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Withdrawal:

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Title: Neuroprotection and Enhancement of Learning and Memory by BF-7
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Authors' Comments:

We deeply regret that part of the experimental data we previously published in the Korean Journal of Physiology and Pharmacology, 8, 173-179, August (2004) was included in the above-mentioned article by mistake.
Therefore, we would like to withdraw this article written by Dao Kyong Kim, Yong Koo Kang, Moo Yeol Lee, Kwang-Gill Lee, Joo-Hong Yeo, Won Bok Lee, Yong Sik Kim, and Sung Su Kim from the Journal of Health Science.

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Editorial Decision:

The Editorial Committee decided to approve the authors' request to withdraw this article.

Editorial Committee of Journal of Health Science (September 15, 2009)

Neuroprotection and Enhancement of Learning and Memory by BF-7

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To investigate whether BF-7 improves the learning and memory of normal people, the Rey-Kim Memory Test was performed in a placebo group (32 persons), BF-7 200 mg group (33 persons), and BF-7 400 mg group (34 persons). BF-7 significantly enhanced learning and memory function in a dose-dependent manner. Our results show that BF-7 protects neurons from amyloid beta (Aβ) toxicity and reduces oxidative stress. We measured the levels of acetylcholine in the brain in a memory impairment animal model. Administration of BF-7 significantly increased acetylcholine levels. It is assumed that the protection of neurons and maintaining the appropriate acetylcholine levels are mechanisms by which BF-7 improves brain function. The results suggest that BF-7 is effective in improving learning and memory ability.

Key words —— learning, memory, BF-7, acetylcholine, neuroprotection, amyloid beta

INTRODUCTION

Each neuron is in synapse contact through its processes with other neurons, so that each neuron is a segment in the network of which the nervous system is composed. Functional or physiologic damage of a neuron induces impairment of brain functions like learning, memory, *etc*.[1,2] There are many causes of neuronal damaged, for example, endogenous factors like reactive oxygen species (ROS) generated naturally during energy metabolism,[3,4] abnormally regulated calcium,[5,6] accumulated toxic substances like amyloid beta (Aβ),[7] and exogenous toxious materials.[8–10] Thus the brain ordinarily receives chemical, environmental, and psychological stress, which leads to decline of brain function.[11–13]

It is well known that Aβ induces neuronal damage, leading to learning and memory impairment. Although the precise molecular mechanism has not been identified, Aβ-induced apoptotic neuronal cell death is believed to be one of the major reasons for failing memory.[14] Several compounds protect neurons from Aβ damage, including peptide aggregation-blocking agents,[15] neurotrophic factors,[16] and antioxidants.[17,18]

The discovery of natural agents with positive effects on cognitive function and no side effects is important. Previous agents for the improvement of cognition all have side effects such as nausea, vomiting, *etc*.[19] Recently, it has been reported that the BF-7, a protein in the natural extract from *Bombyx mori*, exerted significant neuroprotection, functionally and physiologically.[20] For example, Lee *et al*. showed that BF-7 protected SK-N-SH cells, human neuroblastoma cells, from reactive oxidative stress.[21] Kim *et al*. reported improved cognitive function after intake of BF-7 for 3 weeks in 25 normal elderly individuals.[22] It has been suggested that cholinergic neurons are closely related to cognitive functions like learning and memory.[23,24] Thus the protection of neurons and/or maintaining acetylcholine levels may be used one method for improving cognitive function.[25] Many studies on neuroprotective and anti-aging agents have been performed. Methods for improving brain function have also been studied. However, so far, there is no effective strategy improving learning and memory or protecting the ner-

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vous system. Finding effective agents to improve brain function is thus important in many ways.

In this study, we attempted to determine whether BF-7 is effective in preventing Aβ-induced neuroal damage and improving the learning and memory function of normal individuals.

MATERIALS AND METHODS

Materials —— Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, U.S.A.). Aβ, sodium carbonate (Na_2CO_3), marceillous soap, trypsin, α-chymotrypsin, flavouzyme, Hoechst dye 33258, and fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCF-DA) were purchased from Sigma (St. Louis, MO, U.S.A.). Alkalase, Neutrase, Protamex, Ceramix and Sumizyme LP were generous gifts from Novo Nordisk Pharma Korea (Seoul, Korea). The gel-filtration Sephadex column was purchased from Pharmacia (Sweden). AlamarBlue was purchased from Serotec (Oxford, U.K.).

