Effects of harmalol on scopolamine-induced memory disorders, anxiety and depression like behaviors in male mice: A behavioral and molecular study

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Abstract

Introduction: Harmalol is a dihydrocarboline compound found naturally in several alcoholic beverages and medicinal plants. This study was designed to investigate the effect of harmalol on memory function and its possible mechanisms in a scopolamine-induced memory disorder model.
Materials and Methods: Thirty five male mice were randomly divided into five (n=7) group: Control group (normal saline), scopolamin group (scopolamine at a dose of 1 mg / kg) and treatment groups (harmalol in three doses of 5, 10 and 20 mg / kg with scopolamine) for 21 days. Behavioral tests were performed after the treatment period. The mice were then subjected to deep anesthesia and blood and brain tissue samples were obtained.
Results: Harmalol significantly decreased latency to reach the hidden platform in spatial memory test and secondary latency in passive avoidance memory test and immobility time in the forced swimming test compared to scopolamine group ($P < 0.05$). This compound, at the dose of 20 mg/kg, showed a significant increase in the number of crossing and standing on two legs in open field test ($P < 0.05$). Furthermore, harmalol treatment decreased brain malondialdehyde and nitric oxide levels, enhanced the total antioxidant capacity, and also increased the level of brain-derived neurotrophic factor (BDNF) in the hippocampus ($P < 0.05$).
Conclusion: The present study emphasizes that harmalol improves scopolamine-induced memory loss by modulating acetylcholinesterase activity and increasing BDNF in the hippocampus of mice. Therefore, harmalol may be a promising therapeutic drug to prevent amnesia and cognitive deficits associated with aging or neurodegenerative diseases such as Alzheimer's.

Keywords: Scopolamine, Harmalol, Memory deficits, Mice

Introduction

Peganum harmala L. (*P. harmala)* is a native plant of arid regions from North Africa to China. *P. harmala* is used in traditional medicine for a variety of diseases and its anti-inflammatory activity is highly regarded. Seven alkaloids, harmaline, harmine, harmalol, harmole, vasicine, vasicinone and deoxyvasicine, are abundant in P. harmala (1). β-Carbolines have attracted considerable attention as they have a variety of pharmacological activities such as sedative, hypnotic, anxiolytic, anticonvulsant, anti-tumor, anti-thrombotic, antiseptic, antimicrobial and also antiviral activities. Large groups of natural and synthetic indole alkaloids are widely distributed in nature, including various foods, plants, marine organisms, insects, mammals as well as human tissues. Harmalol (1-methyl-4,9-dihydro-3H-pyrido [3,4-b] indole-7-ol) is one

of the common dihydrocarboline compounds found naturally in several alcoholic beverages and medicinal plants including *P. harmala* (Zygophyllaceae) (2). Beta-carboline alkaloids present in the plant and the seeds of *P. harmala* have been considered for their anti-tumor (anti-cancer) properties(3). *P. harmala* seeds are useful for epilepsy, mania, memory loss, chronic headache, kidney stones, edema, jaundice, colic and sciatica. The aqueous extract of the seeds of this plant is also useful for blood purification (4). Recent research indicates that alkaloids such as harmine, harmaline, and tetra-hydroharmine, which belong to the haloxinogenic classes, are potent reversible inhibitors of monoamine oxidases (5). *P. harmala*-derived beta-carbolines have also been shown to interact with the opioid, dopamine, gamma aminobutyric acid (GABA), 5-hydroxytryptamine, benzodiazepine, and imidazoline receptors in the nervous system, thereby induce many pharmacological effects. In addition, these alkaloids are neuroprotective and are potent inhibitors of monoamine oxidase and this important feature makes them a superior target in the treatment of certain conditions such as depression (6).

Neurochemical analyzes of the brain of patients with Alzheimer's disease (AD) revealed that a significant number of cholinergic neurons in the cortex and hippocampus were lost. Acetylcholinesterase (AChE) is a target for the treatment of AD, and inhibiting its activity helps maintain acetylcholine (ACh) levels in neuronal synapses and has positive therapeutic effects in patients with AD. Recently, it has been suggested that memory impairment caused by cholinergic dysfunction is associated with increased oxidative stress following scopolamine consumption. In addition, decreased activity of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) have been reported in early stages of AD (7).

