significantly improved Scop-induced increments in escape latency on the fourth and fifth day (*P*<0.05 and *P*<0.01, respectively). As indicated in **Figure 5B**, compared with the control group, the percent of time spent in the target quadrant of Scop-treated group showed significant decrease on the fourth and fifth day (*P*<0.001 and *P*<0.01, respectively). However, Rh1 (20 μmol/kg)

and DNP (1.6 mg/kg) administration significantly elevated the percent of time spent in the target quadrant compared with Scop group on the fourth and fifth day (*P*<0.05 and *P*<0.01, respectively).

In probe test, the virtual-platform crossing number and the percent of time spent in the target quadrant were used to evaluate retention of long-term, spatial memory. As shown in **Figure 5C**, the Scop treatment significantly decreased the crossings platform numbers compared to control group (*P*<0.05). Rh1 (40 μmol/kg) and DNP (1.6 mg/kg) treatment groups significantly increased the number of crossings (*P*<0.01 and *P*<0.05, respectively). And there were no significant changes in the percent of time spent in the target quadrant among all groups, though Rh1 (40 μmol/kg) and DNP (1.6 mg/kg) groups had higher the percent of time spent in the target quadrant compared with Scop model group (*P*>0.05). These findings demonstrated that Rh1 (20 and 40 μmol/kg) significantly improved the long-term, spatial memory in Scop-treated mice.