Preparation of BF-7 —— The preparation of BF-7 followed the previous method.[26] In brief, *Bombyx mori* and its cocoon were boiled in solution of 0.03% sodium carbonate and 0.05% marceillous soap for 30 min and dried. It was dissolved with $CaCl_2$ 5 M solution at 98°C for 1 hr. The salt was completely removed by dialysis against distilled water. It was then mixed with mixed proteases solution, followed by separation and purification of the protein portion (M.W. 500–5000 range) using Sephadex G-25 gel-filtration chromatography.

Experimental Animals —— Male Sprague-Dawley rats, 8 weeks old (around 200–250 g), were purchased from Korean BioLink Co. (Chungbuk, Korea). After 1 week for adaptation, the 28 rats were divided into 4 groups of 7 rats each: vehicle control group treated with phosphate-buffered saline (PBS); Aβ (2 nmol)-treated group to lower cognitive function; Aβ (2 nmol) treated with BF-7 (5 mg/kg) group; and Aβ amyloid (2 nmol) treated with BF-7 (10 mg/kg) group.

Healthy Volunteers —— Volunteers covered all adult ages from 19 to 64 years. It included individuals in their 20–30s who show good memory and learning ability, in their 30–40s whose memory has started to decrease , and those in their 50–60s who have a certain degree of decline in memory. This volunteer group was considered to represent the general population. Volunteers were excluded from the study if they were receiving medication and/or functional food, had neuropsychiatric problems, had any serious disease, had neurodegenerative disease, or were deemed not suitable for participation. Written informed consent was obtained from all volunteers. 119 examinees were randomly divided into three groups: 39 in the placebo group, 40 in the BF-7 200 mg group, 40 in the BF-7 400 mg group. The final analysis included 32 in the placebo group, 33 in the BF-7 200 mg group, and 34 in the BF-7 400 mg group, because only 99 examinees completed the test.

Cell Culture —— Human neuroblastoma SKN-SH cells were cultured at 37°C in DMEM supplemented with 10% heat-inactivated FBS in a humidified 95% air, 5% CO_2 incubator. The cells were transferred to low-serum media (1% FBS) 2 hr prior to BF-7 treatment.

Cell Viability Assay —— The cells were plated on 96-well plates (Corning, NY, U.S.A.) at a density of 5×10^4 cells/well in 100 μl of 10% FBS/DMEM and incubated for 24 hr. Two hours before the Aβ treatment, the medium was replaced with 90 μl of 1% FBS/DMEM. After the treatment, 10 μl of alamarBlue was added aseptically. The cells were incubated for 3 hr and the absorbance of the cells was measured at a wavelength of 570 nm using an ELISA Reader (Molecular Devices, CA, U.S.A.). The background absorbance was measured at 600 nm and subtracted. The cell viability was defined as [(test sample count) – (blank count) – (blank count)/(untreated control count) – (blank count)] $\times$ 100.[27]

Hoechst 33258 Staining —— The SKN-SH cells were fixed with 4% paraformaldehyde for 20 min and stained with 8 μg/ml of Hoechst dye 33258 for 5 min. They were washed twice with PBS and analyzed under fluorescent microscopy (Olympus IX 70, Tokyo, Japan). The dead cells were characterized by their fragmented nuclei, and the apoptotic morphologic changes were characterized by chromatin condensation and the formation of apoptotic bodies.

Determination of ROS Generation —— Hydrogen peroxide generation induced by Aβ was measured by incubation with a DCF-DA. The SKN-SH cells were stained with 10 μM of DCF-DA for 30 min. The cells were then collected, and washed twice with PBS. They were then placed on slide-glasses and mounted. Photomicrographs of the mounted cells were taken with a fluorescent microscope equipped with a UV supply system (Olympus

IX 70). Cells stained with DCF-DA were incubated with 100 μl of lysis buffer for 5 min on ice and then measured with excitation at 485 nm and emission at 530 nm with a fluorometer (TECAN, GENios, Maennedort, Switzerland). The intensity of ROS was expressed as an arbitrary unit of relative value.