Neurogenesis in the hippocampus is regulated by normal cholinergic activity by modulating the neurogenic mechanisms in which brain-derived neurotrophic factor (BDNF) and cAMP response element-binding protein (CREB) are involved (8). Evidence suggests that a decrease in BDNF levels in the entorhinal cortex and hippocampus of patients with AD leads to a decrease in the patient score in a mini-mental state. In addition, changes in BDNF levels after scopolamine administration have also been observed (9).

Recent evidence suggests that N-methyl-D-aspartate receptor (NMDA) activity is associated with learning, memory, and cognition through modulation of dendritic column density, synaptic plasticity, and synaptic potency. NMDA receptors have a heterotetrameric structure consisting of two Glutamate [NMDA] receptor subunit epsilon-1 (GluN1) subunits and two additional GluN2 or GluN3 subunits that together contribute to the receptor function. The GluN2A and GluN2B NMDA receptor subunits are involved in the development of long-term potentiation (LTP) in hippocampal-dependent spatial memory and increase the expression of the GluN2B variant in adult mice (10). Evaluation of changes in GluN2B expression in memory-impaired mice may indicate NMDAR's role in learning and memory, and may also serve as an assessment of the molecular mechanism of the effects of herbal neuromodulatory drugs.

People used aromatic and medicinal plants for centuries to treat their ailments. Given that the treatment of AD represents a great challenge for healthcare systems, much research has been conducted to find phytopharmaceutical alternatives that are widely available and accessible at lower cost. Therefore, in the present study, we examined the effects of harmalol on memory formation and hippocampal oxidative status in a

scopolamine-induced model. In addition, we examined hippocampal BDNF and GluN2B mRNA in scopolamine-treated mice.

Material and Methods

Animals

Thirthy five mice, weighing 20-30 g, were purchased from the Pasteur Institute of Iran. Animals were kept in special cages under 12 hours of darkness and 12 hours of light and 23± 2 °C. There was no restriction on animals' access to food and water.

Experimental Groups

The animals were randomly divided into 5 groups (n=7) in order to investigate the effect of different concentrations of harmalol on scopolamine-treated mice:
1) The control group received saline; 2) The scoplamine group received scopolamine at the dose of 1 mg/kg for three weeks; and 3) The treatment group consisting of three subgroups that received 5, 10 and 20 mg/kg harmalol, respectively, and 1 mg/kg scopolamine (11). Harmalmol and scopolamine were administered to animals for 21 days (i.p.) after dissolving in 0.9 % saline.

Behavioral Tests

Behavioral tests were performed after the treatment period. The mice were then subjected to deep anesthesia (800 mg/kg chloral hydrate i.p injection) and blood and brain tissue samples were obtained. The hippocampus was isolated from brain tissue for biochemical and molecular tests were performed.

Spatial Memory Test through the Morris Water Maze

It is used to evaluate the learning, memory and motor performance of rats. A plexiglass round platform with a diameter of 10 cm is placed in the center of the southwest quarter and about 1 cm under water. Infrared light source, special imaging camera and the computer is connected to the camera. The maze is in a room surrounded by external signs (such as clocks, posters, desks, etc.) and measures different training protocols and different indicators such as learning, memory and motor performance. In each attempt, the mouse is given 60 seconds to find the platform. If the mouse does not find the platform, the researcher will direct it to the platform. Between the two attempts, the mouse is given a 30-second rest period to examine the surrounding environment. Between the two experiments, mice were ejected from water for about 10 minutes and rested in a cage. The factors of movement velocity and visual-motor coordination and spatial learning are evaluated using this device. Each mouse was trained four times a day for four days and the fifth day was a probe and performed once without the platform (12).

