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Nelumbo nucifera semen extract improves memory in rats with scopolamine-induced amnesia through the induction of choline acetyltransferase expression

Ji Hoon Oh, Bong Jae Choi, Mun Seog Chang, Seong Kyu Park*

Department of Prescriptionology, College of Oriental Medicine, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea

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ABSTRACT

Nelumbo nucifera semen (NNS) is a traditional herb with anti-diarrheal, anti-ganacratia, and tranquilizer-like pharmacological activities. In this study, we examined the anti-amnesic effect of NNS on rats with scopolamine-induced amnesia. Passive avoidance tests, acetylcholinesterase (ACHE) activity, and choline acetyltransferase (CHAT) expression were used to evaluate the NNS anti-amnesic effects. The rats were divided into five groups: the normal group, scopolamine-treated group (1 mg/kg; control), NNS (1 g/kg) and scopolamine (1 mg/kg) co-treatment group, and the ARICEPT (1 mg/kg) and scopolamine (1 mg/kg) co-treatment group (positive control). The rats were administered the compounds orally for 14 days. The latency time of passive avoidance significantly increased by 54% in the NNS-treated group compared to the scopolamine-treated group. The ACHE activity in the NNS-treated group significantly decreased to 7.35% than that of the control group. CHAT-positive neurons increased by 51.02% in the NNS group compared to the control group. These results suggest that NNS extract improves scopolamine-induced dementia by inhibiting ACHE activity and inducing CHAT expression.

Alzheimer's disease (AD) is a progressive human neurodegenerative disorder characterized by neurofibrillary tangles, amyloid plaques, and severe neuronal degeneration in certain regions of the brain [29]. A cholinergic deficit has been shown to be associated with memory loss and the severity of AD. Cholinergic neurons in the central nervous system (CNS) are believed to be involved in learning and memory of both human and animals [1]. Loss of the cholinergic markers choline acetyltransferase (CHAT) and acetylcholinesterase (ACHE) are neurological changes consistently found in the brains of AD patients [4]. Postsynaptic receptors may be of the muscarinic M_1 subtype and are largely spared following presynaptic cholinergic deafferentiation [9]. Muscarinic M_1 receptors have been shown to enhance synaptic plasticity and long-term potentiation and are therefore attractive targets for restoration of cognitive function [17]. Many studies have shown that cholinergic antagonists mediate behavioral impairment in learning tasks. Anti-muscarinic drugs were shown to interfere with the activity of cholinesterase inhibitors in patients with dementia [20]. Specifically, scopolamine, a cholinergic receptor antagonist, is widely used to study cognitive deficits in experimental animals [13]. This and similar anti-muscarinic drugs have been used extensively in animals to mimic the cognitive dysfunction observed in dementia and AD. The majority of studies have focused on scopolamine-reversal as an initial screening method to identify therapeutic candidates for cognitive disorders [6]. In this study, rats with scopolamine-induced amnesia were used as an animal model for screening anti-amnesic drugs. Acetylcholinesterase inhibitors are the most effective pharmacotherapy for AD. These compounds indirectly elevate acetylcholine concentrations in the AD-affected brain, thereby enhancing cholinergic function. However, these drugs also cause undesired gastrointestinal, cardiorespiratory, extrapyramidal, genitourinary, and musculoskeletal side effects and sleep disturbances [2]. Therefore, ACHE inhibitors with fewer side effects are necessary. *Nelumbo nucifera* semen (NNS) is a popular medicinal herb in Korea. It has anti-diarrheal, anti-ganacratia, and tranquilizer-like pharmacological activities. In a previous study, Mukherjee et al. reported that *N. nucifera* rhizome extract inhibited ACHE activity [18]. Also, Yang et al. reported that *N. nucifera* rhizome extract improved learning and memory by enhancing neurogenesis in the dentate gyrus of the hippocampus [32]. However, the effect of NNS on scopolamine-induced dementia has not been investigated in an animal model. In this study, we examined the anti-amnesic effect of NNS in rats with scopolamine-induced amnesia.

