Ameliorating Effects of HX106N, a Water-Soluble Botanical Formulation, on Aβ25-35-Induced Memory Impairment and Oxidative Stress in Mice

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It is well-established that amyloid β (Aβ)-induced oxidative stress plays a crucial role in Alzheimer’s disease (AD) and its cognitive deficits. HX106N is a water-soluble extract prepared from a mixture of the plants Dimocarpus longan, Liriope platyphylla, Salvia miltiorrhiza, and Gastrodia elata. These ingredients are traditionally used in various plant-based medicines for the treatment of neurological disease. In this study, we examined the effects of HX106N on memory impairment and oxidative stress caused by the intracerebroventricular injection of Aβ25-35 peptide in mice. For one week prior to Aβ25-35 peptide injection and 8 d after, mice were given oral HX106N. HX106N treatment reversed the Aβ25-35-mediated decrease in alternation percentage and latency time in the Y-maze and passive avoidance tests. Mice treated with HX106N showed decreased levels of thiobarbituric acid reactive substances (TBARS), a lipid peroxidation marker. Quantitative reverse transcription polymerase chain reaction (RT-PCR) demonstrated that HX106 treatment increased levels of heme oxygenase-1 (HO-1) in the hippocampus of Aβ25-35-injected mice, while having little effect on the expression of tumor necrosis factor (TNF)-α and interleukin (IL)-1β. In the murine hippocampal neuronal cell line HT22, HX106N was found to upregulate HO-1 expression at the RNA and protein levels as well as to protect cells from glutamate-induced oxidative stress. Taken together, our data suggest that HX106N may potentially act as a preventive and/or therapeutic agent for AD.

Key words HX106N; amyloid β25-35; memory impairment; oxidative stress; heme oxygenase-1; Alzheimer’s disease

Dementia is a clinical syndrome characterized by the progressive deterioration of global cognitive function that is severe enough to interfere with daily activities. Alzheimer’s disease (AD) is the most common type of dementia. The amyloid cascade hypothesis, one of the leading theories for the pathology of AD, proposes that amyloid-β (Aβ) peptide, which is derived from the proteolytic cleavage of the amyloid precursor protein, accumulates in the brain and plays a crucial role in the pathology of AD. 1–2) In fact, there have been many reports demonstrating that high levels of the Aβ peptide are neurotoxic and that its deleterious effects are mediated by the generation of reactive oxygen species, 3) neuroinflammation, 4) and a dysregulation of ionic homeostasis. 5) Consequently, much of the focus of AD research has been placed on controlling brain levels of Aβ and preventing Aβ-induced neurotoxicity.

Heme oxygenase-1 (HO-1) is an inducible enzyme that catalyzes the degradation of heme to carbon monoxide, biliverdin, and free iron. 6) HO-1 expression is increased in many neurodegenerative diseases, such as AD and Parkinson’s disease (PD), and it is correlated with oxidative damage and inflammation. 7,8) Elevated levels of HO-1 are believed to produce antioxidative responses, restore redox homeostasis, and confer neuroprotection against various oxidative stimuli. 9–11) Given the beneficial effects of HO-1, the pharmacological modulation of HO-1 levels has been considered an important therapeutic approach to counter neurodegenerative diseases.

HX106N is a water-soluble extract prepared from four plants: Dimocarpus longan Lour. (Sapindaceae), Liriope platyphylla Wang et Tang. (Liliaceae), Salvia miltiorrhiza Bunge (Lamiaceae), and Gastrodia elata Blume (Orchidaceae). These plants were selected based on previous publications describing their activities and functions and based on information accumulated during the practice of traditional phyto-medicine in Asia. For example, Dimocarpus longan is known to have anti-amnesic and anxiolytic effects and has been reported to enhance cognitive performance in a mouse passive avoidance test. 12–14) Liriope platyphylla has been described as effective in the treatment of inflammatory diseases such as bronchitis and pneumonia 13,14) and it contains a compound, spicatoside A, that has been shown to have neurotrophic activities. 15) The aqueous extract of Salvia miltiorrhiza has antioxidative and anti-inflammatory properties, as reported by Zhao et al. 16) and Kim et al. 17) Gastrodia elata has been used for the treatment of central nervous system diseases including headaches, tetanus and epilepsy. 13,14) The ether fraction of its methanol extracts has also been reported to be neuroprotective in kainic acid-treated rats. 18)