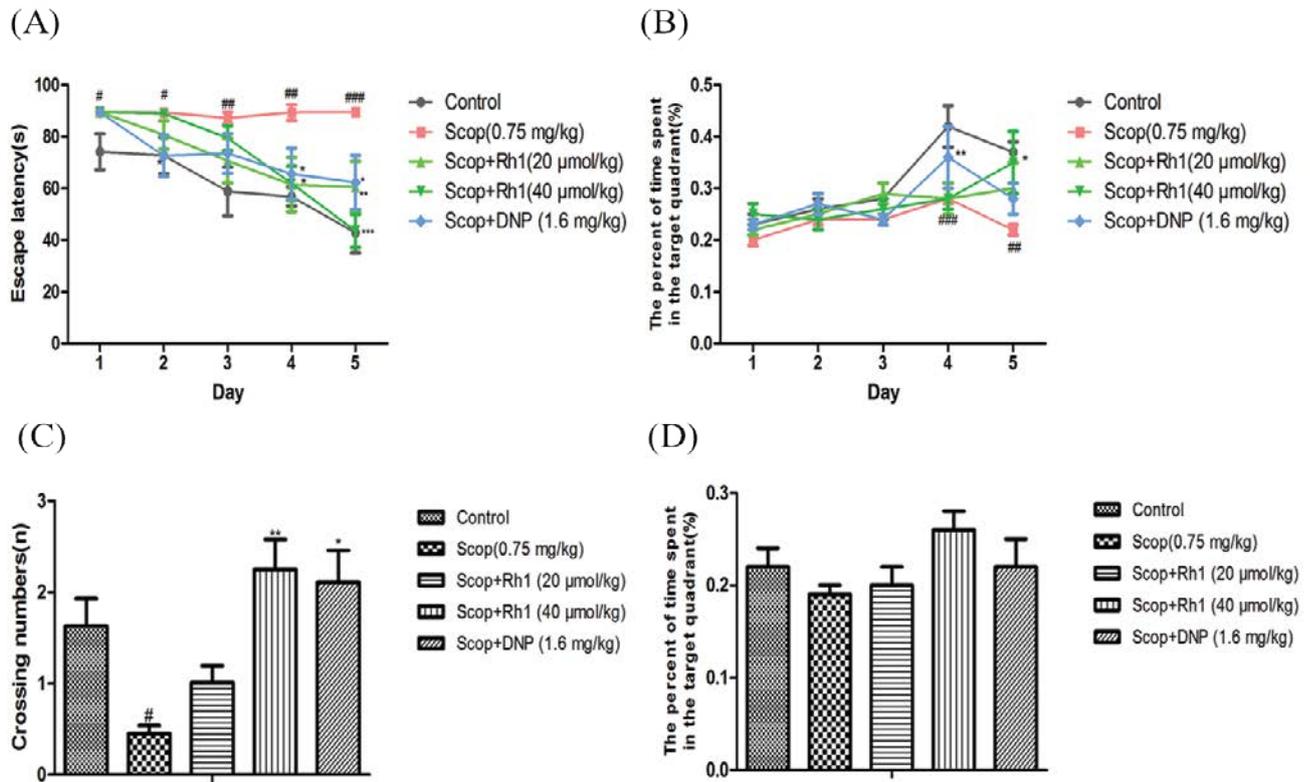


Figure 5: Effect of Rh1 on Scop-induced memory deficit in MWM task. (A) Escape latency in the acquisition phase, (B) The percent of time spent in the target quadrant in the acquisition phase, (C) The crossing numbers in the probing trial and (D) The percent of time spent in the target quadrant in the probing trial. Data are expressed as means \pm SEM ($n=12$). Significant differences # $p<0.05$, ## $p<0.01$, ### $p<0.001$ compared with control group; * $p<0.05$, ** $p<0.01$ compared with the Scop group.

■ Rh1 improved Scop-induced memory impairment assessed in the PA task

The Scop-induced memory impairment of mice were indicated by significant shorten the latency into the dark chamber ($P<0.01$) and more time in dark chamber ($P<0.05$) than that of control mice (Figure 6A). The Rh1 (20 $\mu\text{mol/kg}$) and DNP (1.6 mg/kg) treatment both reversed the decrease of the latency into the dark chamber significantly ($P<0.05$). Meanwhile, treatment with Rh1 (40 $\mu\text{mol/kg}$) significantly decreased the time in the dark chamber compared with Scop model group ($P<0.05$) (Figure 6B).

■ Rh1 decreased AChE activity and increased ChAT activity and Ach level of Scop-treated mice

As shown in Figure 7, AChE activity in the hippocampus of the Scop-treated group was significantly increased as well as ChAT activity and Ach level in the hippocampus of the Scop-treated group were lower than those of control group ($P<0.05$ and $P<0.001$, respectively). Rh1 (20 $\mu\text{mol/kg}$) decreased the AChE activity in Scop-treated

mice with significance ($P<0.01$). Moreover, the treatment of Rh1 (40 $\mu\text{mol/kg}$) and DNP (1.6 mg/kg) showed a significant increase of ChAT activity and Ach level in the Scop-treated mice ($P<0.05$ and $P<0.01$, respectively).

■ Rh1 increased SOD activity and decreased MDA level of Scop-treated mice

As shown in Figure 8A, Scop treatment significantly decreased the SOD activity compared to the control group ($P<0.01$). Treatment with Rh1 (40 $\mu\text{mol/kg}$) and DNP (1.6 mg/kg) significantly increased the SOD activity compared with the Scop group ($P<0.05$ and $P<0.01$, respectively). Figure 8B showed that compared to control group, the level of MDA in the hippocampus of Scop-treated mice was significantly increased ($P<0.01$). However, treatment with Rh1 (40 $\mu\text{mol/kg}$) significantly ameliorated this increase ($P<0.05$).

■ Rh1 up-regulated the expression of CREB, Egr-1, c-Fos and c-Jun in the Scop-treated mouse hippocampus

