Korean J Physiol Pharmacol
Vol 8: 173–179, August, 2004

The Role of BF-7 on Neuroprotection and Enhancement of Cognitive Function

Hee-Sun Chae[1], Yong Koo Kang[4], Yong Kyu Shin[2], Hyun Jung Lee[1], Ji-In Yu[1], Kwang Gill Lee[7], Joo Hong Yeo[7], Yong Sik Kim[5], Dong-Suep Sohn[3], Kyung Yong Kim[1], Won Bok Lee[1], Sang Hyung Lee[6], and Sung Su Kim[1,4]

Departments of [1]Anatomy, [2]Pharmacology, [3]Thoracic & Cardiovascular Medicine, College of Medicine, [4]Biogrand Inc., Chung-Ang University, Seoul, Korea, Departments of [5]Pharmacology, [6]Neurosurgery, Seoul Municipal Boramae Hospital, College of Medicine, Seoul National University, Seoul, Korea, [7]Department of Sericultural and Entomology, National Institute of Agriculture Science Technology, RDA, Suwon, Korea

Amyloid β-peptide (Aβ) contributes to the pathogenesis of Alzheimer's disease (AD), causing neuronal death through apoptosis. In this study, the neuroprotective role of BF-7, extracted form sericultural product, was examined against Aβ -induced toxicity in cultured human neuronal cell SKN-SH. In order to know if the BF-7 has positive role on the cognition and memory in human, the mixture of BF-7, DHA and EPA (BDE) was examined using Rey Kim and K-WAIS test with 50 healthy high school student. We report here that BDE significantly attenuated Aβ-induced apoptosis through the reduction of ROS accumulation, and diminished caspase-like protease activity. Moreover, the memory index and memory preservation, and attentative concentration of BDE treated group for 1 month were significantly improved, in contrast to the case of placebo control treated with DHA and EPA. This result represent that the BF-7 play significant positive role on learning memory. Taken together, our result suggested the natural product BF-7 is a good substance for the brain functionally and physiologically.

Key Words: Brain, Learning and memory, BF-7, Natural product, Amyloid

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with dementia. The brain of AD patients is characterized by deposition of amyloid β-peptide (Aβ), a 39- to 43-amino acid peptide (Yankner, 1996). The precise mechanism of its neurotoxic action is not clearly know, although Aβ has been implicated as the most important cause of neuronal degeneration (Mattson, 1997). However, Aβ induces apoptosis in neurons is believed to be a major reason of AD. It has been identifed that several compounds able to protect neurons from Aβ damage. They include peptide aggregation-blocking agents (Soto et al, 1998; Permanne et al, 2002), neurotrophic factors (Guo & Mattson, 2000), antioxidants (Behl et al, 1992, Goodman & Mattson, 1994; Kumar et al, 1994), drugs that affect calcium signals (Weiss et al, 1994), and estrogens (Goodman & Mattson, 1994). Several peptides were reported to protect Aβ-induced neuronal degeneration. Interestingly, peptide mixture named BF-7, extract from sericultural product was effective to block ceramide induced neuronal damage. To obtain advanced therapeutic drugs that possess both high efficacy and safety, new active extracts or components derived from various natural sources have been studied in the treatment of brain diseases. Although the action mechanisms of natural extracts that have been used medicinally and traditionally should be investigated further, it is thought that they might have various active components responsible for the prevention of diverse brain diseases. Based on these ideas we have investigated whether the BF-7, natural peptide extract from Bombyx mori, has protective roles against Aβ toxicity and effects on learning and memory, using Rey Kim and K-WAIS test with 50 healthy high school student. We report that BF-7 enhances memory and cognitive function and protects neurons against Aβ, suggesting that BF-7 might be beneficial on the cognition function of human and treating against neurodegenerative disease including for AD.