Animal Model for Cognitive Malfunction —— Aβ 2 nmol was injected into the region of the hippocampus. In group 1, the vehicle control group, PBS was injected into the ventricle and PBS was fed orally for 2 weeks, in group 2, Aβ 2 nmol was injected into the ventricle and PBS was fed orally for 2 weeks, in group 3, Aβ 2 nmol was injected into the ventricle and BF-7 5 mg/kg was fed orally for 2 weeks, and in group 4, Aβ 2 nmol was injected into the ventricle and BF-7 10 mg/kg was fed orally for 2 weeks.

Determination of Acetylcholine Concentration —— The acetylcholine level in the brain was determined using the chemiluminescence method.[28] This method was based on the reaction of hydrolysis of acetylcholine by acetylcholinesterase and conversion reaction of choline with H_2O_2 into betaine. The composition of the reaction reagent was 10 ml of 0.2 M sodium phosphate buffer (pH 8.6), 25 μl of choline oxidase (100 units/ml in water), 10 μl of peroxidase type II (2 mg/ml in water), and 50 μl of luminol. Brain tissue (100 mg) was immersed and extracted in 5% trichloroacetic acid (TCA) solution. After centrifugation, it was extracted with 5–10 ml of ether, and the ether was discarded. Then ether extraction was performed an additional 5–6 times, and 20 μl of 0.5% sodium metaperiodate was added to 200 μl of the aqueous layer. Two hundred microliters of sample was mixed with 285 μl of reaction reagent, and the intensity of chemiluminescence was measured.

Clinical Experiments —— Volunteers were randomly distributed into the placebo and test groups in sequence. The test was performed in double-blind manner. Before taking the test material (BF-7), a baseline assessment (vital signs, weight, general health) and the Rey-Kim Memory Test were performed. Then volunteers received two capsules of BF-7 b.i.d., *p.o.*, for 3 weeks. The baseline assessment and Rey-Kim test were performed again to evaluate changes after administration. During the study period, volunteers were instructed to continue their usual meal and smoking patterns, but drinking alcohol was prohibited.

Rey-Kim Memory Test —— All volunteers were administered the Rey-Kim Memory Test[29] to evaluate enhancement after administration of BF-7. In the Rey-Kim Memory Test, verbal memory performance is assessed in the K-Auditory Verbal Test (KAVLT), a Korean version of the Rey Auditory Verbal Learning Test,[30] and nonverbal memory performance in the K-Complex Figure Test (KCFT), a Korean version of the Rey Complex Figure Test.[31] The KCFT is essentially identical to a standard version of the Rey Complex Figure Test.[32] All KCFT tests were scored according to the standard 36-point scoring system.[32]

Statistical Analysis —— The data are expressed as a mean ± SEM. The paired *t*-test was used to analyze the relationship of scores between scores before and after test agent administration. One-way of analysis of variance (ANOVA) (Tukey post-hoc comparison test) was used to analyze the relationship of difference of scores in the placebo, BF-7 200 mg, and BF-7 400 mg groups.

RESULTS

BF-7 Inhibited Apoptotic Cell Death Induced by Aβ

The Aβ-induced morphologic characteristics of apoptotic cell death was observed under phase-contrast (Fig. 1a–1c) and staining fluorescence microscopy with Hoechst 33258 (Fig. 1d–1f), respectively. Exposing the SKN-SH cells to Aβ 20 μM for 24 hr gave rise to membrane blebbing, cell shrinkage, and increased number of apoptotic nuclei with Hoechst 33258 staining. Positive staining indicates nuclear condensation and DNA fragmentation. BF-7 treatment prior to Aβ was found to block the morphologic and apoptoptic characteristics observed when the cells were exposed to Aβ alone (Fig. 1d–1f). As shown in Fig. 1g, Aβ 20 μM induced approximately 40% neuronal cell death for 24 hr. Treatment with BF-7 alone had no effect on cell viability. Pretreatment with BF-7 attenuated Aβ-induced apoptosis and cell viability was similar to that in controls.

