The Korean J. Anat. 37(6), 519-527, 2004

The Role of BF-7 on Enhancement of Memory And Cognitive Function

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< Abstract >

Various factors such as senescence, stress, neurodegenerative diseases including Alzheimer's disease (AD) contribute to the impairments of organs, especially brain. Also, they should be negative factors on normal brain function, like as memory and cognition. In this study, the neuroprotective role of BF-7, extracted from *Bombyx mori*, was examined agaist scopolamine-induced neurotoxicity in SK-N-SH cells. In order to know if the BF-7 has positive role on the cognition and memory, we examined using SD rat model and human. Scopolamine-induced memory impairments were observed, as measured by the passive avoidance and water maze tests, but treatment with BF-7 significantly improved memory and cognitive function. Moreover, the memory index and memory preservation of clinical experiments using MMSE-K tests were significantly improved memory and cognitive function. This results strongly represent that the BF-7 play effectively positive role in the improvement of brain function including learning and memory. Taken together, our results suggested that the BF-7 should be useful for developing strategies protecting nervous system and improving brain function.

Key words : Memory, Cognitive function, BF-7, Scopolamine, Water maze, MMSE-K

Introduction

With rapid changes in modern society, the amount of information to acquire is also increasing. Especially, needs for maximizing brain function for adolescents and adults are rising. In addition, dementia in which learning and memory abilities are first affected is becoming financial and social problems in modern society, with the increase of aging population. The prevalence of dementia is currently estimated to be approximately 10 % in the elderly people above 65 years and 50% in the elderly people above 80 years and it is expected that the increase will continue in the future due to aging population. The most common cause of dementia is known to be Alzheimer's disease (Selkoe, 1993; Hendrie, 1997). It is estimated that about 4 million people in the United States currently suffer from this condition (Launer *et al.*, 1999; Lobo *et al.*, 2000) and this figure will exceed 14 million by 2040, becoming a major cause of death. Therefore, it is the most problematic incurable disease that we have to cope with.

It is known that memory impairment and cognitive dysfunction, the first symptoms of dementia are closely associated with the cholinergic system. Scopolamine (the antagonist of muscarinic receptor at the postsynapse) temporally interrupts information transfer by interfering acetylcholine (the neurotransmitter diffused from the presynapse) from binding to muscarinic receptors. Thus, scopolamine is usually used to develop animal models of memory dysfunction for researches on the improvement of learning and memory (Shudo *et al.*, 2004; Tanabe *et al.*, 2004).

In recent years, a number of studies have been conducted to develop management strategies and effective therapeutic agents that improve cognitive dysfunction and deteriorated learning ability induced by dementia. Agents developed to date include acetylcholine precursors, receptor agonists and acetylcholine esterase inhibitors

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(AchEIs) but the use of these agents is controversial because their effects are temporary and unsatisfactory and severe toxicities have been reported. (Amenta *et al.*, 2001; Fayuk and Yakel, 2004; Liu and Zhao, 2004). Thus, development of effective agents for memory impairment and dementia with minimal side effects that can improve brain lesions is needed.

With the intention to develop agents with low toxicity that strongly enhance the cholinergic system and improve learning and memory impairments by supplementing mentioned weaknesses, this study evaluated the suppression effects of BF-7, extracted from *Bombyx mori* (Chae *et al*., 2004), on the cell death of SK-N-SH (a human neuroblastoma cell line) and neuronal cell death induced by scopolamine in animal models as well as its effects on the improvement of memory and cognitive function. Furthermore, clinical effects of BF-7 on the improvement of cognitive function were evaluated with randomly selected populations with various backgrounds.

Materials and Methods

1. Cell culture

SK-N-SH, a human neuroblastoma cell line, was cultured in RPMI 1640 medium (Life Technologies, USA) with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies) at temperature 37℃, humidity 95% and CO_2 5%. Two hours prior to the $FeSO_4$ (Sigma, USA) treatment, the culture medium was changed to the medium with 1% FBS and SK-N-SH was pretreated to evaluate cellular protective effects of BF-7.

2. Preparation of animal models and breeding conditions

Eight-week-old healthy male Sprague-Dawley rats weighing 200-250g were used for this study. A week of adaptation period was allowed and rats with abnormal behaviors were excluded from this study. All the rats were kept in a room with accurate control of temperature and humidity and regular cycles of 12/12 hour light and dark. Water and food were supplied at all times. Rats were handled and treated according to NIH guideline. A total of 30 rats were used and 10 rats were assigned to each groups; the control group, the scopolamine(Sigma, USA) group, and the scopolamine + BF-7 group.