Corresponding to: Sung Su Kim, Department of Anatomy, College of Medicine, Chung-Ang University, 221 Heukseok-dong, Dongjak-gu, Seoul 156-756, Korea. (Tel) +82-2-820-5690, (Fax) +82-2-821-8286, (E-mail) sungsu@cau.ac.kr
Sang Hyung Lee, Department of Neurosurgery, College of Medicine, Neuroscience Research Institute, Seoul National University, Seoul 100-799, Korea. (Tel) +82-2-840-2481, (Fax) +82-2-831-2826, (E-mail) nslee@ plaza.snu.ac.kr or nslee@brm.co.kr

METHODS

Cell culture

Human neuroblastoma SKN-SH cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Life

ABBREVIATIONS: K-WAIS, K-Wechsler adult intelligence scale; Ac-DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-7-maino-4-methylcoumarin; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Technologies, ON, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies) in a humidified 95% air, 5% CO_2 incubator. The cells were transferred to low serum media (1% FBS) 2 h prior to the BF-7 treatment (BF-7 was presently gifted from NIAST).

Cell viability assay

The cells were plated on 96-well plates (Corning, NY, USA) at a density of 5×10^4 cells/well, in 100 μl of 10% FBS/DMEM and incubated for 24 h. Two hours before the Aβ (Sigma, St. Louis, MO, USA) treatment, the media was replaced with 90 μl of 1% FBS/DMEM. After the treatment, 10 μl of alamarBlue (Serotec, Oxford, UK) was aseptically added. The cells were incubated for 3 h and the absorbance of the cells was measured at a wavelength of 570 nm using an ELISA Reader (Molecular Devices, CA, USA). 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)]×100 (Shimoke & Chiba, 2001).

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 (Sigma, St. Louis, MO, USA) for 5 min. They were washed twice with phosphate-buffered saline (PBS) and analyzed by fluorescent microscopy (Olympus IX 70, Tokyo, Japan). The dead cells were characterized by their fragmented nuclei, and the apoptotic morphological 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 fluorescent probe 2',7'-dichlorofluorescin diacetate (DCF-DA) (Sigma, MO, USA). 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 a UV supply system (Olympus IX 70, Tokyo, Japan). Also the cells stained with