BF-7 Decreased ROS Generation Induced by Aβ

Oxidative stress is the main factor in neuronal damage and aging. The level of ROS was measured using DCF fluorescence detection because the level of DCF fluorescence is an indicator of ROS production. Strong fluorescence was detected in Aβ 20 μM-treated cells. The level of fluoroscence was greatly reduced by pretreatment with either BF-7 (Fig. 2c) or *N*-acetyl cysteine (NAC) (Fig. 2d), a well-known

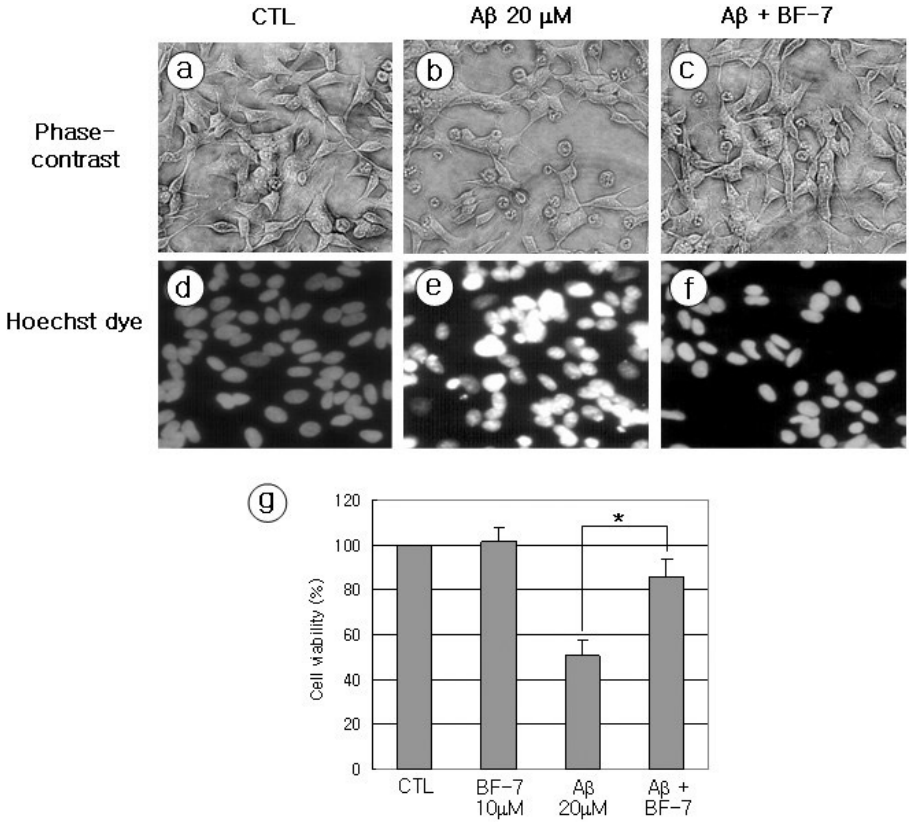

Fig. 1. Attenuated Aβ-induced Apoptosis BF-7 was Determined Morphologically and Biochemically

The photographs represent the optical phase-contrast microscopic morphology (a–c), and nuclear morphology, stained with Hoechst dye 33258, detected with fluorescence microscopy (d–f). The SKN-SH cells were either not treated (a, d) or treated (b, e) with 20 μM of Aβ for 24 hr. Rats were pretreated with BF-7 (10 μM) was pretreated 2 hr before Aβ (c, f). The photographs are representative of three different experiments. Cell viability was determined biochemically, using the Alarmablue assay, 24 hr after treatment with 20 μM of Aβ. Rats were pretreated with 10 μM of BF-7, 2 hr before Aβ (g). The values are expressed as mean ± SEM of four independent experiments. The difference from the cells incubated with Aβ alone was statistically significant ($p < 0.05$).