As displayed in Figure 9, Scop treatment

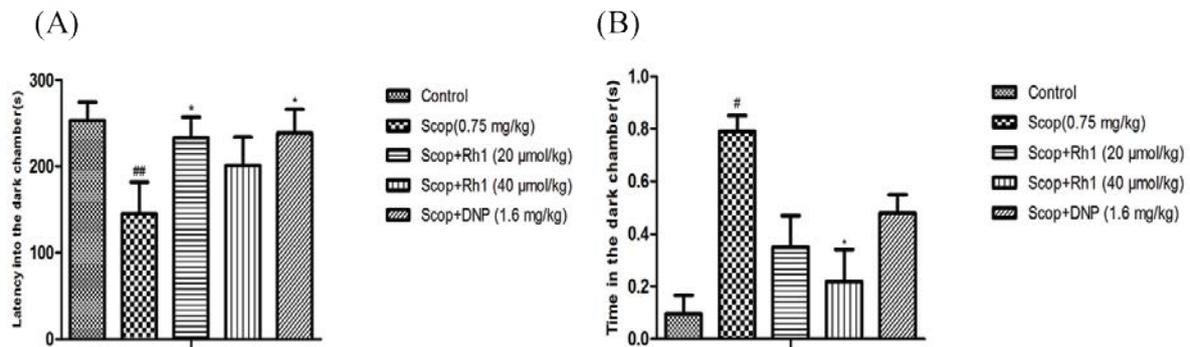


Figure 6: Effect of Rh1 on Scop-induced memory deficit in PA test. (A) Latency into the dark chamber and (B) Time in the dark chamber. Data are expressed as means ± SEM (n=12). Significant differences #*p*<0.05, ##*p*<0.01 compared with control group; **p*<0.05 compared with the Scop group.

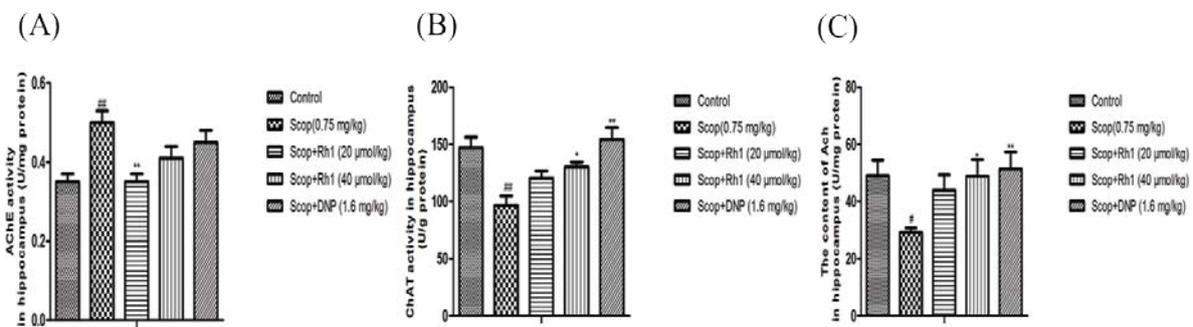


Figure 7: Effects of Rh1 on (A) AChE activity, (B) ChAT activity and (C) Ach level in the Scop-treated mouse hippocampus. Data are expressed as means ± SEM (n=12). Significant differences #*p*<0.05, ##*p*<0.01 compared with the control group; **p*<0.05, ***p*<0.01 compared with the Scop group.

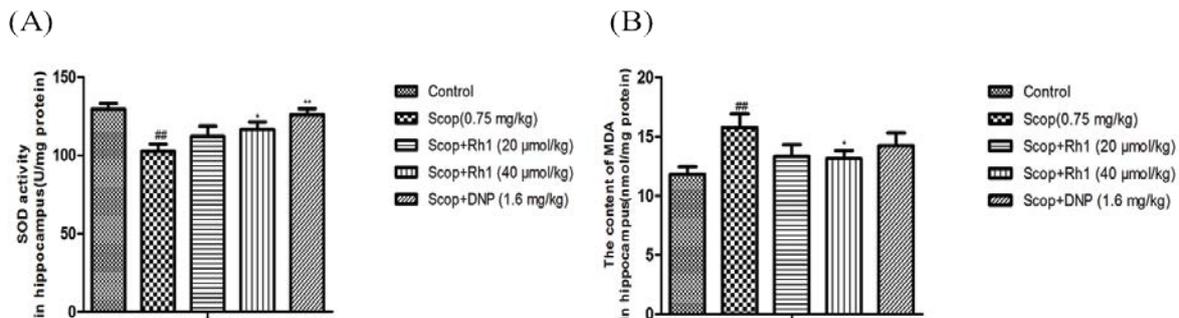


Figure 8: Effects of Rh1 on (A) SOD activity and (B) MDA level in the Scop-treated mouse hippocampus. Data are expressed as means ± SEM (n=12). Significant differences ##*p*<0.01 compared with the control group; **p*<0.05, ***p*<0.01 compared with the Scop group.