3. Preparation of materials

BF-7 used for cognitive function test was supplied from rural development administration. BF-7 was separated and purified from *Bombyx mori* and used after being dissolved in normal saline.

4. Animal models of learning and memory impairments

When scopolamine, the antagonist of muscarinic receptors, is administered to rats, learning and memory impairments are induced because binding of acetylcholine (neurotransmitter) diffused from the presynapse to muscarinic receptors from the postsynapse is temporarily interrupted. With this principle, animal models of memory impairment are usually developed for researches examining learning and memory improvement.

5. Human subjects

Subjects were voluntarily recruited among elderly people who visit a day care center in Seoul dementia. The following volunteers were excluded from this study; 1) one receiving treatments for any diseases, 2) one who took medications that may affect cognitive function within 4 weeks prior to the clinical trial, 3) one who had health functional foods that may affect cognitive function within 4 weeks prior to the clinical trial, 4) one who has a difficulty in having an everyday conversation, 5) one who cannot read or see pictures due to visual impairment, 6) one who cannot freely write due to physical disability, 7) one who was judged to be inappropriate to participate in the clinical trial. A total of 30 people were initially enrolled but 5 people were dropped during the study and 25 people were included in the analyses. The characteristics of the subjects were demonstrated in Table 1.

Table 1. Characteristics of volunteers

	Characteristics
Age	72±5.1*
Education (year)	6.8±4.7*
Number of Male	9
Number of Female	16

* expressed as mean and S.D.

6. Cell viability (MTT assay)

For the evaluation of cell viability in this study, we slightly modified the previously reported MTT reduction assay method (Kim SS et al., 2002). The cultured cells were treated with scopolamine and incubated at 37°C with 5% CO_2. After 48 hours, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) was added to each well until the concentration reached to 0.5 mg/ml and incubated for another 4 hours and a half. The formazan precipitate produced by MTT reduction was dissolved in a solution (0.1N HCl in absolute isopropanol) and absorbance was measured at 570 nm using an ELISA reader. Each sample value was relatively

given; the control value produced by the addition of the solvent was 100 % and the level of MTT reduction when cells were completely destructed by 0.9% Triton X-100 was 0%.

7. Passive avoidance test

Passive avoidance test is a widely used tool to assess the working memory ability of rodents (Van der Zee EA. et al., 2004) and has been adopted in many studies on learning and memory impairments. The experiment was conducted as followed. For the test, a shuttle box (50×15×40cm, electric grid floor) divided into two rooms (25×15cm) by a connecting guillotine door (10×10cm) was used. Each room was lighted with a 20W bulb. The noise level was kept under 60dB and the laboratory light was dimmed. BF-7 (10 mg/kg, p.o) that strongly suppresses the activation of acethylcholin esterase (AChE) was administered to the rats and the improvement in scopolamine-induced amnesia was evaluated by comparing with the control group via in vitro test. BF-7 was dissolved in 5% DMSO (10 mg/ml) and orally administered to the rats weighing 200-250 g (10 mg/kg). After 30 min, scopolamine dissolved in normal saline (1 mg/kg) was administered and foot-shock was given in the shuttle box after another 30 min. Rats were first placed in one of the two rooms divided by the connecting guillotine door (A) and the door was opened as a 1500 Lux light was turned on to the room. When the rats moved to the other room without a light (B), the door was automatically closed. The latency from the opening of the connecting guillotine door to its closing was checked. When this was repeated 5-6 times and the rats successfully managed to move from room A to room B within 20 seconds with very few exceptions. On the 6^{th} trial, when the rats moved to room B, the room was kept dark and foot-shock was given by running through 3 mA current to the stainless grid under the floor for 3 seconds. The rats would relate the dark room with the foot-shock. After 24 hours, when they were placed in the room A, they would hesitate to move from room A to room B even if the light in the room A was turned on. The latency during this time was compared with that of the scopolamine group.