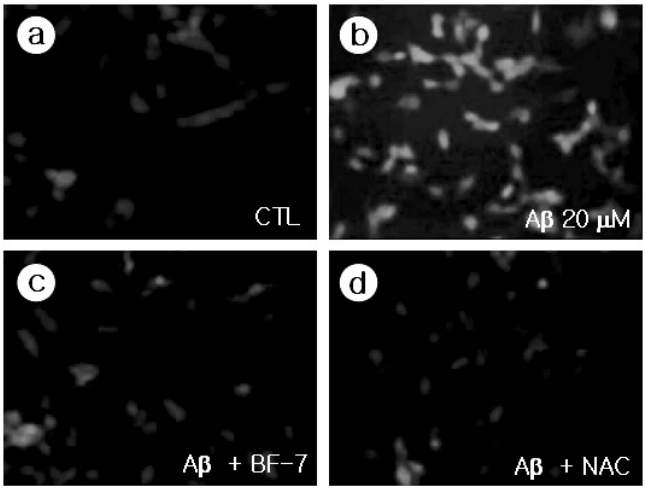

Fig. 2. Downregulated ROS by BF-7 Detected Morphologically and Biochemically

The cells generating ROS were visualized using fluorescence with DCF-DA under fluorescence microscopy (Olympus IX 70). The SKN-SH cells were treated with 20 μM of Aβ for 3 hr (b). Cells were pretreated with either 10 μM of BF-7 (c) or 1 mM of NAC (d) 2 hr before Aβ treatment. The quntitative level of ROS was measured using a fluorometer, with excitation at 485 nm and emission at 530 nm. The figures are representative of four independent experiments. The difference from the cells treated with Aβ alone was statistically significant ($p < 0.05$).

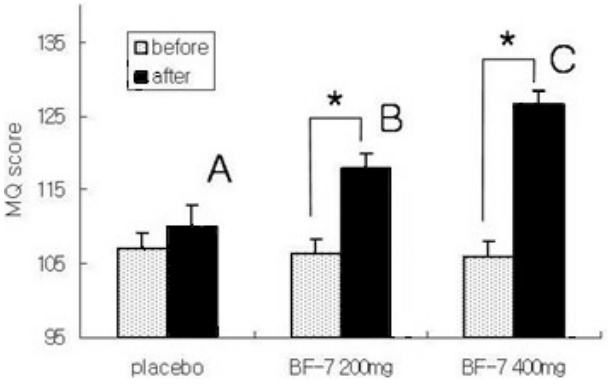

Fig. 3. Enhanced Memory Quotient in BF-7 Groups
MQ scores are expressed as mean ± SEM. Scores before and after administration of BF-7 were compared using paired *t*-tests. *Significantly different ($p < 0.05$).

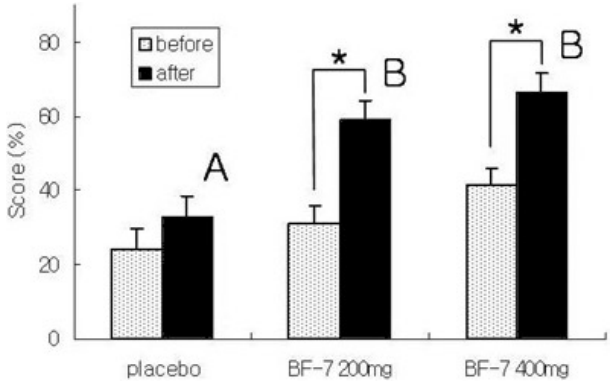

Fig. 4. Increased Memory Recall Efficiency in BF-7 Groups
Values are expressed as mean ± SEM. Scores before and after administration of BF-7 were compared using paired *t*-tests. *Significantly different ($p < 0.05$).

antioxidant. Interestingly, the effects of BF-7 were almost the same as those of NAC. Thus BF-7 was able to down-regulate ROS.

BF-7 Enhanced Memory Quotient BF-7

The memory quotient (MQ), is the most direct index reflecting memorizing ability. The average MQ of all 98 volunteers was around 105. Interestingly, the average MQ was significantly increased to about 126.6 after receiving of BF-7 for 3 weeks, but not in the placebo group (Fig. 3). To determine whether BF-7 can enhance memorizing ability, we analyzed improved MQ score differences. The increased MQ score increased significantly from 3.1 to 20.6 in a dose-dependent manner (Fig. 3). These results suggest that BF-7 enhances memorizing ability.

BF-7 Increased Memory Recall Efficiency

Memory recall efficiency indicates how much and how precisely individual recall preserved memory. A higher percentage score represents better efficiency. As shown in Fig. 4, the memory recall efficiency score was significantly increased from 31 to 58.9% and from 41.5 to 66.5% in the BF-7 200 mg/day and BF-7 400 mg/day for 3 weeks group, respectively, but not in the placebo group. The results indicate that the memory recall efficiency was improved by BF-7.