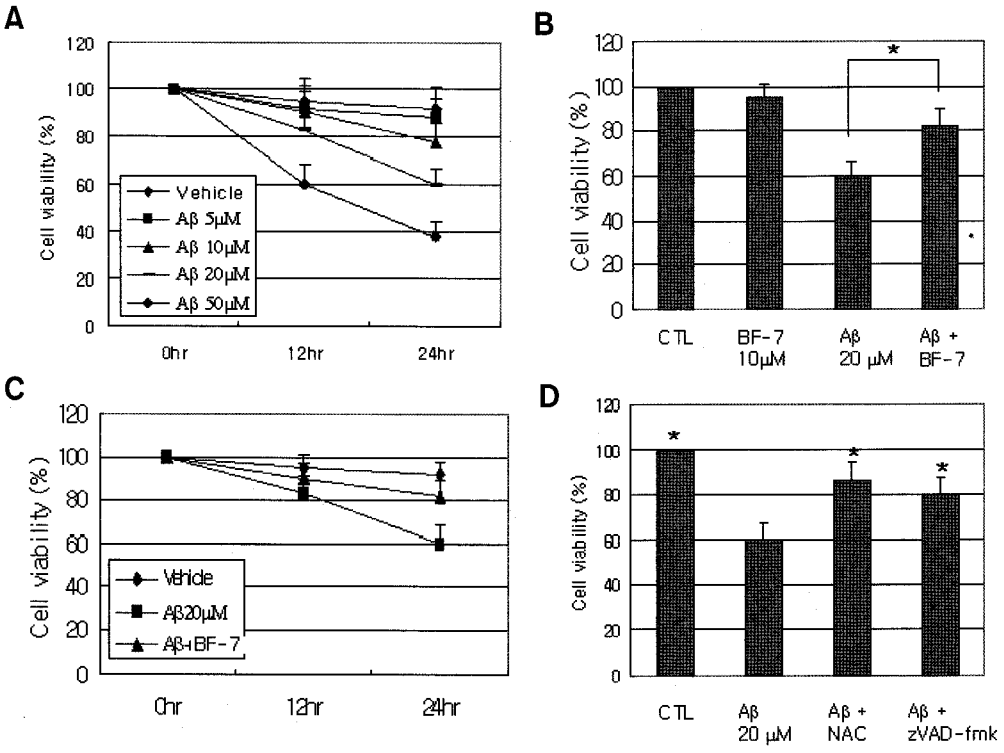

Fig. 1. Effect of BF-7 on Aβ-induced neuronal cell death. (A) SKN-SH cells were replaced with 1% FBS/DMEM 2 h prior to the Aβ treatment. The cells were treated with various doses of Aβ. (b) The cells were pretreated with various doses of BF-7 for 20 μM of Aβ were then added. The Aβ was dissolved in DMSO (final concentration <0.1%). (c) The cell viability was determined at indicated time points. (d) SKN-SH cells were pretreated with 10 μM of zVAD-fmk or 1mM of NAC for 2 h and then treated with 100 μM of Aβ. A bar graph of cell viability by the alarm a blue assay expresses the result at 24 h after 100 μM Aβ treatment. The values are a mean S.E.M. of three separate experiments. The difference from the cells incubated with Aβ alone was statistically significant ($p<0.05$).

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 by fluorometer (TECAN, GENios). Intensity of ROS was expressed as arbitrary unit of relative value.

Caspase substrate cleavage assay

The SKN-SH cells were collected by centrifugation at 150 *g* for 10 min and the washed cell pellet was resuspended in 100 μl of a lysis buffer containing 50 mM Tris, pH 7.5, 0.03% NP-40, and 1 mM dithiothreitol (DTT). The lysates were incubated on ice for 20 min with intermittent stirring and then centrifuged at 15,800 *g* for 5 min. The supernatant was analyzed for its protein content using a protein assay kit (Bio-Rad, CA, USA). To assess the extent of caspase cleavage, 20 μg of the cellular extracts were incubated with 0.25 mM Ac-DEVD-AMC (Pharmingen, CA, USA), which is a substrate for caspase-3, in a total volume of 100 μl at 37°C for 1 h. The caspase-3 activity was measured with excitation at 380 nm and emission at 460 nm using TECAN GENios fluorescence microplate reader (TECAN, Maennedorf, Schweiz). The enzyme activity is expressed as arbitrary units of a relative value.

Statistical analysis

The data is expressed as a mean S.E.M. values. The Student's *t*-test was used to analyze the relationship between the different variables. A $p<0.05$ was considered to be significant.

Treatment

For in vitro measurement of protective effects of BF-7 against Aβ, the rat primary cortical neurons were pretreated with BF-7 (0.5, 3, and 5 g/ml) for 12 h before the treatment with Aβ. Cell viability assays were carried out 12 h after the treatment with Aβ. For the clinical trial the with human, mixture of 400 mg of BF-7, 132 mg of DHA, and 206 mg of EPA (BDE) per day was treated orally for 30 days. As a placebo, a mixture of 132 mg of DHA and 206 mg of EPA (DE) was treated.

Intellectual learning tests

Rey-Kim test (Min et al, 2001) and number-memorizing test of K-WAIS (Min et al, 2001) were performed to random-sampled 40 high school students before administration. Test groups were divided by 30 people for BF-7, DHA, EPA mixture and 10 people for placebo. Indication was two capsules b.i.d., p.o., three weeks and tests were performed again to evaluate changes after administration.

RESULTS

BF-7 attenuated the decreased cell viability induced by Aβ

As shown in Fig. 1A, exposure to Aβ decreased the SKN-SH cell viability dose dependently. Aβ 20 μM was induced neuronal cell death by approximately 40% for 24 h. Treatment with BF-7 alone had no effect on the cell viability although a combination of Aβ and BF-7 was found to block approximately 20% of the cell death induced by Aβ (Fig. 1B). Fig. 1c showed the time-dependent inhibitory effect of BF-7 on the cell death induced by Aβ. The anti-toxic effect of 10 μM BF-7 against Aβ was lasted for 24 h.