8. Water maze test

Water maze test is a widely used tool to evaluate spatial learning and memory using rodents. The water maze and a data analyzer (Ethovision, Netherland) are needed for the test. The test was performed as follows. A circular pool with a diameter of 140 cm and a depth of 45 cm was 70% filled with water and milk was added to disturb the vision. A circular platform with a diameter of 15 cm and a height of 35 cm was submersed 1.5 cm under the water surface. The water temperature was kept between 21-32 ℃. The room light was dimmed and rats were monitored with a video camera under the 50W light.

- **Training trial**: The white rats were placed in the pool and the time consumed to get to the platform was checked for. The maximum time allowed for them was 90 seconds. If the rats failed to reach to the platform within 90 seconds, they were removed from the pool and left on the platform for 15 seconds. After 30 seconds, they were placed in the pool again and the time consumed to get to the platform was recorded. This trial was repeated two times a day (one in the morning and one in the afternoon) and when the rats succeeded to get to the platform within 15 seconds, it was considered that training trial was completed.
- **Test trial**: Once training was completed, the rats were assigned to the PBS group(the control group), the scopolamine group (only scopolamine 1 mg/kg was administered) and the scopolamine + BF-7 group (both scopolamine and BF-7 (10 mg/kg) were administered) and escape time was checked. The escape time was checked 2 times a day for 5 days by the same method used for the training trial and the maximum time limit was 90 seconds.

9. Clinical trial

A clinical trial was conducted as a double blinded, placebo-controlled study and MMSE-K was used as an assessment tool. The subjects were randomized into the placebo group and the experimental group, and the trial was conducted for 3 weeks. A total of four capsules of BF-7, two capsules of BF-7 (100 mg BF-7 per capsule) in the morning and in the evening were administered. The subjects were instructed to keep their usual diet and smoking habit but alcohol was prohibited. The effects of BF-7 was evaluated using MMSE-K prior to BF-7 intake and 3 weeks after BF-7 intake. MMSE-K is the most widely used tool for quantitative evaluation of cognitive function. This tool is also useful to monitor changes of cognitive function with repetitive measurement. In this study, cognitive function was assessed by evaluating orientation, memory registration, memory retrieval, attention, concentration, calculation, spatial and temporal organization and linguistic ability.

Trial monitoring staff monitored changes of the subjects' weight, alcohol intake, changes in diet, the presence of side effects, enthusiasm of the subjects and the compliance on weekly basis.

10. Statistical analyses

All statistical analyses were performed using SPSS

program and p <0.05 was considered statistically significant. Data of cellular experiment was presented as means ± standard deviations, and t test was used to examine correlations and differences between the groups. In the clinical trial, student's t-test or one-way ANOVA (Tukey's multiple comparison test) was used to compare statistical significance for the difference in cognitive function improvement among the groups.

Results

1. Suppression effects of BF-7 on cellular damages in SK-N-SH cell death induced by scopolamine

Cell death of SK-N-SH was induced once they were treated with cytopermeable scopolamine (10 μM). Cellular morphology was examined to identify the patterns of SK-N-SH cell death induced by scopolamine. Observation of changes in cellular morphology for 24 hours with a phase contrast microscope revealed typical apoptosis such condensation and segmentation of the cell body, the loss of neuritis, membrane blebbing (Fig. 1B). On the other hand, apoptosis was suppressed by BF-7 when the cells were pre-treated with 10 μM BF-7 two hours before and cellular damages were also hardly observed, remaining alike to that of the control group(Fig. 1C). To quantify the cell death induced by scopolamine, MTT reduction assay was performed. The cell viability after 24 hours was about 50 % comparing to that of the control group when the cells were treated with 10 μM scopolamine but the cell viability increased above 90 % when the cells were pre-treated with 10 μM BF-7 two hours before(Fig. 1D).