Passive Avoidance Memory Test

The shuttle box was used to perform this test. The machine is a plexiglass box with two sections of light and dark. These two parts are connected by a guillotine valve. The bottom of the two rods is about one millimeter in diameter and about one centimeter apart, and the electric shock is applied to the animal foot through the same rods in the dark section. At first, the animal was placed in the shuttle box's light section for familiarization with the instrument, and after 30 seconds the valve was opened to allow the animal to enter the darkness as it would normally. In this test, the initial delay in entering the dark room was recorded. Immediately after the animal entered the dark section, a shock (1 mA, 1 second, 1 time) was entered from the foot to the animal and returned to the corresponding cage from the dark section. 24 hours later, each animal was placed in the bright room for

further testing, the time interval between being in the bright room and entering the dark room was measured and expressed as the secondary latency (maximum 60 s) (13).

Rotarod Test

Power to maintain balance and motor resistance of mice were evaluated using a rotarod device. It contains a caster whose speed is 0-40 rpm. Rotarod device has a belt that can adjust the speed of the caster. At first, the animals were placed on rotarod roll bars and move on based on the original protocol (rotational speed: 10 rpm, acceleration: 7 rpm^2) was taught to them. 30 minutes after training, the test was performed. The maximum test time for each animal in was 300 seconds (13).

Open Field Test

The open field is an 80×80×60 cm glass enclosure divided into 16 squares, located in the middle of a quiet room. Animals were placed in the test room at least one hour before the test. One day before the test, each animal was placed in the apparatus for 5 minutes to become familiar with it. The next day, each animal was placed in the center square of the device and the behavior of the animal was evaluated for 5 min. In this test, the number of squares crossed by the animal, the number of standing on two legs, and the number of scratches were examined (14).

Forced Swimming Test

This test is one of the most valid and common animal tests for depression. To measure the immobilization time, they record the total time the animal is immobilized over a specified time limit. At first the glass container fill with 25 ° C water (25cm length, 12cm width and 15cm high), then the animal gently placed in the water. Conventionally, stopping the hand and foot movements of the mice is considered as immobilization. All tests are taken by one person. The whole forced swimming test is 7 minutes and the first two minutes are set to match the animal with the current conditions and the immobility time is not recorded but the immobility time was measured for the next 5 minutes (15).

Measurement of Total Antioxidant Capacity of Serum and Brain Tissue

Total antioxidant capacity, serum and homogenate brain tissue were determined by FRAP method. The FRAP solution was prepared by adding 2.5 mL of 0.25 mM acetate buffer with pH = 3, 2.5 mL of 10 mM TPTZ prepared in 40 mM hydrochloric acid and 2.5 mL of 20 mM iron chloride 6-hydrate. 25 µL of serum or tissue homogenate sample was mixed with 1.5 ml of FRAP solution and read for 10 min at 37 °C at 593 nm by spectrophotometer (16).

Measurement of Malondialdehyde in Serum and Brain Tissue

200 µl of serum / homogenate brain tissue were mixed with 1.5 ml of 20% acetic acid, 1.5 ml of thiobarbituric acid (TBA, 0.8%) and 200 µl of 8.1% SDS solution. The samples were then placed in Bain Marie for 60 minutes. The samples were then cooled and 1 ml of distilled water and 5 ml of N-butanol-pyridine mixture were added and shaken. The mixture was then centrifuged at 4000 rpm for 10 min and the optical absorbance of the supernatant was recorded at 523 nm (16).

Measurement of Nitric Oxide in Serum and Brain Tissue

The amount of nitric oxide was determined by measuring its nitrate and nitrite products using a calorimetric kit. Nitrate was converted to nitrite by nitrate reductase enzyme and then the amount of nitric oxide

was determined by measuring the nitrite with a griess reagentat 570 nm (17).