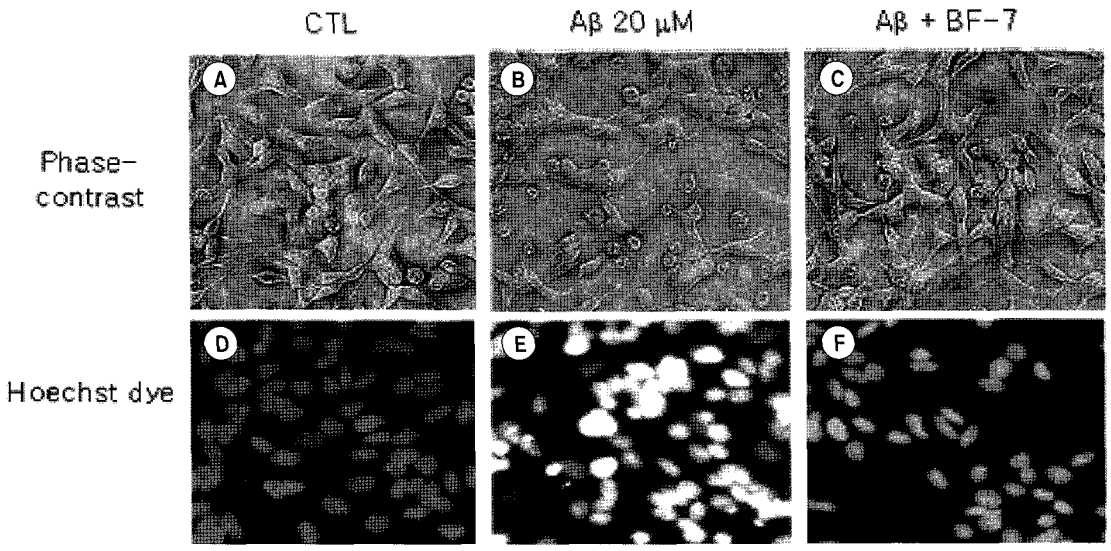

Fig. 2. Morphological assessment of apoptosis by phase-contrast and fluorescence microscopy. The SKN-SH cells were either not treated (A, D) or treated (B, E) with 20 μM of Aβ for 24 h. The BF-7 pretreated (10 μM of BF-7 for 2 h cells were treated with 20 μM of Aβ for 24 h (C, F). The figures show the optical microscopic morphology (A~C) and Hoechst 33258 stained nuclear morphology over fluorescence microscope (D~F). The figures are representative of three different experiments. Scale bar indicates 10 μm.

