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Effects of HX106N, a Water-Soluble Botanical Formulation on Scopolamine-Induced Memory Impairment in Mice

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식물성 열수 추출물 HX106N이 스코폴라민으로 유도한 생쥐 기억력 저하에 미치는 효과에 관한 연구

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국문요약

HX106N은 용안육, 맥문동, 단삼 및 천마 등의 4가지 식물로 구성된 추출물로서, 선행 연구에서 amyloid β peptide에 의한 생쥐의 기억력 저하 및 산화 손상을 억제하는 것으로 밝혀졌다. 이 연구에서는 HX106N이 비선택적 무스카린 수용체 길항제로 잘 알려진 스코폴라민(scopolamine)으로 유도한 콜린성 건망증(cholinergic amnesia)에 어떤 영향을 미치는지를 평가하였다. ICR 생쥐에게 스코폴라민(1 mg/kg body weight, i.p.)을 주입하기 1시간 전에 HX106N(100 mg/kg body weight, p.o.)을 투여하였다. 30분 후 수행한 Y-미로 시험(Y-maze test) 및 수동 회피 시험(passive avoidance test)에서 HX106N는 스코폴라민에 의해 감소되는 자발적 변경 행동(spontaneous alternation) 및 지체시간(step-through latency)을 유의미하게 억제하여 건망증을 개선시키는 것으로 나타났다. 또한 HX106N을 투약 1시간 후 생쥐의 해마와 대뇌피질 부위의 아세틸콜린에스테라제(acetylcholinesterase; AChE)의 활성을 측정한 결과 통계적으로 유의미한 정도의 활성 감소가 관찰되었다. 이러한 결과들을 종합할 때 HX106N은 AD에서 관찰되는 콜린성신경전달 장애로 인한 기억력 저하 억제에 사용될 수 있는 가능성을 가진 것으로 판단된다.

Keywords: HX106N, memory impairment, scopolamine, cholinergic amnesia, Alzheimer's disease

Introduction

The cholinergic hypothesis suggests that the dysfunction of the central cholinergic system significantly contributes to the cognitive decline observed in Alzheimer's disease (AD) patients (Francis et al. 1999; Terry & Buccafusco 2003). Therefore, the prevention of acetylcholine deficiency through the inhibition of acetylcholinesterase (AChE) has been explored as one of the therapeutic approaches to treat AD. Drugs currently used for AD patients such as donepezil, rivastigmine, and galantamine belong in this category. Although these drugs have shown to improve cognitive functions (Rogers et al. 1998; Rosler et al. 1999; Tariot et al. 2000), they often cause adverse side-effects and are ineffective at preventing the progression of AD (Benzi & Moretti 1998). As a result, there are still needs for the development of safer and more effective therapeutic approaches.

HX106N is an aqueous extract prepared from four plant sources, *Dimocarpus longan* Lour. (Sapindaceae), *Liriope platyphylla* Wang et Tang. (Liliaceae), *Salvia miltiorrhiza* Bunge (Lamiaceae), and *Gastrodia elata* Blume (Orchidaceae). We previously demon-

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strated that HX106N could ameliorate the memory impairment induced by amyloid β (Aβ) peptide with the inhibition of oxidative stress (Lee et al. 2014). In this study, we tested whether HX106N has an anti-amnesic effect against memory impairment caused by cholinergic dysfunction. Scopolamine, a nonselective antagonist for muscarinic acetylcholine receptor, was delivered intraperitoneally to mice to induce cholinergic amnesia. In both the Y-maze and passive avoidance tests, memory impairment was rescued when mice were pre-treated with HX106N one hour before injecting scopolamine. Because of the important role of AChE for cholinergic neurotransmission, we examined the effect of HX106N on AChE activity. The level of AChE activity was significantly reduced in the hippocampus and cortex of mice treated with HX106N. The *in vitro* AChE activity assay showed that the IC_{50} value of HX106N was 4.7±0.1 mg/mL. Our results suggest that HX106N could improve the cholinergic amnesia, most likely through the inhibition of AChE activity, and might be used to alleviate memory impairment caused by cholinergic dysfunction such as those found in AD patients.