Measurement of GluN2B, BDNF Gene Expression

Samples of mice brain were frozen in an adequate volume of acid guanidium thyocianate solution and kept at -80°C until RNA extraction. Total cellular RNA was extracted by the method of acid guanidium thyocianate phenol/chloroform extraction. Total tissue RNA concentration was measured by spectrophotometric absorbance (260 nm) and the quality of isolated RNA was verified by agarose gel electrophoresis with ethidium bromide staining. One µg of purified total RNA was used as substrate for reverse transcription. The reaction was performed by incubation of RNA with 1 µM oligo(dT) and 200 units of MMLV reverse transcriptase from a Clontech first strand cDNA synthesis kit. An aliquot (5 µl of a 1/10 dilution) of the cDNA of each sample was used for RT-PCR. The PCR primers used was shown in Table 1. DNA amplification was carried out in 1 x Taq polymerase buffer, 1.5 mM MgCI2 supplemented with 50 µM dNTP, 0.25 µM of 5' and 3'-specific primers, 1 µCi of [α-32p] and 2 units of Taq polymerase (Promega C) in a final volume of 50 µl. The mixture was overlaid with mineral oil and amplified for 30 cycles (each consisting of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, extension for 1 rain at 72°C) then extension for 7 min at 72°C and storage at 4°C in a Triothermoblock. Ten µl of cDNA products were size-separated by electrophoresis on a 10% acryl/bisacrylamide gel and stained with ethidium bromide (15 µg/ml). Each band was excised from the gel and the quantity of 32p incorporated was measured in a scintillation counter (Table1).

Table 1. The forward and reverse primers used in the study.

Gens	Forward primer	Reverse primer
GluN2B	CTACTGCTGGCTGCTGGTGA	GACTGGAGAATGGAGACGGCTA
BDNF	ATCGGCTTCACAGGAGACATC	CAGAACAGAACAGAACAGAACCAG

GluN2B, glutamate [NMDA] receptor subunit epsilon-2. BDNF , brain-derived neurotrophic factor.

Data analysis was conducted by SPSS version 22. First, normal distribution of the data was investigated by Kolmogorov-Smirnov test and variance homogeneity was studied by Levene's test. Then, to investigate the significance of difference between the treatments, one-way ANOVA was used, and to compare the mean values, Tukey's test. The data were expressed as mean (standard. deviation) and $P < 0.05$ was considered statistically significant.

Results

The results of the latency to reach the hidden platform and probe test are shown in Figure 1. The results of this study show that there was no statistically significant difference between the control group and scopolamine group in the delay to reaching the hidden platform on the training days. According to the results of the spatial memory test, the delay in reaching the hidden platform was significantly reduced in the third day of the treatment group received 5, 10 and 20 mg/kg doses of harmalol in compared to the scopolamine group ($P < 0.05$). According to the results on the fourth day of the experiment, the Harmalol group received 20 mg/kg has less delay in reaching the hidden platform in compared to the scopolamine group ($P < 0.05$). The results showed that the scopolamine group spends less time in the target quadrant in compared to the control group ($P < 0.05$). Swimming time in the target quadrant was statistically increased in the groups received 5, 10 and 20 mg/kg harmalol in compared to the scopolamine group ($P < 0.05$). Frequency of crossing the

target quadrant was significantly increased in the 5 and 10 mg/kg harmalol received groups in compared to the scopolamine group ($P < 0.05$).

The results of the passive avoidance memory in the shuttle box test are shown in Figure 2 (A, B). According to the results of this study, there was no significant difference between experimental groups at the time of initial delay. Secondary latency to dark chamber in the scopolamine-treated group showed a statistically significant decrease compared to the control group ($P < 0.05$). The group received harmalol at dose of 20 mg/kg showed a significant increase in delayed entry into the dark chamber compared to the scopolamine group ($P < 0.05$).

The results of the forced swimming test are shown in figure 3. The results showed that immobility time was significantly increased in the scopolamine group in compared to the control group ($P < 0.05$). Treatment of the mice with harmalol at doses of 10 and 20 mg/kg showed a significant decrease in immobility time compared to scopolamine group ($P < 0.05$).