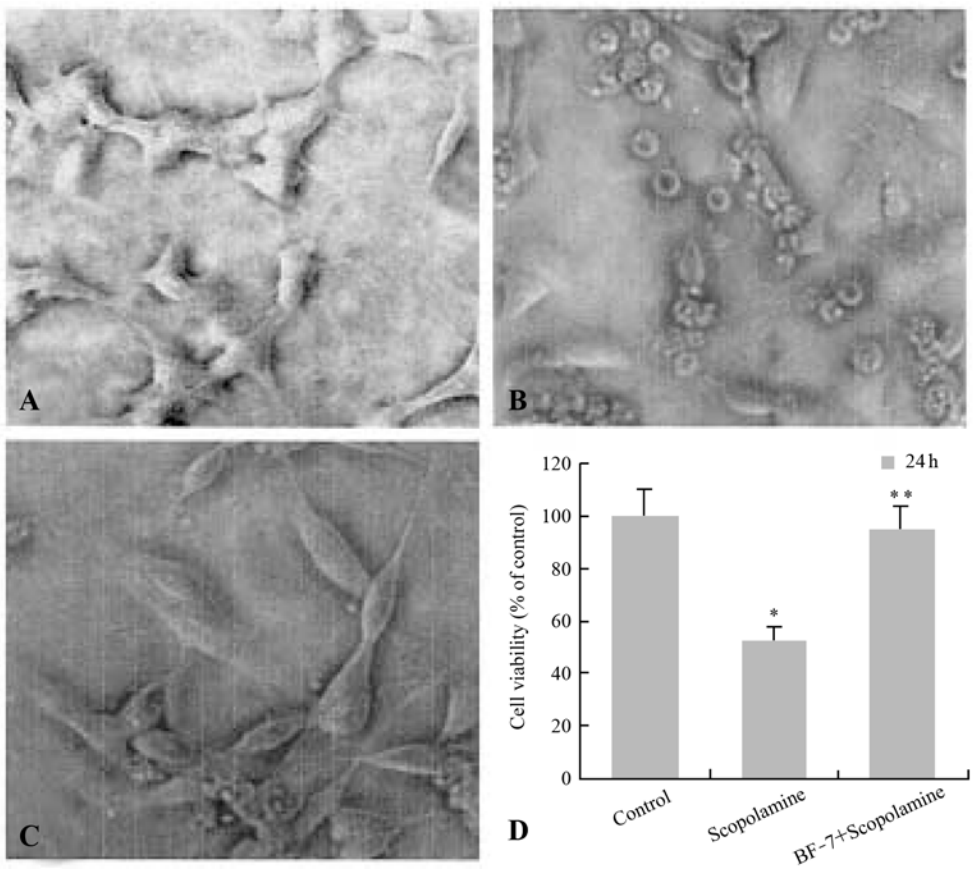

Fig. 1. The effect of BF-7 on scopolamine-induced neuronal cell death in SK-N-SH neuroblastoma cells. SK-N-SH cells were pretreated with 10 μM of BF-7 for 2 h and then treated with 10 μM of scopolamine. SK-N-SH cells were untreated control (A) or treated with 10 μM of scopolamine for 24 h (B). Pre-treatment of SK-N-SH cells with 10 μM of BF-7 were treated with 10 μM of scopolamine for 24 h (C). BF-7 and scopolamine were dissolved in PBS. The figures were shown that Light microscopic morphology and are representative for three different experiments. Cell viability was determined by MTT assay at 24 h after 10 μM of scopolamine treatment (D). *, $p < 0.05$ from control; **, $p < 0.05$ from scopolamine.

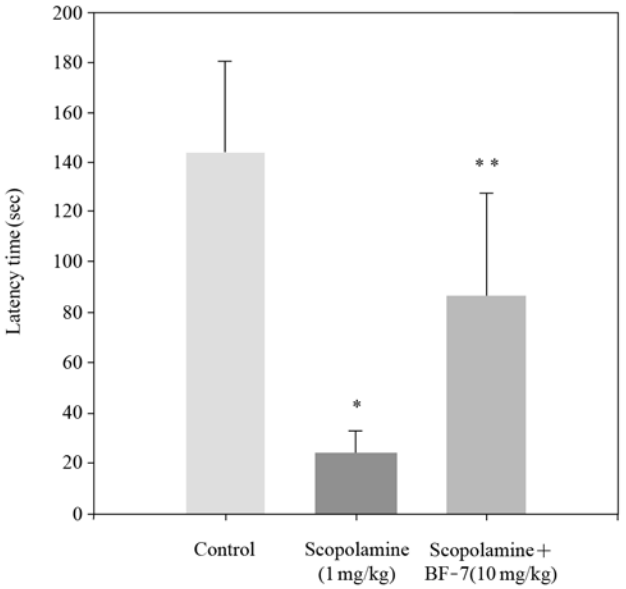

Fig. 2. The effect of BF-7 on the performance of scopolamine-treated mice in the retention trial of the passive avoidance task. The task was performed on days 3∼4 after the i.c.v. ingection of scopolamine. Each value is the mean±S.E.M. of 10 animals. *, $p<0.01$ vs. control group; **, $p<0.001$ vs. scopolamine only by one-way ANOVA.