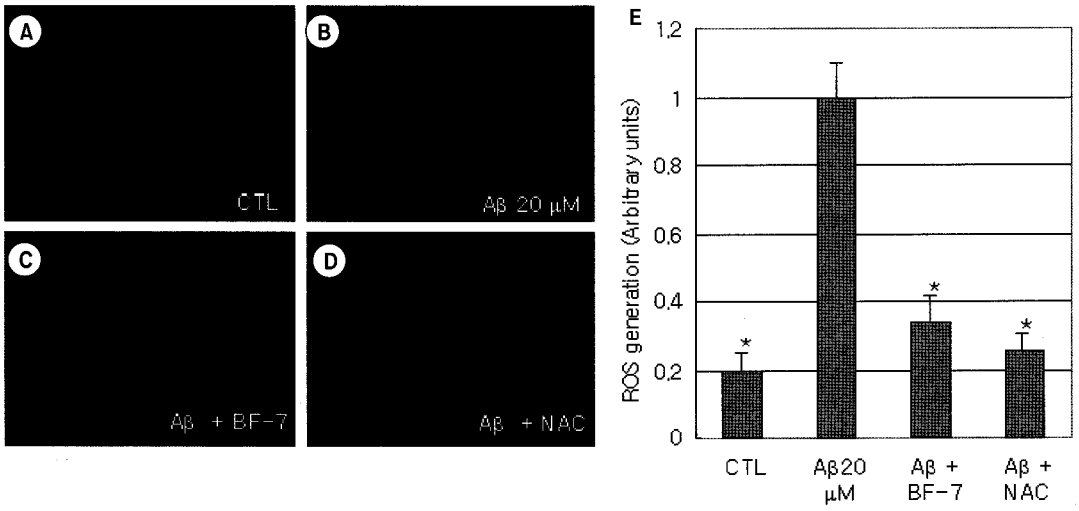

Fig. 3. Determination of ROS generation by Aβ after pretreatment with BF-7. (A) Levels of ROS generation were measured using the fluorescent probe 2',7'-dichloroflorescein-diacetate (DCF-DA). The effect of pretreatment of BF-7 is presented. For detecting ROS generation, the cells were incubated with 10 μM DCF-DA for 30 min. And excitation at 485 nm and emission at 530 nm were measured with fluorometer (Section 2). The levels of hydrogen in SKN-SH cells were determined with fluorescence microscopy (Olympus IX 70, Japan). The cells were untreated (B) or treated (D) with 20 μM of Aβ for 3 h. The SKN-SH cells pretreated with 10 μM of BF-7 were either not treated (C) or treated (E) with 20 μM of Aβ for 3 h. The figures are representative of four independent experiments. The difference from the cells treated with Aβ alone was statistically significant ($p<0.05$). Scale bar indicates 10 μm.

BF-7 inhibited the apoptotic cell death induced by Aβ

The Aβ induced morphological characteristics of apoptotic cell death over the phase-contrast (Fig. 2A~C) and the staining fluorescence microscopy with Hoechst 33258 (Fig. 2D~F), respectively. Exposing the SKN-SH cells to 20 μM Aβ for 24 h gave rise to membrane blebbing, cell shrinkage in the cell morphology and an 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. 2D~F).

BF-7 had an inhibitory role on ROS generation by Aβ

ROS are the main factor that causes oxidative stress, which results in cytotoxicity. Aβ 20 μM was able to produce these toxic species in the SKN-SH cells (Fig. 3B). The level of DCF fluorescence is an indicator of ROS production. The elevated fluorescence in the cells exposed to Aβ was attenuated by a pre-treated with BF-7 to the cells (Fig. 3C). Quantitative level of ROS was measured with excitation at 485 nm and emission at 530 nm by fluorometer (Fig. 3E). The intensity of DCF fluorescence showed significant, above seven-fold increases, than those in untreated cells. These results implicate involvement of ROS in Aβ-induced apoptosis and antioxidant effect of BF-7 in SKN-SH cells.

BF-7 inhibited the increase in caspase-3 activity after Aβ exposure

To determine whether caspase activation, which is an enzyme of the cysteine protease family that is involved in neuronal delayed death, is involved in Aβ-induced apo-

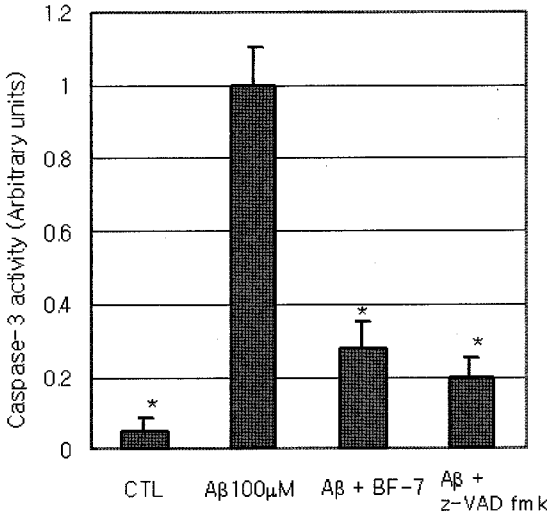

Fig. 4. Effect of BF-7 on Aβ-induced caspase-3 activity. CTL was vehicle-treated SKN-SH cells (control). The cells were either not incubated (Aβ) or incubated (BF-7+Aβ) with 10 μM of BF-7 for 2 h which was then followed by treatment with 20 μM of Aβ for 6 h. Fifty micrograms of the cellular extracts were incubated with 0.5 mM AC-DEVD-AMC in a total volume of 100 μl at 37°C for 1 h. Excitation at 380 nm and emission at 460 nm were measured with a Fluorescence microplate reader (TECAN, GENios). The enzymatic activity is expressed as arbitrary units of relative values. The values are reported as a mean S.E.M. of three separate experiments (*$p<0.05$, **$p<0.01$).

ptosis, cells were pre-incubated with the pan-caspase inhibitor, zVAD-fmk. The pre-treatment with 10 μM of zVAD-fmk significantly prevented cell death following a 24 h exposure to 20 μM of Aβ (Fig. 1D). And then, in order to explore the influence of BF-7 on caspase-3, the caspase-3 activity was determined by the extent of Ac-DEVD-AMC cleavage. Although Aβ increased the caspase-3 activity by up to two times, a pretreatment with either BF-7 or zVAD-fmk, a pancaspase inhibitor attenuated the Aβ-induced caspase-3 activity by either one half or abolished the activity completely, respectively (Fig. 4).