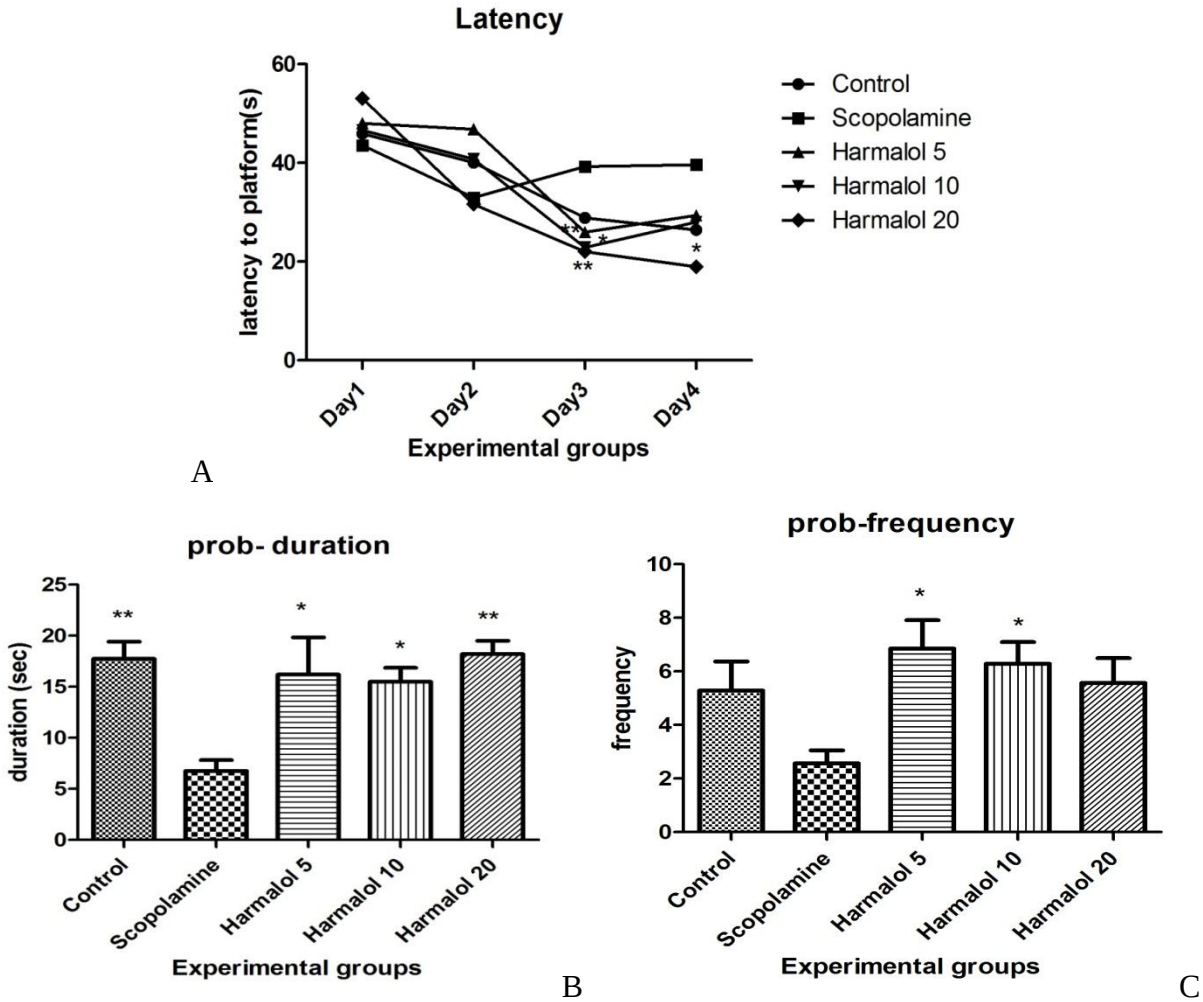
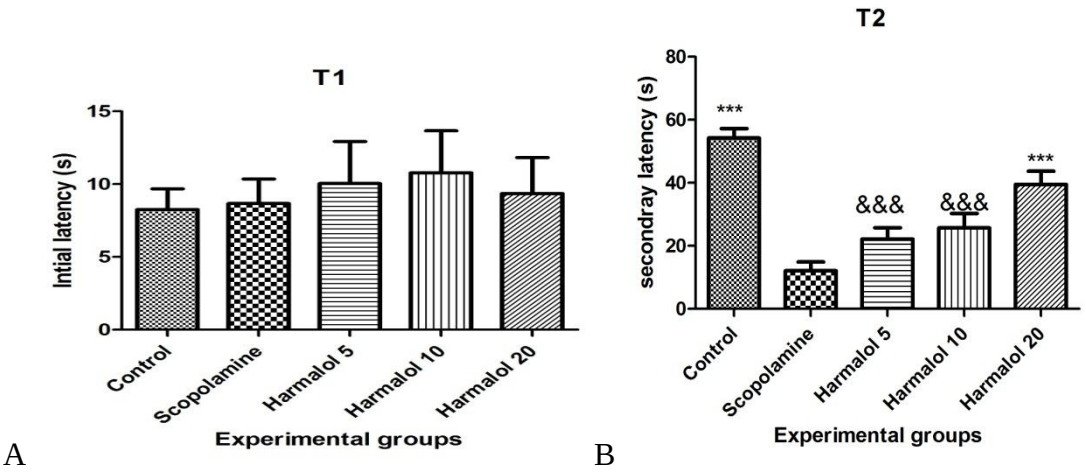