NNS was purchased from Muan Herb (Muan, Korea). A 350-g sample of dried NNS was boiled in 7 l water for 2 h at 100 °C, and the suspension was filtered and concentrated under reduced pressure. The filtrate was lyophilized, yielding 76.60 g (26%) of powder, which was stored at 4 °C. Before each experiment, the

* Corresponding author. Tel.: +82 2 961 0330; fax: +82 2 961 0536.
E-mail address: comskp@khu.ac.kr (S.K. Park).

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dried extract was dissolved in distilled deionized water and vortexed for 2 min at room temperature. Male Wistar rats (5 weeks old) were purchased from Japan SLC (Shizuoka, Japan). They were housed in a specific pathogen-free environment with a 12 h/12 h light/dark cycle and with free access to standard rodent pellets (Purina, Seongnam, Korea) and water. Animal care and experimental procedures followed requirements put forth in the Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare, National Institutes of Health, 1996), which was approved by the Institutional Review Board of the College of Oriental Medicine at Kyung Hee University. After 7 days of adaptation, the rats were randomly divided into five groups: the normal group, scopolamine-treated group (1 mg/kg; control), NNS (1 g/kg) and scopolamine (1 mg/kg) co-treatment group, and the ARICEPT (1 mg/kg) and scopolamine (1 mg/kg) co-treatment group (positive control). The NNS extract and ARICEPT (Eisai Korea Co., Ltd., Korea) were dissolved in distilled water, and the rats were administrated NNS and ARICEPT for 14 days. Rats in the normal and control groups were similarly treated with corresponding volumes of distilled water. All rats except those in the normal group received scopolamine (1 mg/kg body weight, i.p.) 30 min prior to consecutive trials of the passive avoidance test. Scopolamine (Sigma–Aldrich Fine Chemicals Korea Co., Ltd., Korea) was dissolved in distilled water. Passive avoidance behavior was evaluated by a step-through method. A modified passive avoidance test was used to assess the effect of NNS on scopolamine-induced dementia. The step-through passive avoidance apparatus contained one light chamber equipped with an illuminator and one dark chamber. The chambers were separated by a guillotine door. During the training phase, a rat was placed in the light chamber with the door open. When the rat entered the dark chamber, the guillotine door was closed immediately. After being allowed to explore the dark chamber for 1 min, the rat was removed from the apparatus and placed in its cage. This training protocol was repeated until the rat entered the dark chamber within 20 s of being placed in the light chamber. Animals that did not enter the dark chamber were excluded from the experiment. At 24 h after training, the rat was placed in the light chamber. Immediately after the rat entered the dark chamber (all four paws), the door was closed and an electric shock (2.0 mA) was delivered to the feet for 3 s. The testing phase to evaluate memory retention commenced 24 h later. The rat was placed in the light chamber, and the latency time before it entered the dark compartment was measured. If the rat did not enter the dark chamber within a cut-off period of 300 s, a value of 300 s was used as the latency time.

The whole hippocampus of each rat was dissected on ice after the passive avoidance test and homogenized in 2 ml of ice-cold phosphate-buffered saline (PBS; 0.1 M, pH 7.4). The homogenate was centrifuged at 3000 rpm for 15 min at 4 °C and then pre-incubated at 37 °C for 5 min with ethopropazine (0.1 mM), a selective inhibitor of butyrylcholinesterase (bCHE). The ACHE activity assay was performed using a colorimetric method with minor modifications. A 2-ml volume that contained 0.1 ml acetylcholine iodide (12 mM), 1.8 ml sodium phosphate buffer (0.1 mM, pH 7.4), and 0.1 ml homogenate was incubated at 37 °C for 8 min. The reaction was terminated by adding 1 ml of 3% (w/v) sodium lauryl sulphate (SLS), and then 1 ml of 0.2% (w/v) 5,5-dithiobis (2-nitrobenzoic) acid (DTNB) was added to produce a yellow complex. The reaction was measured spectrophotometrically at 412 nm. The ACHE inhibition was calculated by the following equation

$$\text{ACHE inhibition} = -\left(1 - \frac{\text{OD}_{(\text{test})}}{\text{OD}_{(\text{normal})}}\right) \times 100\%,$$