In this study, we investigated the therapeutic potential of HX106N using mice injected with Aβ25-35 peptide. HX106N produced anti-amnesic and antioxidative effects in Aβ25-35-injected mice. In HT22 cells, a murine hippocampal neuronal cell line, HX106N increased HO-1 mRNA and protein and provided a protective effect against glutamate-induced oxidative stress. Our results show that HX106N may be useful in developing preventative and/or therapeutic agents for the treatment of AD.

MATERIALS AND METHODS

Preparation of HX106N All plants used in the preparation of HX106N were purchased from Humanherb (Gyeongsan, Korea) and authenticated by Juwon Shim, a pharmacist at ViroMed Co., Ltd. (Seoul, Korea). The following plants were used: Dimocarpus longan Lour. (Longanaceae Fructus,
6 g); Liriope platyphylla Wang et Tang. (Liriopes Radix, 10 g); Salvia miltiorrhiza Bunge (Salvia miltiorrhiza Radix, 6 g); and Gastrodia elata Blume (Gastrodiae Rhzoma, 2 g). The plants (total dry weight, 24 g) were mixed, minced using a grinder (Rong Tong Iron Works, Taichung, Taiwan), and extracted by boiling in distilled water for 3 h. The extract was filtered with 10-µm cartridge paper and concentrated using a rotary evaporator (Eyela, Tokyo, Japan), followed by a freeze-drying process. This process generally produced approximately 10–11 g of brown powder. The voucher specimens (HX106N-110624) used in this study have been deposited in the herbarium of ViroMed Co., Ltd. (Seoul, Korea). To validate the quality of HX106N, high-performance liquid chromatography analysis was employed, using salvianolic acid B, glycoside, and spicato-side A for Salvia miltiorrhiza, Gastrodia elata, and Liriope platyphylla, respectively. For Gastrodia elata, liquid chromatography-tandem mass spectrometry analysis was performed, using ellagic acid as a marker (Supplemental information). A cell-based bioassay was also used to prepare HX106N in a solution. In the preliminary experiments, the injection site was returned to normal behavior within 1 min following the injection. The mice was delivered over an approximately 30-s interval. The mice were initially placed in the clear chamber. After 30 s, the guillotine door was opened to allow the mouse to enter the dark chamber. Once all four 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. Twenty-four hours after the acquisition trial, mice were returned to the clear chamber for the retention trial. Latency was defined as the time required for a mouse to enter the dark chamber and was recorded for up to 300 s.

Measurement of Lipid Peroxidation After the retention trial of the passive avoidance test, the mice were sacrificed, and the hippocampus and cortex were isolated. The hippocampal and cortical tissues were homogenized in ice-cold phosphate-buffered saline (PBS) (10% w/v) containing 0.05% butylated hydroxytoluene (BHT) and centrifuged at 10000×g for 10 min at 4°C. The thiobarbituric acid-reactive substances (TBARS) levels of the homogenates were determined using the OxiSelect TBARS Assay Kit (Cell Biosabs, San Diego, CA, U.S.A.), according to the manufacturer’s protocol.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis Mice were sacrificed at the indicated time points after Ap25-35 injection. The hippocampus was dissected on ice and stored at -80°C. The tissue was homogenized, and total RNA was isolated using TRIzol reagent, following the manufacturer’s instructions (Invitrogen, Carlsbad, CA, U.S.A.). First-strand cDNA was synthesized using an oligo(dT) primer and AMV reverse transcriptase (TaKaRa, Shiga, Japan) and amplified by PCR using a specific primers for tumor necrosis factor (TNF)-α, interleukin (IL)-1β, HO-1 and glyceroldehyde-3-phosphate dehydrogenase (GAPDH) using SYBR Premix Ex TaqTM (TaKaRa). The primer sequences were as follows: TNF-α (forward, CCC TCA CAC TCA GAT CAT CTT CT; reverse, GCT ACG TGG GCT ACA G), IL-1β (forward, GCA ACT GTT CCT GAA CTC AAC T; reverse, ATC TTT TGG CGG TCG TCA ACT), HO-1 (forward, GAT AGT GAG CGT CCA CAG C; reverse, TGG TGG CCT CCT TCA AGG), and GAPDH (forward, AGC CTC GTC CGG TAG ACA A; reverse, AAT CTC CAC TTT GCC ACT GC). The reaction was started at 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 20 s. The threshold cycle (Ct) of each gene was determined as PCR cycles during which an increase in reporter fluorescence was observed above a baseline. GAPDH was amplified simultaneously as an internal control. The relative expression levels of the target genes were calculated using the 2-ΔΔCt method, as described previously.