Materials and Methods

1. Preparation of HX106N

HX106N was prepared using a protocol described previously (Lee et al. 2014). The following 4 plants were used to prepare HX106N: *Dimocarpus longan* Lour. (*Longanae Fructus*); *Liriope platyphylla* Wang et Tang. (*Liriopes Radix*); *Salvia miltiorrhiza* Bunge (*Salvia miltiorrhiza Radix*); and *Gastrodia elata* Blume (*Gastrodiae Rhizoma*). In an effort to make HX106N in a consistent manner at different times, high-performance liquid chromatography analysis was employed, using salvianolic acid B, gastrodin, and spicatoside A for *Salvia miltiorrhiza*, *Gastrodia elata*, and *Liriope platyphylla*, respectively. For *Dimocarpus longan*, liquid chromatography-tandem mass spectrometry analysis was performed, using ellagic acid as a marker. The cell-based bioassays, as reported by Lee et al. (2014), have also been used to control the quality of HX106N in consistent manner.

2. Animals and reagents

Male ICR mice at 5-weeks-old were purchased from Samtako, Bio Korea (Kyoung-Ki, Korea). The mice were housed under a 12:12 h light-dark cycle (light on from 08:00 to 20:00 h) with access to food and water *ad libitum*. All experimental procedures were conducted in compliance with the guidelines set by the Institutional Animal Care and Use Committee of Seoul National University. Donepezil hydrochloride monohydrate and scopolamine hydrochloride were purchased from Sigma (St. Louis, MO, USA).

3. Scopolamine-induced amnesia model

Mice were orally administered with HX106N (100 mg/kg body weight) or donepezil (1 mg/kg body weight). One hour later, amnesia was induced by scopolamine (1 mg/kg body weight, i.p.). The mice were introduced to the Y-maze test and the acquisition trial of the passive avoidance test 30 min after the injection.

4. Y-maze test

The apparatus was made of dark opaque polyvinyl plastic and placed horizontally, with the 3 arms at 120°. Each arm was 40 cm long, 13 cm high, 3 cm wide at the bottom, and 10 cm wide at the top. The mice were initially placed at the end of one arm and allowed to move freely for 7 min. The series of arm entries was recorded manually. Spontaneous alternation was defined as successive entries into the 3 arms in overlapping triplet sets. The alternation percentage was calculated as the ratio of actual alternations to maximum alternations (defined as the total number of arm entries minus 2) multiplied by 100.

5. Step-through passive avoidance test

The apparatus was composed of clear and dark chambers that were identical, separated by a guillotine door. The clear chamber contained a 15 W bulb, and the floor of both chambers consisted of 2-mm stainless steel rods that were spaced 1 cm apart. For the acquisition trial, the mice were initially placed in the clear chamber. Thirty seconds later, the guillotine door was opened to allow the mice to enter the dark chamber. When all 4 limbs of the mouse were inside the dark chamber, the door was closed, and an electric foot shock (0.5 mA, 3 s) was delivered through the stainless steel rods. Twenty-four hours after the acquisition trial, the mice were placed back into the clear chamber for the retention trial. Latency was defined as the time it took for a mouse to enter the dark chamber, and was recorded up to 300 s.

6. Measurement of acetylcholinesterase activity

For the ex vivo assay, the mice were sacrificed 1 h after the oral administration of HX106N (100 mg/kg body weight) or donepezil (1 mg/kg body weight). The hippocampal and cortical tissues were isolated and homogenized in ice-cold sodium phos-

phate buffer (100 mM, pH 7.0). The homogenates were centrifuged at 10,000 × *g* for 10 min at 4℃, and the supernatant was assayed for the determination of AChE activity using the Amplex Red Acetylcholine/acetylcholinesterase assay kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

The assay for *in vitro* AChE activity was conducted using the modified method described by Ellman et al. (Kosasa et al. 1999). Briefly, a reaction buffer containing 134 μL of sodium phosphate buffer (100 mM, pH 7.0), 1 μL of acetylthiocholine iodide (75 mM), and 5 μL of 5,5-dithiobis-2-nitrobenzoate (10 mM) was mixed with 50 μL of diluted HX106N solution and incubated for 10 min at 25℃. Then, 10 μL of mouse brain homogenates (100 mg brain/mL of 12.5 mM sodium phosphate buffer, pH 7.0) was added as an enzyme source. The absorbance was measured at 405 nm after 30 min of incubation at 25℃. AChE activity was expressed as the percentage of absorbance relative to the control (50 μL of distilled water in reaction buffer).