significantly decreased the expression levels of CREB, Egr-1, c-Fos and c-Jun (*P*<0.001). Meanwhile, treatments with Rh1 (20 and 40 μmol/kg) and DNP (1.6 mg/kg) significantly ameliorated this decreases of CREB, Egr-1 and c-Fos protein levels in the hippocampus compared to the Scop group (*P*<0.05, *P*<0.01, *P*<0.001, respectively). Beside this, the obvious increase of c-Jun expression level was also observed in Rh1 (40 μmol/kg) and DNP (1.6 mg/kg) groups

(*P*<0.05 and *P*<0.001, respectively), whereas a non-significant enhancement was shown in Rh1 (20 μmol/kg) administration group compared with the Scop-treated group.

Discussion

In the present study, we demonstrated that treatment with Ginsenosides Rh1 ameliorated Scop-induced cognitive dysfunctions. This effect

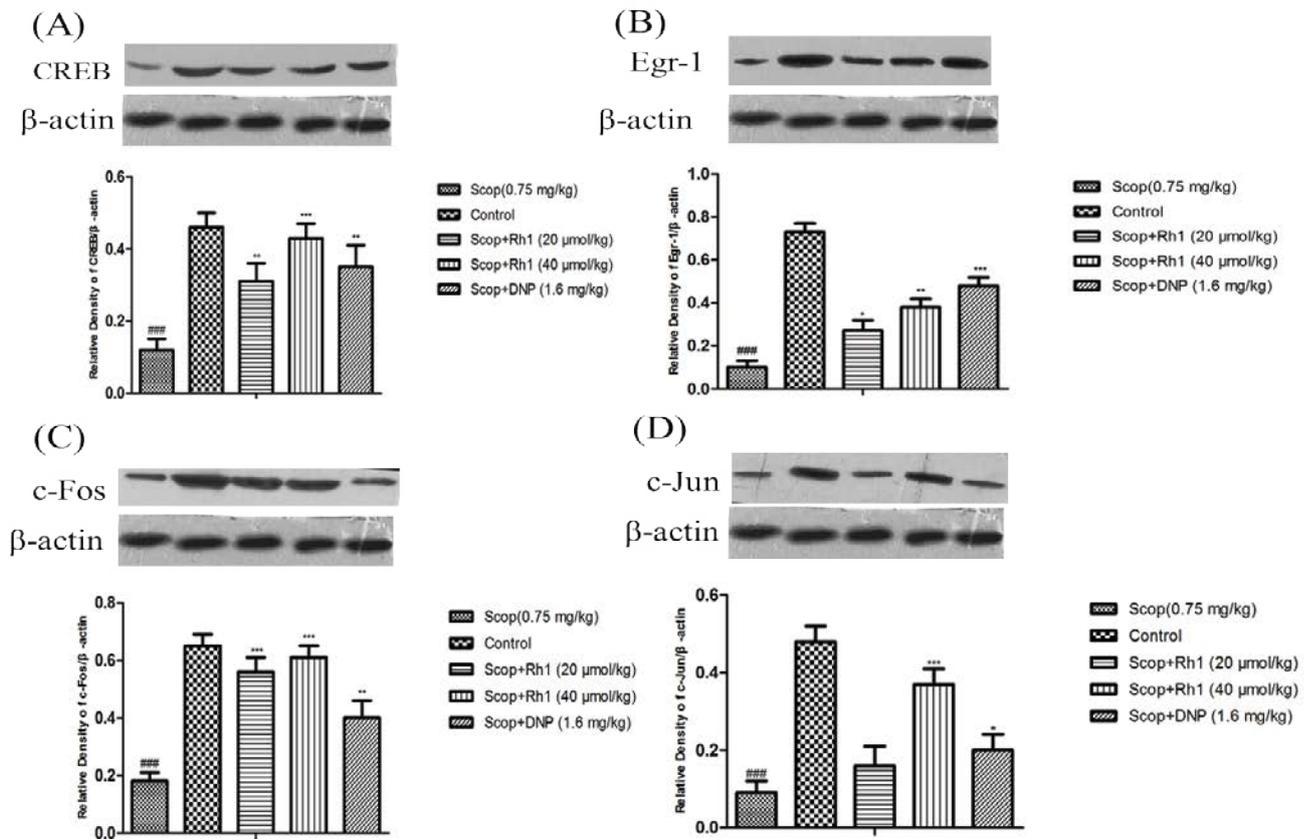


Figure 9: Effects of Rh1 on mice hippocampal expression levels of CREB, Egr-1, c-Fos and c-Jun. (A): Western blot of CREB and Gray intensity analysis of CREB, (B): Western blot of Egr-1 and Gray intensity analysis of Egr-1, (C) Western blot of c-Fos and Gray intensity analysis of c-Fos and (D) Western blot of c-Jun and Gray intensity analysis of c-Jun. All data were expressed as means \pm SEM (n=3). Significant differences ### p <0.001 compared with the control group. * p <0.05, ** p <0.01, *** p <0.001 compared with the Scop model group.

was observed in the object location recognition (OLR), novel object recognition (NOR), Morris water maze (MWM) and passive avoidance (PA) tasks. Furthermore, we found that Rh1 reversed the increased AChE activity and MDA level, decreased ChAT and SOD activities as well as Ach level induced by Scop treatment in the hippocampus. Moreover, the protein expression levels of hippocampal CREB, Egr-1, c-Fos and c-Jun were remarkably elevated in Rh1-treated mice.

Scopolamine, a non-selective muscarinic antagonist block cholinergic signaling and produce cognitive dysfunctions, including short-term and long-term memory impairment [26,27]. In the present study, memory tasks were carried out to evaluate the effect of Rh1 on scopolamine model of AD in mice. OLR and NOR were respectively employed to assess short-term spatial and non-spatial recognition memory, based on the rodent spontaneity to explore a novel object [28,29]. In agreement