Figure 1. Effect of scopolamine and different doses of harmalol on the delay time parameter to reach the hidden platform on test days (A), swimming time in target quadrant (B), and frequency of crossing target quadrant (C) in Morris Water Maze test. The differences were between experimental and scopolamine group. $^{*}P < 0.05$, $^{**}P < 0.01$.



A B

Figure 2. Effect of scopolamine and different doses of harmalol on the parameter of the primary delay time (A), and secondary delay time (B) in shuttle box test. ***indicates significant difference between experimental groups and scopolamine group. $^{\&}$indicates significant difference between experimental groups and control group ($P < 0.01$).

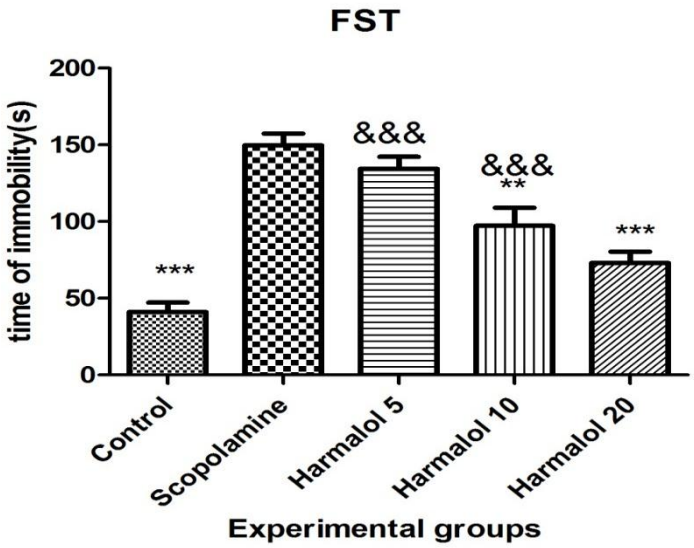


Figure 3. Effect of scopolamine and different doses of harmalol on immobility duration parameter in forced swimming test. $^{**}P < 0.01$, $^{***}P < 0.001$ and the differences were between experimental groups and scopolamine group. $^{\&}$indicates significant difference between experimental groups and control group.

The results of the open field test are shown in figure 4 (A, B, C). The results of this study showed that the number of crossing and the number of standing on two legs were significantly decreased in the scopolamine group in compared to the control group ($P < 0.05$). The group received harmalol at a dose of 20 mg/kg showed a significant increase in the number of crossing and the number of standing on two legs in compared to the scopolamine group ($P < 0.05$). The scopolamine group showed a significant decrease in the number of itching in compared to the control group, and harmalol at three doses of 5, 10 and 20 mg/kg increased the number of itching in compared to the scopolamine group ($P < 0.05$).