7. Statistics

The data are presented as the mean±S.D. or ±SEM. Significant differences between the experimental groups were analyzed using a one-way analysis of variance (ANOVA), while Dunnett's multiple comparison test was employed for multiple comparisons. *P*-values less than 0.05 were considered as significant.

Results and Discussion

Cognitive decline in patients with AD is known to be strongly correlated with decreased cholinergic neurotransmission (Francis et al. 1999; Terry & Buccafusco 2003). Scopolamine-induced amnesia is used as a model for AD because it causes cognitive deficits by blocking cholinergic signaling (Klinkenberg & Blokland 2010). The effect of HX106N on the working memory impairment induced by scopolamine was examined using a Y-maze spontaneous alternation test. The animals were orally administered with HX106N (100 mg/kg body weight) or donepezil (1 mg/kg body weight) 1 h before an intraperitoneal (i.p.) injection of scopolamine. The Y-maze test was conducted 30 min after the injection. The percentage of spontaneous alternation was significantly lower in scopolamine-injected mice compared to control mice, but this effect was prevented by the oral administration of HX106N (Fig. 1A). The administration of donepezil also increased the alternation behavior in scopolamine-injected mice, but not in a statistically significant manner (Fig. 1A). No difference was

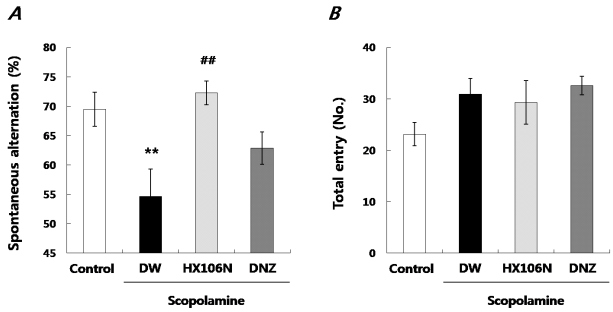

Fig. 1. The effects of HX106N on scopolamine-induced working memory impairment in the Y-maze test. Mice were orally treated with HX106N (100 mg/kg body weight) or donepezil (1 mg/kg body weight) 90 min before the test. Memory impairment was induced by the i.p. injection of scopolamine. After 30 min, mice were subjected to the Y-maze test. The percentages of spontaneous alternation (A) and number of total entry (B) are shown as indicated. The data are presented as the mean±SEM (n=7~8). ***P*<0.01 vs. the PBS-injected, DW-treated control group; ##*P*<0.01 vs. the scopolamine-injected, DW-treated group. DNZ, donepezil.

observed in the total number of entries between the groups (Fig. 1B). These data showed that HX106 could improve scopolamine-induced impairment of working memory in this model.

To evaluate the effect of HX106N on the impairment of long-term memory, a passive avoidance test was performed in scopolamine-injected mice. The animals were trained for the passive avoidance test 30 min after the injection of scopolamine. One day later, step-through latency was measured as an index of long-term memory in the retention trial. Scopolamine-injected mice showed significantly decreased latency times in the retention trial compared to control mice (Fig. 2). However, a significant increase in step-through latency was observed when the mice were treated with HX106N (100 mg/kg body weight) 1 h before the injection of scopolamine, and this effect was comparable to that of donepezil (Fig. 2). These results demonstrated that HX106N can attenuate the long-term memory deficit induced by scopolamine in the mouse.

Enhancing cholinergic neurotransmission by inhibiting AChE activity is a major strategy for improving cognitive function in patients with AD (Hirai S 2000). To examine whether AChE activity was affected by treatment with HX106N, AChE activity in the hippocampus and cortex of mice was measured one hour after the oral administration of HX106N (100 mg/kg body weight). HX106N inhibited AChE activity by 42.5%±3.1% in the hippo-