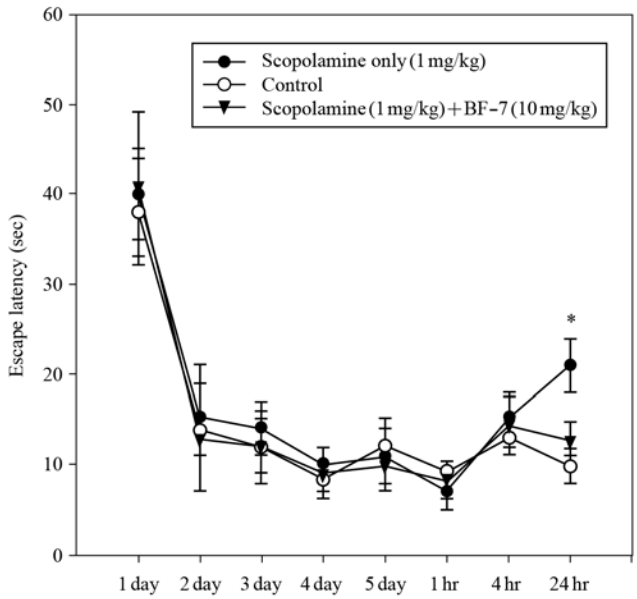

Fig. 3. The positive effect of BF-7 on the performance of scopolamine -treated mice in the working memory trial of the water maze test. The training traials were carried out on days 3∼6 (four per day) and the working memory trials (five per day) were carried out on days 8∼10 after the i.c.v. injection of scopolamine. Each value is the mean±S.E.M. of 10 animals. The statistical significance of the working memory trials were calculated using one-way ANOVA. *, $p<0.05$ vs. control and scopolamine+BF-7 (10 mg/kg).

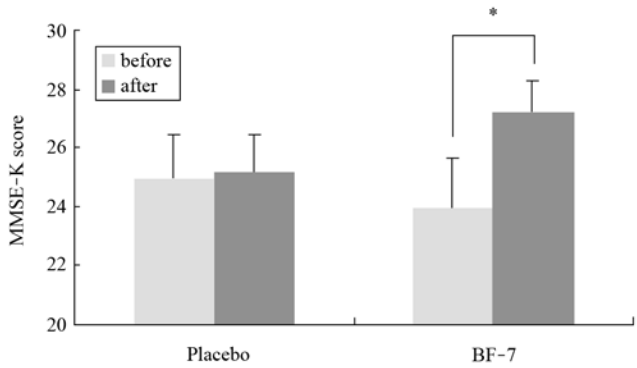

Fig. 4. Improvement effects of BF-7 on cognitive function. Volunteers were randomly divided into placebo and BF-7 group. MMSE-K tests were performed again to evaluate chages after administration. The values were presented as mean±S.E.M. The student's t-test was used to analyze the relationship between the different variables. * indicates statistically significant ($p<0.05$).

2. Improvement in learning and memory impairments (evaluation using animal models of memory impairment)

2-1. Passive avoidance test

The suppression effects of BF-7 was examined with animal models of memory impairment developed by a single administration of scopolamine (1 mg/kg) using passive avoidance test, a memory evaluation tool for animal models. The latency time was reduced by scopolamine administration(1 mg/kg) with a statistical significance (*, $p < 0.05$ vs. the control group), comparing to the positive control group. But, passive avoidance test confirmed that the latency time was recovered to the normal level by the single dose of oral administration of BF-7 (10 mg/kg) (**, $p < 0.05$ vs. the scopolamine group) (Fig. 2). A longer latency time means the restoration of learning and memory impairments. This implies that BF-7 actually gets into the brain and enhances the actions of the neurotransmitter, acetylcholine (ACh), improving learning and memory although further studies to identify pharmacological mechanisms of BF-7 is required.