with the previous studies [12,30], our results demonstrated that scopolamine significantly decreased the DI in both OLR and NOR tests, indicating impairment of learning and memory. Rh1 treatment significantly ameliorated the scopolamine induced cognitive impairment both in OLR and NOR task by elevating the DI, showing short-term memory enhancing effect. MWM task is generally accepted as an indicator of spatial learning and reference memory, which reflects long-term memory [31,32]. To evaluate the effect of Rh1 on long-term memory, the MWM task was carried out. Consistent with previous studies [33,34], Scop-treated mice exhibited poor cognitive performance both in the acquisition phase and the probe phase of MWM test. Our results showed that treatment of Rh1 (40 μ mol/kg) shortened the escape latency to find platform, elevated the percent of time spent in the target quadrant in memory acquisition process, and increased the crossing numbers in probe test when compared with Scop-treated mice. The spatial memory improving effect of

Rh1 was in agreement with the previous report [19], which demonstrated that the long-term administration of Rh1 enhanced spatial memory of mice in MWM task. Moreover, the step-through passive avoidance task is a fear motivated inhibitory avoidance test indicating non-spatial learning and memory, which depends on the ability of retaining and recall information [25]. The cognitive effect of Rh1 was further evaluated in PA test. Our results showed that Rh1 administration effectively increased the latency into the dark chamber and decreased the time in the dark chamber of Scop-treated mice. The non-spatial memory improvement effect of Rh1 was consistent with the previous report [18], which found Rh1 administration improved Scop-induced memory impairment in passive avoidance task. Considering Rh1 did not influence the locomotor activity of mice, it suggested that the cognitive effect of Rh1 was to be mnemonic in origin, rather than induced by sensorimotor effects. Taken together, these behavioral tests demonstrated that the Rh1 treatment could effectively improve the impaired learning and memory in Scop-treated mice.