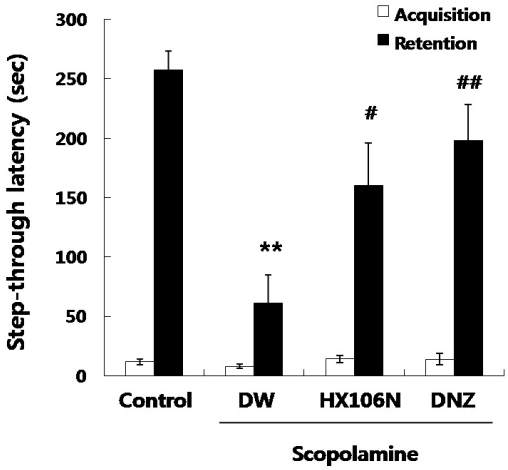

Fig. 2. The effects of HX106N on long-term memory impairment induced by scopolamine in the passive avoidance test. Scopolamine was delivered to mice by an i.p. injection, one hour after oral administration of HX106N (100 mg/kg body weight) or donepezil (1 mg/kg body weight). Thirty minutes later, mice were subjected to the acquisition trial of step-through passive avoidance test, and retention trial was performed 24 h after the acquisition. The data are presented as the mean±SEM (n = 8~10). $^{**}P<0.01$ vs. the PBS-injected, DW-treated control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. the scopolamine-injected, DW-treated group. DNZ, donepezil.

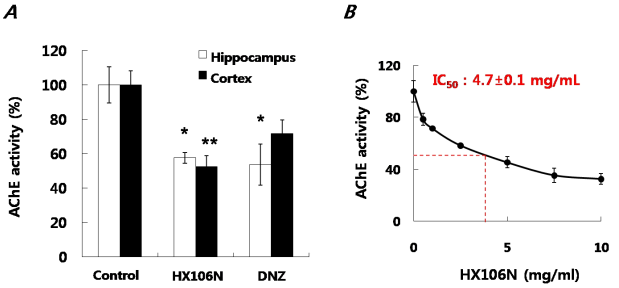

Fig. 3. The effects of HX106N on AChE activity. (A) The mice were orally treated with HX106N (100 mg/kg body weight) or donepezil (1 mg/kg body weight). After 1 h, the hippocampus and cortex were isolated, total proteins were prepared, and AChE activity was measured. The data are presented as the mean±SEM (n=3). $^{*}P<0.05$, $^{**}P<0.01$ vs. the DW-treated, control group. (B) Various concentrations of HX106N were added to mouse brain homogenates, which were used as a source of the enzyme. AChE activity was measured using the modified Ellman's method as described in the Materials and Methods section. The data are presented as the mean±SD of triplicate samples from a representative experiment. DNZ, donepezil.

campus and 47.5%±6.3% in the cortex compared to control mice (Fig. 3A). The AChE inhibitor donepezil also decreased AChE activity (Fig. 3A). Indeed, the inhibitory effect of HX106N at 100 mg/kg was comparable to that of the donepezil.

The effect of HX106N on AChE activity was also examined, using mouse brain homogenates. When the homogenates were treated with various concentrations of HX106N, the level of AChE activity decreased in a dose-dependent manner with an IC_{50} value of 4.7±0.1 mg/mL (Fig. 3B). These results suggest that HX106N might improve cognitive deficits by inhibiting AChE activity in amnesia animal model.

In a previous study, oral administration of HX106N ameliorated memory impairment and reduced the level of thiobarbituric acid-reactive substances, which is an indicator for lipid peroxidation, in the brain of $A\beta_{25-35}$-treated mice (Lee et al. 2014). This suggests that the memory-enhancing effects of HX106N might be accomplished by its antioxidative activity. Amnesia caused by $A\beta$ toxicity has been reported to be associated with the damage in cholinergic neuron that can be improved by enhancement of cholinergic neurotransmission through the inhibition of AChE activity (Maurice et al. 1996; Tran et al. 2002). Based on our data it can be thought that the improvement of memory functions in $A\beta_{25-35}$-injected mice might be the result of AChE inhibitory activity of HX106N.

Because acetylcholine is known as a critical neurotransmitter involved in attentional process, AChE inhibitors including donepezil and galantamine, were explored as possible therapeutics for individuals with attention deficit hyperactivity disorder (Biederman et al. 2006; Cubo et al. 2008). HX106N (100 mg/kg) was shown to contain AChE inhibitory activity comparable to that of donepezil. In the Y-maze test, which tests for working memory requiring attention for appropriate operation, the process was ameliorated by oral administration of HX106N in scopolamine-treated mice. These results suggest that HX106N might also be useful in improving attention problem.

Conclusion

We showed that working memory and long-term memory deficits resulting from cholinergic dysfunction were rescued by oral treatment with HX106N, at least in part by inhibiting AChE activity. HX106N may have the potential to become a therapeutic

for cholinergic amnesia found in AD.

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