Cell Culture HT22 cells (a gift from Dr. Yong-Keun Jung, Seoul National University, Seoul) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Gibco, Grand Island, NY, U.S.A.) with 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C under 5% CO2.

Western Blot Analysis After treatment, whole cell lysates were prepared for general western blot analysis. Membranes containing the blotted proteins were incubated with antibodies against HO-1 (1 : 2000; Stressgen, Victoria, Canada) and β-actin (1 : 5000, Sigma). For visual detection, a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG antibody (1 : 100000, Sigma) was used with chemiluminescence reagents (Millipore, Billerica, MA, U.S.A.).
Northern Blot Analysis  Total RNA was isolated using TRIzol reagent (Invitrogen). The RNA was subjected to 1% agarose-formaldehyde gel electrophoresis and then transferred to a nylon membrane (Hybond-N; Amersham, Buckinghamshire, U.K.). The blot was then washed and exposed to autoradiography film. Specific probes for mouse HO-1 and GAPDH were labeled by random priming using the Klenow fragment of DNA polymerase I (Stratagene, La Jolla, CA, U.S.A.) and \( ^{32}P \) dCTP (PerkinElmer, Inc., Norwalk, CT, U.S.A.). The PCR primer sequences were as follows: HO-1 (forward, TAC ACA TCC AAG CCG AGA AT; reverse, GTT CCT CTG TCA GCA TCA CC) and GAPDH (forward, ACG GCA AAT TCA ACG GCA CAG; reverse, GGC GGC ACG TCA GAT CC).

Determination of Cell Viability  HT22 cells were plated at 3×10^3 cells/well in 96-well plates. After 24 h, cells were treated with 4 mM glutamate (Sigma) and various concentrations of HX106N for 18 h. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay kit, according to the manufacturer’s protocol (Roche, Mannheim, Germany).

Measurement of Reactive Oxygen Species (ROS)  Intracellular levels of ROS were measured using the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Invitrogen). HT22 cells were treated with 4 mM glutamate in the absence or presence of 1 mg/mL HX106N for the indicated times. Cells were then washed twice with phosphate-buffered saline (PBS) and incubated with 5 mM CM-H_2DCFDA for 30 min at 37°C. The fluorescence was determined by flow cytometry using a FACS Calibur (BD, San Jose, CA, U.S.A.).

Statistics  The data are presented as the mean±S.D. (for in vitro experiments) or±S.E.M. (for in vivo experiments). Behavioral tests and biochemical analysis, with the exception of the ROS measurements, were analyzed using a one-way ANOVA, with Dunnett’s multiple comparison test employed for multiple comparisons. The data from the ROS analysis were analyzed with a two-way ANOVA followed by Bonferroni’s post-hoc test. The p-values of less than 0.05 were considered significant.