where $\text{OD}_{(\text{test})}$ and $\text{OD}_{(\text{normal})}$ are the absorbance values of the samples obtained from the tested and normal groups, respectively. Results were compared after normalization to the untreated normal group (100% of inhibition). Rats were anesthetized with ether, and the hearts were perfused with 25 ml of 0.1 M PBS, followed by 250–300 ml of 4% paraformaldehyde (PFA) in PBS (50 mM, pH 7.4). The brains were rapidly removed, post-fixed for 4 h in PFA (4 °C), and cryoprotected in 30% sucrose with PBS. Brains were sliced (30 μm coronal sections) using a cryostat. The free-floating sections were then stained by immunohistochemistry. The sections were washed in PBS containing 0.3% Triton X-100 and 1% rabbit serum, and they were incubated in the CHAT primary antibody (Chemicon, USA) that was diluted 1:100 in the same buffer at 4 °C for 72 h. After washing, the sections were incubated in biotinylated anti-sheep serum and ABC complex (Vectastain Elite Kit; Vector Lab., Burlingame, CA, USA) for 2 h. The ABC complex was visualized using 0.05% diaminobenzidine containing 0.02% H_2O_2. The images were captured using an Axio Vision 3.0 imaging system (Zeiss, Oberkochen, Germany), and these images were processed using Adobe Photoshop. For measuring CHAT-positive cells, the grid was placed on the medial septum areas of the rat brain. The number of cells was counted at 100× magnification using a microscope rectangle grid that measured 100 mm × 100 mm. Raw data from the passive avoidance test were analyzed using the non-parametric Mann–Whitney test. The immunohistochemistry data were analyzed using Student's *t*-test. All data are expressed as means ± the standard error of the mean (SEM).

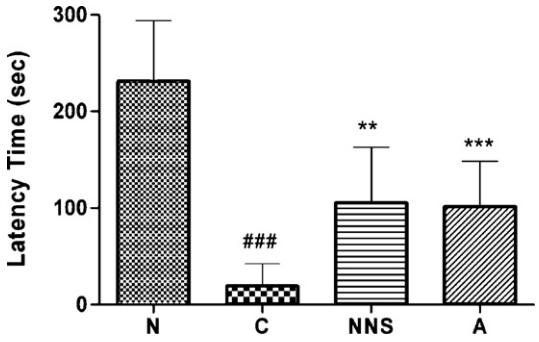

Fig. 1. Effects of NNS on the response latency of rats in the passive avoidance test. In the passive avoidance test, the mean response latency of the NNS-treated rats (106 ± 56.9 s) was significantly greater than that of the control rats (19.8 ± 22.5 s) ($p < 0.01$). Detectable irritation or sedation was not observed following vehicle treatment. Compared to the positive control group (102.1 ± 45.9 s), the effect of NNS was significant. $^{###}p < 0.001$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the control group.

Scopolamine-treated rats showed a significantly decreased latency of reaction (19.8 ± 22.5 s) compared to the normal group (232.5 ± 61.7 s; $p < 0.001$). The latency time of NNS-treated rats was greatly increased compared to the control group (106.0 ± 56.9 s vs. 19.8 ± 22.5 s; $p < 0.01$). The improvement mediated by NNS was comparable to the positive control, ARICEPT, which is widely used to treat mild cognitive impairment (102.1 ± 45.9 s; $p < 0.001$) (Fig. 1). We measured ACHE activity in the hippocampus. Scopolamine, which is a known muscarinic receptor (mACR) antagonist, significantly reduced the level of ACHE inhibition compared to the normal group (90.3 ± 1.1%; $p < 0.001$). The inhibition rate of ACHE activity in the NNS-treated group was significantly increased compared to that of the scopolamine-treated group (90.3 ± 1.1% vs. 97.7 ± 0.9%; $p < 0.001$). NNS was shown to inhibit ACHE activity to a similar level as the ARICEPT positive control (97.0 ± 0.5%; $p < 0.001$) (Fig. 2). CHAT degradation is related to acetylcholine deficiency. We investigated the CHAT activity in various groups after treatment. As shown in Fig. 3, CHAT-positive neurons were significantly reduced by treatment with scopolamine compared to the normal group, whereas the NNS-treated group showed increased CHAT expression compared to the control group (7.4 ± 0.7 vs. 4.9 ± 0.4; $p < 0.01$). This improvement by NNS was similar to that of the ARICEPT group (7.6 ± 1.1; $p < 0.01$) (Fig. 3).