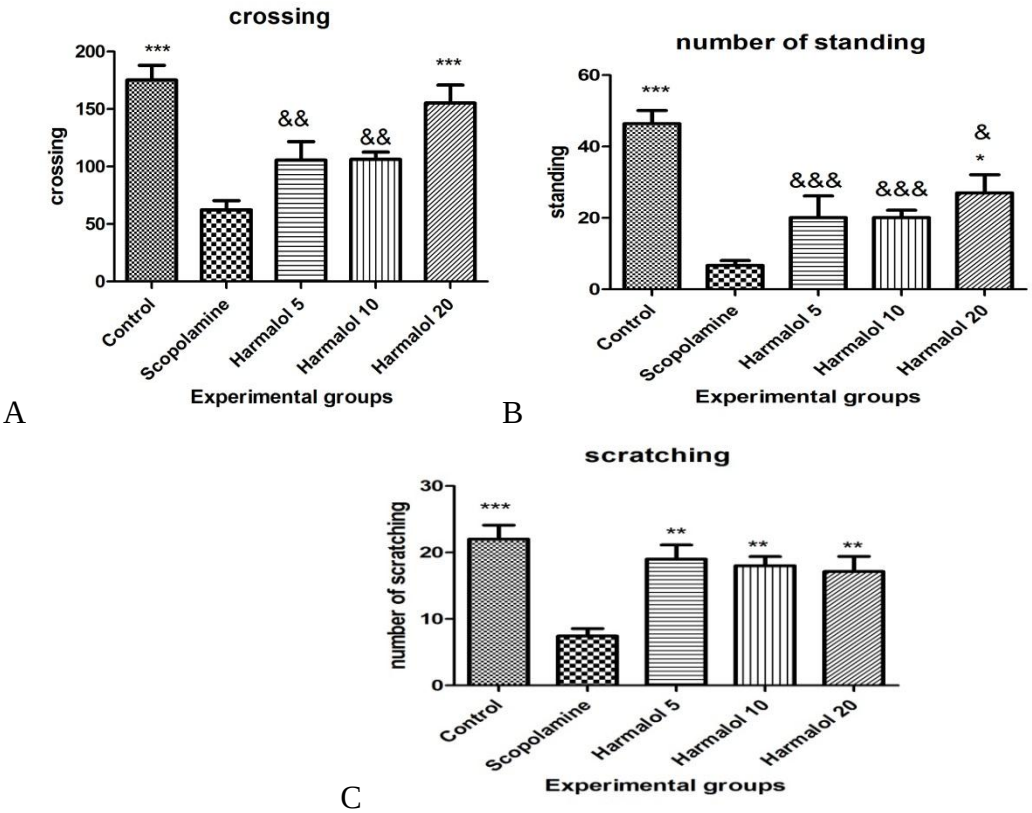


Figure 4. Effect of scopolamine and different doses of harmalol on the number of crossing (A), standing on two legs (B), and scratching (C) in the open field test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *indicates significant difference between experimental groups and scopolamine group. $^{\&}$indicates significant difference between experimental groups and control group.

In the rotarod test, the scopolamine-treated group showed a shorter balance time on the spinning rod than the control group. Treatment with 20 mg/kg harmalol significantly increased resistance and balance time on the spinning rod in comparison with scopolamine group ($P < 0.05$). Scopolamine treatment of mice was significantly increased in brain MDA ($P < 0.001$). Treatment of mice received scopolamine by harmalol at dose of 20 mg/kg significantly decreased brain MDA ($P < 0.01$).

According to the results, scopolamine treatment of mice significantly decreased the antioxidant capacity of brain and serum ($P < 0.001$). Intraperitoneal administration of Harmalmol at doses of 10 and 20 mg/kg increased brain antioxidant capacity in compared to the scopolamine group ($P < 0.001$).

Treatment of mice received scopolamine with harmalol at dose of 20 mg/kg significantly decreased serum NO in comparison with scopolamine group ($P < 0.01$). According to the results, scopolamine treatment significantly increased brain NO in mice ($P < 0.001$). Intraperitoneal administration of harmalol at doses of 5, 10 and 20 mg/kg significantly decreased NO in the brain in compared to the scopolamine group ($P < 0.001$).

The expression levels of BDNF and GluN2B genes in the brain were significantly decreased in compared to the control group ($P < 0.05$). In the group received Harmalol at doses of 5 and 20 mg/kg, BDNF gene

expression was significantly increased in compared to the scopolamine group ($P < 0.05$). The results of this study showed that harmalol did not significantly alter GluN2B gene expression at three doses.