RESULTS  

Effects of HX106N on Memory Impairment in Aβ_{25-35}-Injected Mice  The Aβ peptide levels in the brain of AD patients strongly correlate with cognitive decline.21) Because i.c.v. injection of Aβ peptides has been shown to induce memory deficits in mice, it is considered a useful means of evaluating therapeutic agents.22) The effect of HX106N on working memory was examined using a Y-maze spontaneous alternation test in Aβ_{25-35}-injected mice. Mice were orally

![Fig. 1. Effects of HX106N on Memory Impairment Induced by Aβ_{25-35} in the Y-Maze and Passive Avoidance Tests](image-url)

The mice were orally treated with HX106N (50, 100, or 200 mg/kg body weight) or donepezil (1 mg/kg body weight) once daily from 7 d before and 8 d following induction of amnesia by an i.c.v. injection of Aβ_{25-35} peptide. (A) Spontaneous alternation behavior was examined as an index of working memory using the Y-maze test on day 7. (B) The mice were trained for the acquisition of passive avoidance response on day 8, and step-through latency was measured in a retention trial 24 h after acquisition. The data are presented as the mean±S.E.M. (n=10–11). ** p<0.01 vs. the vehicle-injected, DW-treated control group; * p<0.05, *** p<0.01 vs. the Aβ_{25-35}-injected, DW-treated group. DNZ, donepezil.
administered three different doses of HX106N (50, 100, or 200 mg/kg body weight) or donepezil (1 mg/kg body weight) on a daily basis from 7 d before to 7 d after Aβ25-35 peptide delivery. Control mice were administered distilled water and injected with a vehicle solution (distilled water). The Y-maze test was performed on day 7. Following the i.c.v. injection of Aβ25-35, the alternation behavior of mice was significantly decreased, indicating that Aβ25-35 impaired their working memory (p<0.01, Fig. 1A). Treatment with HX106N inhibited the alternation behavior impairment in a dose-dependent manner [F(5, 54)=4.841, p=0.001, Fig. 1A]. The effect of HX106N at 200 mg/kg was comparable to that of donepezil. Based on this result, HX106N at 200 mg/kg was used for subsequent experiments. The total number of entries into the three arms was similar among the groups, suggesting that neither HX106N nor donepezil affected locomotor activity [F(5, 54)=1.211, p=0.317, data not shown].

To evaluate the effect of HX106N on the long-term memory impairment induced by Aβ25-35, the mice were subjected to an acquisition trial of the step-through passive avoidance test on...
day 8. One day later, the step-through latency was measured as an index of long-term memory in a retention trial. The step-through latency time was significantly lower in the Aβ25-35-injected mice than in the control mice (p<0.01, Fig. 1B) and was dramatically increased following the daily administration of 200mg/kg HX106N or 1mg/kg donepezil [F(3, 37)=5.643, p=0.003, Fig. 1B]. The latency times during the acquisition trial were similar across all groups [F(3, 37)=0.364, p=0.779, Fig. 1B]. The effect of HX106N was comparable to that of donepezil. These results demonstrate that HX106N may help ameliorate the memory impairment induced by Aβ25-35 in mice.

Effects of HX106N on Lipid Peroxidation in the Hippocampus and Cortex of Aβ25-35-Injected Mice It has been reported that oxidative stress plays an important role in Aβ-induced neurotoxicity. To investigate whether HX106N had any effect on the oxidative stress induced by an Aβ25-35 injection, lipid peroxidation levels were measured in the hippocampus and cortex using a TBARS assay. The level of TBARS in the hippocampus of Aβ25-35-injected mice was increased by 2.7-fold compared with that of the control mice (p<0.05, Fig. 2A). This increase was prevented by the oral administration of HX106N or donepezil [F(3, 12)=7.145, p=0.005, Fig. 2A]. The TBARS production induced by Aβ25-35 injection was also inhibited in the cortex of the mice treated with HX106N and donepezil [F(3, 16)=44.24, p<0.001, Fig. 2B]. These results suggest that the ameliorating effect of HX106N, as observed in the present experiments, might be due to its ability to suppress oxidative stress induced by Aβ25-35.