The central cholinergic system plays a critical role in learning and memory functions [35]. Cholinergic deficit is a major neuropathological feature that is associated with memory loss and closely correlated with the severity of cognitive dysfunction in AD [36]. AChE, a marker of cholinergic system, is an important regulatory enzyme that rapidly hydrolyzes Ach which plays a vital role in cognitive functions, while ChAT is an enzyme that associated with the synthesis of Ach [37]. Thus, in order to elucidate the underlying mechanism of cognitive effect of Rh1 in Scop-treated mice, AChE and ChAT activities as well as Ach level in the hippocampus were firstly assessed. Our results together with those of other reports [38,39] showed that increased AChE activity and decreased ChAT activity, which resulted in the decrease of Ach level in the hippocampus were observed in the Scop-treated mice. Rh1 treatment demonstrated significant inhibition of AChE activity and an obvious increase of ChAT activity and elevation of Ach level in Scop-treated mice, indicating the ability of enhancing the cholinergic system function. Furthermore, both preclinical and clinical studies have shown increased level of oxidative stress during latent period of the disease, which often leads to sudden onset of symptoms of AD, including cognitive decline and post-mortem studies have confirmed elevated levels of MDA,

an index of lipid peroxidation in AD brains, which further supports the role of oxidative stress in the pathogenesis of the disease [40]. SOD, an antioxidant enzyme, plays a positive role in the reduction of oxidative stress by inducing increased free radical generation [41]. Our present results showed that Rh1 (40 $\mu\text{mol/kg}$) treatment significantly restored the reduced SOD activity and attenuated the elevated MDA level in the hippocampus of Scop-treated mice. It is well consistent with our previous finding that Rh1 had neuroprotective effect in sleep deprivation-induced mouse memory impairment model via reducing oxidative stress [20]. Hence, the modification of cholinergic system function and the reduction of oxidative stress could be related to the cognitive effect of Rh1 in Scop-treated mice.

cAMP response element-binding (CREB) protein is a cellular transcription factor necessary and required for hippocampus-dependent long-term memory formation [42] and CREB activation has a significant impact on synaptic plasticity and memory formation [43]. Scop-induced memory deficits were associated with a significant reduction in CREB expression in the hippocampus and disruption in hippocampal function [44]. Immediate early genes (IEGs) such as Egr-1, c-Fos and c-Jun play important role in learning and memory. Particularly, the expression of these IEGs has been widely used as a molecular marker for synaptic plasticity and long-term memory [45,46]. Recently, accumulating evidence indicated that downregulation of the expression of IEGs at protein levels in the hippocampus of scopolamine-induced amnesic mice was observed [47-49]. Additionally, Egr-1 interacts with CREB and regulates the expression of synaptic plasticity genes [50]. Thus, in the present study, we performed western blot analysis to examine whether Rh1 affected the activation levels of those memory-associated molecules aforementioned above in the hippocampal region. Our results for the first time showed that Rh1 administration significantly increased the expression levels of CREB and IEGs (Egr-1, c-Fos and c-Jun) in the hippocampus of Scop-treated mice.

Conclusion

In conclusion, the findings of the present study indicated that Rh1 treatment ameliorated Scop-induced cognitive dysfunction in the object location recognition test, the novel object

recognition task, the Morris water maze task and the passive avoidance test. Furthermore, the ameliorating effect of Rh1 was, in part, related to modification of cholinergic system function, the reduction of oxidative stress and the activation of memory-associated molecules, including CREB, and immediate early genes (IEGs) such as Egr-1, c-Fos, and c-Jun in the hippocampus. Therefore, our findings suggested that Rh1 may be a potential therapeutic agent for treating cognitive dysfunction, such as that presented in AD.

Conflicts of Interest

The authors have declared that there is no

conflict of interest.

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