Clinical study results

The memory index MQ, which is the most direct reflective index of memorizing ability. The average MQ of all 40 one was around 100. Interestingly, the average MQ was significantly increased to about 122 after intaking of DBE for a month, but not in placebo cases (Fig. 5A). To investigate if DBE can help the efficiency to memorize something, the number of how many times one should repeat learning to memorize something (learning gradient test) was performed. As shown in figure, the learning gradient was declined from 47% to 18% at DBE treated case. This result represents that DBE was of help to enhance learning efficiency (Fig. 5B).

The memory preservation is an index of how well one maintains once memorized matter. It was shown that the DBE was effective for prolong of memory, as the memory preservation index was increased from 58% to 72% (Fig. 5C).

The attentive concentration and short-term memory are very important in brain cognitive functions. To evaluate how much the DBE exert positive role on the attention and short-term memory, the DBE was addressed to a part of IQ test (K-WAIS), memory and recall many numbers in

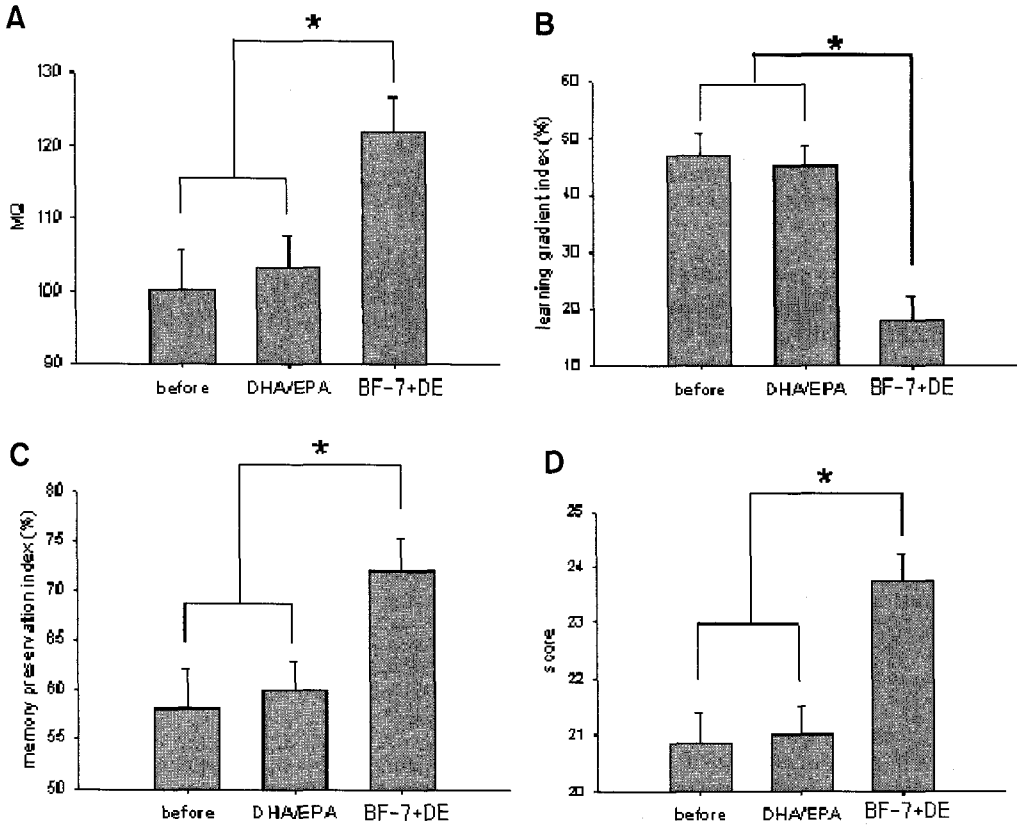

Fig. 5. Effects of BF-7 on learning and short term memory activity. a. Rey-Kim test was performed as follows: The number of recalled words was counted after 15 words were presented to examinee. This test was repeated 5 times. After 20 min, the words that examinee could remember was counted without presenting words in this case. Visual memory test was performed after verbal test. Examinees were asked to duplicate a complex geometrical figure and draw the same figure without reference immediately. After 20 min, it was asked to draw the same figure without reference. All indexes were calculated as described (Kim, H.G (1999) Rey-Kim memory test Neuropsychology Press). A memory part of K-WAIS test was also tested as follow: Examinees were asked to recall the numbers forward and backward after listening several (from 3 to 8) numbers. The scores were calculated as described (Yeom, T.H. et al (1992) Guidance of K-WAIS Korea Guidance Press). The values are reported as meanS.E.M. ($p<0.05$).

order and in reversed order. With one month treatment of DBE, epoke-making score was improved significantly from 21 to 24 (Fig. 5D). the result represents that DBE is very effective on enhancing short-term memory and attentive concentration.

DISCUSSION

Since, the therapy for neurodegenerative disease like as AD, PD has a many limits so far, there has been a lot of effort to develop effective materials for those disease. It may be one of the reasonable way to develop or find effective material screened from natural products. Fotunately, we have found out the BF-7, a natural peptide mixture from sericultural product has protective effect against ceramide-induced neuronal damage (unpublished data). The overproduced