2-2. Water maze test

In addition to the improvement of simple memory confirmed by passive avoidance test, the effects of BF-7 on learning and memory were examined using water maze test which can evaluate a higher level of memory (spatial memory). After repetitive training trials which were conducted two times a day (more than 12 rats per group), most rats were able to reach to the platform within 10 seconds from the 5th day. This means that there

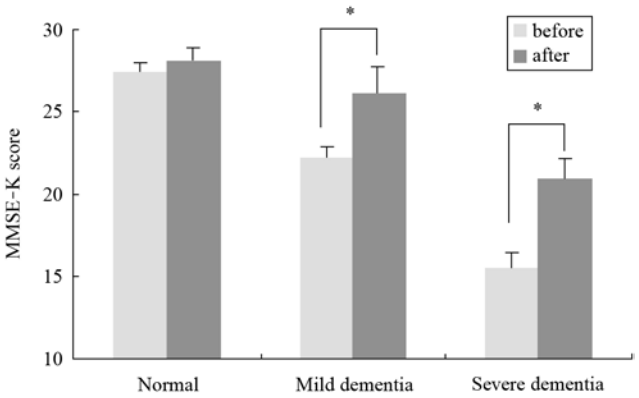

Fig. 5. Higher improvement of cognitive function in severe dementia by BF-7. Examinees were divided into three groups, normal (>25), mild dementia (20~25), and severe dementia (<20) by first MMSE-K test score. Indication was two capsules b.i.d., p.o., three weeks and MMSE-K tests were performed again to evaluate chages after administration. The values were represented as mean±S.E.M. The student's t-test was used to analyze the relationship between the different variables. * indicates statistically significant ($p<0.05$).

is no deficit in learning ability of the white rats. In this study, most of the rats successfully reached to the platform within 10 seconds on the 5th day of learning trials. The rats were given either scopolamine (1 mg/kg) alone or both scopolamine (1 mg/kg) and BF-7(10 mg/kg), and latency time was recorded at 1 hour, 4 hours and 24 hours after the administration. In the scopolamine group, the latency time increased after 1 hour and was not recovered. On the other hand, in the group both scopolamine and BF-7 were given, the latency time tended to increase up to 1 hour after the administration but was recovered to the normal level between 4 ~ 24 hours (Fig. 3). In addition, there was a statistically significant difference in the latency time after 24 hours between the scopolamine group and the BF-7 group (*****, $p < 0.05$ vs. the scopolamine group by one-way ANOVA test). This implies that BF-7 enhances the action of acetylcholine in the brain and suppresses spatial memory impairment.

3. Effects of BF-7 on cognitive function in the clinical trial (MMSE-K test)

A dementia assessment tool, MMSE-K, evaluating memory, attention, concentration, calculation, linguistic ability and spatial organization was used to examine the effects of BF-7 on the improvement of cognitive function in this study. Dementia was significantly improved after taking BF-7. Whereas there was no difference in the MMSE-K score of the control group, that of the experimental group improved 13 % in average (*****, $p <0.05$) (Fig. 4). This improvement in cognitive function was more apparent in the subjects with severe dementia (Fig. 5). In the subjects with mild dementia, the improvement was 17.3% in average and in the subjects with severe dementia, it was 35% in average (*****, $p <0.05$).

Discussion

Learning and memory functions are the indications for the effective functioning of the brain. Damages in the neuronal cells of the brain lead to learning and memory impairments. Thus, discovering methods to improve brain function has a medical value because it can also protect the brain. Furthermore, with rapid development of modern society and needs for abundant amount of information acquisition, effective improvements of the brain function are needed for adolescents who have to digest abundant amount of educational materials and adults with active social activities. Besides, excessive stress that is expressed in various forms becomes the main cause of diseases related to brain function as the age of limitless competition begins. The prevalence of dementia is currently estimated to be approximately 10 % in the elderly people above 65 years and 50% in the elderly people above 80 years and it is expected to be increasing in the future due to aging population.

There is abundant evidence that memory impairment and cognitive dysfunction, the first symptoms of dementia, are closely associated with the cholinergic system. For instance, when scopolamine, a muscarinic receptor antagonist, was administered, short-term memory impairment is induced in a healthy person and memory impairment is aggravated in patients with Alzheimer's disease. Furthermore, the number of neuronal cells in the neucleus basalis of Meynert which is the major source of cholinergic innervations of the cerebral cortex is rapidly reduced and the uptake of choline and the composition of acetylcholine are reduced in the hippocampus and cerebral cortex, resulting in the rapid reduction of cholineacetyltransferase (ChAT) activity. In addition, the numbers of nicotinic and muscarinic receptors are also reduced.