Effects of HX106N on the Expression of TNF-α, IL-1β, and HO-1 in the Hippocampus of Aβ25-35-Injected Mice Inflammatory cytokines, such as TNF-α and IL-1β, have been suggested to mediate the deleterious effects of Aβ, including the inhibition of long-term potentiation and production of long-term memory in a retention trial. The step-through latency time was significantly lower in the Aβ25-35-injected mice than in the control mice (p<0.01, Fig. 1B) and was dramatically increased following the daily administration of 200mg/kg HX106N or 1mg/kg donepezil [F(3, 37)=5.643, p=0.003, Fig. 1B]. The latency times during the acquisition trial were similar across all groups [F(3, 37)=0.364, p=0.779, Fig. 1B]. The effect of HX106N was comparable to that of donepezil. These results demonstrate that HX106N may help ameliorate the memory impairment induced by Aβ25-35 in mice.

Fig. 4. Effects of HX106N on HO-1 Expression and Glutamate-Induced Oxidative Neurotoxicity in HT22 Cells

(A) HT22 cells were treated for 12h with various concentrations of HX106N. Total protein extracts were prepared and subjected to Western blot analysis using specific antibodies for HO-1, β-Actin was used as a loading control. Densitometric quantifications were presented as the mean of three independent experiments. (B) HT22 cells were treated for 9h with various concentrations of HX106N. Total RNA was prepared by northern blot hybridization. GAPDH was used as a loading control. Densitometric quantifications were presented as the mean of three independent experiments. *p<0.05, **p<0.01 vs. untreated group. (C) HT22 cells were treated for 18h with 4mM glutamate and various concentrations of HX106N. Cell viability was determined by the MTT assay. The results are presented relative to untreated control cells. The data are presented as the mean±S.D. of replicate samples from a representative experiment. (D) HT22 cells were treated with 4mM glutamate or with 1mg/mL of HX106N for the indicated times followed by incubation for 30min with 5µM of H2DCFDA. Intracellular ROS levels were measured by flow cytometry. The results are presented relative to untreated control cells. The data are presented as the mean±S.D. of replicate samples from a representative experiment. *p<0.05, **p<0.01 vs. glutamate-treated group.
oxidative damage. The expression of TNF-α and IL-1β in the hippocampus of mice were measured at different time points after the injection of Aβ25-35 peptide. The RNA levels of TNF-α and IL-1β were elevated 2 h after Aβ25-35 injection, remained high on day 1, and returned to baseline levels by day 3 (data not shown). Based on this result, the effect of HX106N on the production of these cytokines was analyzed on day 1. Mice were treated orally with 200 mg/kg HX106N once a day for 7 d and injected with Aβ25-35 peptide. One day later, mice were sacrificed, and quantitative RT-PCR was used to measure the hippocampal levels of TNF-α and IL-1β. As shown in Figs. 2A and B, the RNA levels of TNF-α and IL-1β increased 3.0- and 10.1-fold, respectively, compared to the control mice after Aβ25-35 injection. This increase was not attenuated by treatment with HX106N (p > 0.05, Figs. 3A, B).

Because of the critical roles of HO-1 in the cellular defense responses against oxidative stress,9 the effect of HX106N on the expression of HO-1 was tested. The time course of the RNA level of HO-1 in the hippocampus of Aβ25-35-treated mice is shown in Fig. 3C. When Aβ25-35 peptide was injected, HO-1 expression increased more than two-fold on day 1 but decreased on day 3, and it remained relatively low on day 9. However, in HX106N-treated mice, the rate of decrease in the level of HO-1 expression was much lower, and on day 9, the RNA level of HO-1 was significantly higher than in the Aβ25-35-injected, DW-treated mice (p > 0.05, Fig. 3C). These data suggest that HX106N may upregulate the expression of HO-1, and the antioxidative effect of HX106N on Aβ25-35-induced lipid peroxidation may have resulted from the upregulation of HO-1.