ceramide in the human, may be one of the major reasons leading loss of cognitive function, aging and various neurodegenerative disease (Soreghan et al, 2003; Cutler et al, 2004). In this study, the BF-7 was addressed to check if it has positive role on enhancing human brain cognitive function like as learning and memory. Also, it has been studied that the BF-7 blocked Aβ induced neuronal damage. As many previous studies suggested, Aβ-induced neuronal cell apoptosis should be a major pathlogical reason of AD (Boland & Campbell, 2003). The BF-7 significantly blocked Aβ induced damage though attenuating ROS and caspase activation. It is well known that the ROS is a causatative molecule of neurodegeneration (Wang et al, 2003), so diminishing ROS may be very important and effective machanism to protect neuron. Also, caspases are real executors leading to apoptosis (Yakovlev & Faden, 2001), the attenuating caspase activation would be pivotal role of BF-7 for protecting neuronal cell.

It has been known that the cholinergic system is damaged by Aβ, and profound losses in the cholinergic system of brain are associated closely with cognitive deficits observed in AD (Cummings & Kaufer, 1996). The protective role of BF-7 against Aβ lead us to expect the possibility of the BF-7 enhancing cognitive function of human.

Rey-Kim test and number-memorizing test of K-WAIS were performed to check the BF-7 has the fuction or not. Since EPA and DHA has been known as good brain nutrient, those was used as material for placebo (Uauy-Dagach & Valenzuela, 1992). In the case of placebo, no significant enhancement of learning and memory. However, various learning and memory index of the group treated with BF-7, EPA and DHA, was significantly improved. It represent that the positive effect is due to BF-7.

The memory index MQ representing ability of memory directly, was significantly increased by taking DBE for 1 month. Interestingly, our results showed that the number of trial to memory something was decreased. Interestingly, the preservation of memory was prolonged greatly. These results represented that DBE is of help on learning and memory efficiently. Moreover, our result showing increased Epoke-making score represents that DBE is very effective on enhancing short-term memory and attentive concentration. In sumary, our result represented that DBE is a good material for improving learing and memory, and the positive role might be arised from BF-7.

Since, the BF-7 is derived from non toxic natural product, it will be a profer substance for attenuating neurodegenerative disease including AD, and for improving learng and memory.

ACKNOWLEDGEMENTS

This research was supported by grants from the Korea Health 21 R&D Project funded by the Ministry of Health and Welfare of the Korean government (001-PJ8-PG1-01 CN2-0003) and Biogreen 21 funded by Rural Development Administration (02-N-I-02).