Efforts to develop agents for cognitive dysfunction are being made by supplementing and improving the malfunctioning cholinergic system which is closely associated with memory as described. Agents developed to date include acetylcholine precursors, receptor agonists and AChEIs but the use of these agents remain controversial because their effects are temporary and unsatisfactory and they have severe toxicities. Domestically and internationally used agents for memory impairment today include anti-cholinergic agents that control symptoms, metabolic drugs that non-specifically

accelerate metabolism and blood circulation agents that improve blood circulation. However, effects of these drugs have temporary and unsatisfactory. In addition, they induce side effects such as nausea, vomiting, bronchocontriction, depression, insomnia, hypertension and constipation because they stimulate the parasympathetic nerve. Thus, development of effective agents for memory impairment and dementia with minimal side effects that can improve brain lesions is needed. However, researches on the causes of impaired brain function resulted from aging and stress and the development of agents for learning and memory impairments resulted from degenerative brain diseases such as dementia are focused on the supplement of malfunctioning cholinergic system. Systemic treatment with currently used AChEI can cause serious side effects due to the parasympathetic nerve stimulation because they suppress AChE both from the central nerve system and the peripheral nerve system non-specifically. Therefore, efforts are devoted to make more specific agents for AChE from the central nerve system by modifying the structures of existing AChEI or to develop new AChEI from natural substances and use in the clinical practice after toxicity test. Hence, developing agents with minimal toxicity that strongly enhance the cholinergic system and improve learning and memory impairments is a meaningful task.

Recent studies suggested that BF-7 extracted from *Bombyx mori* has a neuroprotective effect. In this study, suppression effects of BF-7 on cell death in SK-N-SH and neuronal cell death induced by scopolamine in animal models were examined. And if BF-7 has suppression effects on cell death, whether BF-7 improves memory impairment and cognitive dysfunction in the animal models of brain dysfunction induced by scopolamine was also evaluated. In addition, this study also aimed to clinically evaluate the positive roles of BF-7 for brain function such as learning and memory in randomly selected population. As shown in results, BF-7 effectively inhibited neuronal cell death induced by scopolamine and maintained the cell viability, leading to effective protection of neuronal cell damages. The protection mechanisms of BF-7 for neuronal cell death induced by scopolamine may be induced by various factors involving in cellular stress and cell death and should be additionally investigated with intracellular studies.

Moreover, in this study, passive avoidance test and water maze test were performed with animal models of memory impairment (developed using scopolamine, the muscarinic receptor antagonist) to examine whether BF-7 actually improves memory impairment. The results suggested that orally administered BF-7 effectively improves memory and spatial perception ability. This implies that BF-7 passes through the blood brain barrier and improves the function of cholinergic system in the brain. There were no side effects or toxicities with the therapeutic dose.

Cognitive function was tested using MMSE-K which is the most widely used tool for quantitative evaluation of cognitive function. This tool is also useful to monitor changes of cognitive function with repetitive measurement. In this study, cognitive function was assessed by measuring orientation, memory registration, memory retrieval, attention, concentration, calculation, spatial and temporal organization and linguistic ability. MMSE-K results suggested that BF-7 improved dementia; there was no difference in the MMSE-K score of the placebo group prior to BF-7 administration and post to BF-7 administration but that of the experiment group was significantly improved. Moreover, this improvement in the cognitive function was more apparent in the severe dementia group. Comparative analysis of MMSE-K scores for each item in the subjects who made improvements showed that the improvement were made in all the areas including memory, attention, concentration, calculation, temporal and spatial organization and linguistic ability rather than occurred in a specific area.

These results suggest that BF-7 is not only effective to improve memory of high school students but also effective to improve complex cognitive function in the elderly people with dementia. It is thought that the role of BF-7 in removal of reactive oxygen that continuously damages the brain also contributed to this improvement of brain function. Thus, BF-7 improves cognitive function including memory by ultimately suppressing the neuronal cell damages and cell death. Nevertheless, other protective mechanisms are thought to be involved in the improvement of brain function. Further studies on the protective mechanisms of BF-7 at the molecular level will have a medical significance in the view of brain protection and function and will provide valuable information for the prevention and treatment of dementia.

In conclusion, BF-7 is a valuable substance that is worth investing to develop an effective and safe agents that improves learning and memory by reinforcing the cholinergic system of the central nerve system.

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— **Abstract** —

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