Effects of HX106N on HO-1 Expression and Glutamate-Induced Oxidative Neurotoxicity in HT22 Cells To understand the mechanism underlying the effect of HX106N on HO-1, the murine hippocampal cell line HT22 was treated with various concentrations of HX106N for 12 h. Whole-cell lysates were prepared followed by Western blot analysis to determine HO-1 levels. As shown in Fig. 4A, the background level of HO-1 was very low but was greatly increased by treatment with HX106N in a dose-dependent manner [F(3, 8) = 24.63, p < 0.001]. To determine whether this regulation occurred at the protein or RNA level, total RNAs were prepared from cells treated for 9 h and subjected to northern blot hybridization. Consistent with the Western blot data, a dose-dependent increase in the RNA level of HO-1 was observed in cells treated with HX106N [F(3, 16) = 120.1, p < 0.001, Fig. 4B].

It has previously been reported that a high concentration of glutamate induces cell death via the accumulation of reactive oxygen species. Therefore, we also tested the effect of HX106N on glutamate-induced oxidative neurotoxicity. HT22 cells were treated for 18 h with glutamate and HX106N, and cell viability was measured using an MTT assay. Treatment with glutamate reduced cell viability by 82.0 ± 13.7% (p < 0.001). HX106N inhibited this cytotoxic effect in a dose-dependent manner [F(5, 12) = 549.1, p < 0.001, Fig. 4C]. The half-maximal effective concentration (EC50) value was 627.9 ± 13.7 μg/mL. Treatment with glutamate increased the level of ROS by 6.7-fold at 12 h (Fig. 4D). However, the presence of 1 mg/mL HX106N significantly slowed the rate of ROS accumulation [F(1, 20) = 213.8, p < 0.001; F(1, 20) = 132.0, p < 0.001; F(4, 20) = 40.55, p < 0.001, Fig. 4D]. These data suggest that HX106N protected HT22 cells from the glutamate-induced oxidative neurotoxicity, possibly by increasing the expression of HO-1.

DISCUSSION

In this study, we demonstrated that the botanical formulation HX106N has ameliorating effects on the memory impairment and oxidative stress in Aβ25-35-injected mice. The oral administration of HX106N significantly improved deficits in alternation behavior and passive avoidance, which coincided with a marked reduction in the level of TBARS, an indicator for lipid peroxidation, in the hippocampus and cortex. Aβ25-35 injection itself was found to briefly and transiently increase the level of HO-1 expression as a part of cellular defense mechanisms. Treatment with HX106N appears to maintain the increased level of HO-1 for a relatively long time to provide a protective effect against Aβ25-35-induced oxidative damage. In cell culture, HX106N protected HT22 cells from glutamate-induced oxidative neurotoxicity, and the antioxidative effect of HX106N appears to have resulted from upregulation of HO-1 expression. As the neuroprotective effect of HO-1 has been demonstrated in previous studies, it has been proposed as a new therapeutic target for neurodegenerative diseases. In light of this finding, HX106N might be a useful starting point for the development of appropriate therapeutic agents for various neurodegenerative diseases, including AD.

One of the greatest challenges in the field of botanical extracts is the difficulty associated with preparing such reagents in a reproducible manner due to the lack of understanding of the active compounds. In this study, we attempted to overcome this problem by employing a cell-based bioassay, as described in the materials and methods. The effects of HX106N on NO production, AChE activity, and cell viability following glutamate exposure were used as markers. The IC50 or EC50 values were calculated, and only reagents showing less than 20% variation were used for the experiments. This method appears to be reliable, given that HX106N produced at different times yielded reproducible results in both the in vitro and in vivo experimental systems.

The safety of the plants used for the preparation of HX106N has been established through a long history of human use. Indeed, no toxic effects of HX106N have been observed in acute or repeated-dose toxicity studies involving rats and dogs (unpublished data). Taken together, our results demonstrate that HX106N has the potential to act as a preventive and/or therapeutic agent for AD.

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Conflict of Interest Seon-Hee Kim is an employee of ViroMed, Co., Ltd. Sunyoung Kim hold stocks of ViroMed,
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