REFERENCES

Behl C, Davis J, Cole GM, Schubert D. Vitamin E protects nerve cells from amyloid toxicity. Biochem. *Biophys Res Commun* 186: 944−950, 1992

Boland B, Campbell V. beta-Amyloid (1-40)-induced apoptosis of cultured cortical neurones involves calpain-mediated cleavage of poly-ADP-ribose polymerase. *Neurobiol Aging* 24: 179−186, 2003

Cutler RG, Kelly J, Storie K, Pedersen WA, Tammara A, Hatanpaa K, Troncoso JC, Mattson MP. Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proc Natl Acad Sci USA* 101: 2070−2075, 2004

Cummings JL, Kaufer D. Neuropsychiatric aspects of Alzheimer's disease: the cholinergic hypothesis revisited. *Neurology* 47: 876−883, 1996

Goodman YD, Mattson MP. Staurosporin and K-252 compounds protect hippocampal neurons against amyloid β-peptide toxicity and oxidative injury. *Brain Res* 650: 170−174, 1994

Guo ZH, Mattson MP. Neurotrophic factors protect terminals against amyloid- and oxidative stress-induced impairment of glucose transport, glutamate transport and mitochondrial function. *Cereb Cortex* 10: 50−57, 2000

Kumar U, Dunlop DM, Richardson JS. The acute neurotoxic effect of β-amyloid on mature cultures of rat hippocampal neurons is attenuated by the anti-oxidant U-78517F. *Int J Neurosci* 79: 185−190, 1994

Mattson MP. Cellular actions of β-amyloid precursor protein and its soluble and fibrillogenic derivates. *Physiol Rev* 77: 1081−1132, 1997

Min SK, Moon IW, Ko RW, Shin HS. Effects of transdermal nicotine on attention and memory in healthy elderly non-smokers. *Psychopharmacology (Berl)* 159: 83−88, 2001

Permanne B, Adessi C, Saborio GPSF, Frossard MJ. Van Dopre J, Dewachter I, Banks WA, Van Leuven F, Soto C. Reduction of amyloid load and cerebral damage in a transgenic mouse model of Alzheimer's disease by treatment with a β-sheet breaker peptide. *FASEB J* 16: 860−862, 2002

Shimoke K, Chiba H. Nerve growth factor prevents 1-methyl-4-penyl-1,2,3,6- tetrahydropyridine-induced cell death via the Akt athway by suppressing caspase-3-like activity using PC12 cells: relevance to therapeutical application for Parkinson's disease. *J Neurosci Res* 63: 402−409, 2001

Soreghan B, Thomas SN, Yang AJ. Aberrant sphingomyelin/ceramide metabolic- induced neuronal endosomal/lysosomal dysfunction: potential pathological consequences in age-related neurodegeneration. *Adv Drug Deliv Rev* 55: 1515−1524, 2003

Soto C, Sigurdsson EM, Morelli L, Kumar RA, Castano EM, Frangione B. β-Sheet breaker peptides inhibit fibrillogenesis in a rat brain model of amyloidosis: implications for Alzheimer's therapy. *Nat Med* 4: 822−826, 1998

Uauy-Dagach R, Valenzuela A. Marine oils as a source of omega-3 fatty acids in the diet: how to optimize the health benefits. *Prog*

Food Nutr Sci 16:199−243, 1992
Wang JY, Shum AY, Ho YJ, Wang JY. Oxidative neurotoxicity in rat cerebral cortex neurons: synergistic effects of H2O2 and NO on apoptosis involving activation of p38 mitogen-activated protein kinase and caspase-3. *J Neurosci Res* 72: 508−519, 2003
Weiss JH, Turetsky D, Wilke G, Choi DW. AMPA/kainate receptor-mediated damage to NADPH-diaphorase containing neurons is Ca^{2+} dependant. *Neurosci Lett* 167: 93−96, 1994
Yakovlev AG, Faden AI. Caspase-dependent apoptotic pathways in CNS injury. *Mol Neurobiol* 24: 131−144, 2001
Yankner BA. Mechanisms of neuronal degeneration in Alzheimer's disease. *Neuron* 16: 921−932, 1996