BF-7 Up-Regulated Acetylcholine Levels

As cognitive function declines the level of acetylcholine in the brain is reduced.[14] Our results showed that the acetylcholine levels in the brain of rats treated with Aβ was reduced to 45% of that in the untreated controls. Interestingly, the concentration recovered with BF-7 administration. In the BF-7 5 and 10 mg/kg groups, acetylcholine level increased to 78 and 80% of that in the controls, respectively (Fig. 5).

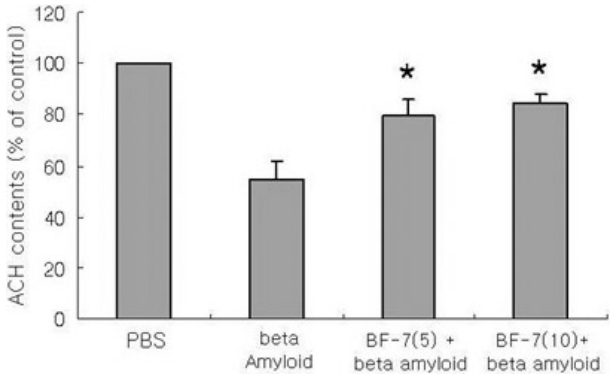

Fig. 5. Positive Role of BF-7 in Maintaining Brain Acetylcholine Levels
The level of acetylcholine is shown as a relative percentage compared with the control group. The values are expressed as mean ± SEM. Differences among the PBS, BF-7 5 mg/kg, and BF-7 10 mg/kg groups were analyzed using one-way ANOVA. *Statistically significant differences using the Tukey post-hoc comparison test are shown ($p < 0.05$).

DISCUSSION

Many factors lead to the decline in brain function.[12,33] Beyond transient functional declines, severe physiologic, chemical, and environmental stress gives rise to anatomic and physiologic brain damage.[34,35] The discovery of methods to protect the brain would thus be meaningful. In this study, the BF-7 was investigated to determine whether it has a

role in enhancing human learning and memory. As many previous studies suggested, Aβ-induced neuronal cell apoptosis should be a major pathologic reason for Alzheimer's disease.[36] Our results show that the BF-7 significantly inhibited Aβ-induced damage by attenuating ROS. It is well known that ROS cause neurodegeneration,[37] and thus decreasing ROS may be an effective mechanism to protect neurons.

In human volunteers, BF-7 significantly increased MQ in a dose-dependent manner.[38–40] The significantly increased scores in memory recall test suggest that BF-7 might be helpful in recalling and using specific memories precisely.

The positive role of BF-7 would result from various molecular mechanisms, including neuroprotection from various insults. Since BF-7 protected against the neurotoxicity of Aβ and ROS, the protective role might enhance brain function. Other mechanisms could improve learning and memory. Hypothetically, first, BF-7 possibly induces neurite spins, allowing neurons to connect more with others, which resulting in reinforcement of the neural network.

Another possibility is that BF-7 contributes to neurogenesis. Generally, it has been through that severely damaged neurons can not recover. However several reports have shown that neurogenesis occurs. For example, neurogenesis in the hippocampus is induced by estrogen.[41] If some neurons are damaged, then others could compensate functionally, and therefore BF-7 may contribute to the compensation. This hypothesis is supported by our previous results showing that BF-7 protected neurons efficiently against various insults. The other possibility is that the BF-7 contributes to the maintenance of homeostasis of various signal molecules like calcium, cAMP, *etc.*, as well as neurotransmitters like acetylcholine.

Among the possible mechanism, in this study, the maintenance of acetylcholine levels was examined in a Aβ-induced memory-impaired rat model.[42,43] The results showed that BF-7 helped to maintain acetylcholine levels similar to those in the controls. It is suggested that the ability of BF-7 to maintain acetylcholine levels is one mechanism for its role in enhancing brain function.

When neuroprotective and neuropromoting agents are screened, safety is an important consideration, since they must be administered long term. Since BF-7 is derived from a natural product, it appears to be both safe and useful in protecting the nervous system and improving learning and memory.

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