Discussion

The present study showed that scopolamine treatment impaired memory in mice. Different behavioral and molecular experiments were performed and a decrease in memory function, a change in the oxidant/antioxidant balance along with a change in the expression of BDNF and GluN2B genes were observed in the hippocampus of mice. Treatment with scopolamine significantly decreased swimming time and swimming frequency in the target quadrant in probe test and also significantly decreased the secondary latency in passive avoidance memory test. Treatment with scopolamine for 21 days also increased anxiety-related behaviors in the open field test and decreased balance power in the rotarod test. Harmalol significantly improved memory, anxiety and balance ability of scopolamine received mice. The results showed that harmalol reduced the latency to reach the hidden platform in the Morris Water Maze test and increased the secondary delay in the shuttle box test.

Jaqueline Moura et al showed that acute pretraining systemic administration of aromatic β-carboline alkaloids, harmine and harmalol, improved novel recognition tested 1.5 h (STM) after training in mice, while the dihydro-β-carboline harmaline principally enhanced 24 h memory (LTM)(18). Harmaline and harmalol reduced the catecholamine- induced loss of the transmembrane potential and of cell viability in PC12 cells(19).

Scopolamine-induced amnesia is a classic model to investigate the effect of nootropic factors on learning and memory disorders including Alzheimer's disease. Cholinomimetic agents have been shown to reverse the effects of scopolamine-induced amnesia in animals and humans. Thus acetylcholinesterase inhibitors that improve access to acetylcholine in the synaptic cleft reverse the cognitive deficits induced by scopolamine (20). Zhao et al.'s study shows that beta-carbonyls, including harmalol, have great inhibitory potency against acetylcholinesterase in vitro and, given their potent inhibition, these alkaloids may be used as candidates for the treatment of Alzheimer's disease (21).

Evidence suggests that scopolamine-induced memory impairment, which is associated with a decrease in cholinergic neurotransmission, is associated with decreased antioxidant enzyme activity and increased levels of radicals (22). In this study, administration of scopolamine decreases the antioxidant capacity and NO levels of brain tissue and this abnormality is markedly improved by harmalol. As expected, injection of scopolamine causes oxidative stress in the hippocampus, which is evident with increasing MDA levels. These changes were significantly reduced by pretreatment with harmalol.

Evidence suggests that BDNF dysfunction plays an important role in the pathology and manifestation of AD symptoms. BDNF is a protein that plays an important role in neuroprotection, neurogenesis, and synaptic plasticity and can therefore play an important role in learning (23). It has been suggested that suppression of BDNF gene expression and its receptors in the hippocampus results from scopolamine and leads to altered memory function in mice(24). In our study, scopolamine suppresses BDNF gene mRNA, which is similar to the results of previous studies. As expected, treatment of mice received scopolamine with harmalol increased the mRNA copy number of the BDNF gene. These results suggest that the cognitive enhancing effects of harmalol may

be associated with activation of the BDNF gene. Harmalol at three doses did not significantly alter GluN2B gene expression, suggesting that Harmalol may not alter NMDA receptor activity that is related to learning, memory and cognition.

Although cognitive symptoms are characteristic of AD, non- cognitive symptoms are becoming increasingly important because of the prevalence and dysfunctions they generate. Non- cognitive symptoms, such as agitation, aggression, depression, anxiety and psychosis are often observed in AD patients. These symptoms known as behavioural and psychological symptoms of dementia have been reported to occur in about 20% of AD patients (24). About 40% of AD patients show clinical signs of depression within 5 years (24). In this study, mice treated with harmalol reduced immobility time in forced swimming test.

Conclusion

The present study emphasizes that harmalol improves scopolamine-induced memory loss by modulating AChE activity and increasing BDNF in the mice hippocampus. Therefore, harmalol may be a promising therapeutic drug to prevent amnesia and cognitive deficits associated with aging or neurodegenerative diseases such as Alzheimer's.

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Conflict of interest

The authors declare that there is no conflict of interest.

Ethics approval and consent to participate

All animal procedures were based on the Guideline for the Care and Use of Laboratory Animals.

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