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BF-7 Improved Memory Function and Protected Neuron from Oxidative Stress

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Endogenously or exogenously generated oxidative stress impair organs, especially brain. Also, the oxidative stress appears to be a negative factor on normal brain function, like memory and cognition. Our result showed that BF-7, extracted from *Bombyx mori*, effectively diminished oxidative stress, leading to the protection of neuron from reactive oxygen species donated by $FeSO_4$. Clinical experiments showed that BF-7 significantly improved memory and cognitive functions of normal adults in a dose-dependent manner. Thus, our results suggest that BF-7 play a role in the improvement of brain functions by removing oxidative stress, and provide therapeutic potential role of BF-7 to protect nervous system from oxidative damage.

Key words : Memory, Cognitive function, BF-7, Oxidative stress, K-WAIS, Apoptosis

Introduction

Oxygen is essential for human survival but reactive oxygen species (ROS), the oxygen metabolite, exert potent cytotoxic activity. Most ROS and superoxide anion radical (O_2^-) generated by tissues are produced from the mitochondrial respiratory system. 1-2% of oxygen consumed under the physiological oxygen concentration is converted into ROS.

Especially, the brain is very sensitive to oxidative stress due to a rate of high oxygen consumption. Although the proportion of the brain is relatively small, the brain consumes 20% of the resting total body oxygen consumption. To maintain different ionic concentrations (a high intracellular K^+, low Na^+ and very low Ca^{2+} level), significant amount of ATP and oxygen are consumed. In addition, the brain consumes 4 x 10^{21} molecules of glucose per minute to produce energy. This explains why neuronal cell death is induced by hypoglycemia and inhibitors of ATP synthesis like rotenone or cyanide.

Another cause for neuronal cell damages is nitric oxide (NO^-). This gaseous form of free radical that can easily spread is a vital biological carrier and plays an important role in cerebral physiology. Neuronal NO synthase (nNOS), inducible NO synthase (iNOS) and endothelial NO synthase

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(eNOS) produced by activated microgliacytes are involved in the production of NO. In the brain, nNOS whose expression is regulated by pathophysiological stimulation is involved in the activation of NO^- [Yun 1996]. Peroxynitrite ($ONOO^-$), the highly activated RNS, is formed by rapid reaction of NO^- with O_2^-.

ROS/RNS is the oxidative stress of the nerve system which is largely produced in pathological conditions, and NO^- is, in particular, generated by activated microgliacyte (iNOS) or endothelial cell (eNOS). In inflammatory responses, the major source of ROS is damaged mitochondria or activated microglial cells.

Traditionally, oxidative stress was thought to be occurred by the imbalance between the production and destruction of ROS/ RNS and ROS/RNS was considered to be absolutely harmful. However, in recent years, it is reported that oxidation-reduction regulations including ROS play an important role in the regulation of vital cellular functions such as MAP kinase cascade activation, ion transfer, calcium transport and activation of apoptosis program in astrocytes and microgliacytes. Furthermore, it is believed that oxidative stress is associated with abnormal protein aggregations and induces constant stress to the brain or brain dysfunction by causing abnormality in DNA, protein and lipid.

Thus, this study aimed to identify the mechanisms of nerve cell apoptosis induced by $FeSO_4$ and evaluate suppression effects of BF-7, which is known to be neuroprotective (Chae 2004), on nerve cell apoptosis using SK-N-SH, a human neuroblastoma cell line. Furthermore, whether BF-7 clinically improves cognitive function in randomly selected general population with various backgrounds was also examined.

Table 1. Characters of voluteers

Character	Value
Age (year)	42.9±16*
Education (year)	12.7±2.3*
Number of male	13
Number of female	53

*Expressed as means ±S.D.

Materials and Methods

1. Materials

1) Materials

BF-7 used for the cognitive function test was supplied from rural development administration and was separated and purified from *Bombyx mori*.

2) Subjects

Healthy Korean adults with various backgrounds including university students, housewives, employees, people who visit religious organizations were voluntarily enrolled for this study. 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 80 people were initially enrolled for this study but 14 of them were dropped during the trial and the rest 66 people were included in the analyses. The characteristics of the subjects were demonstrated in Table 1.

2. Methods

1) Cell culture

SK-N-SH, a human neuroblastoma cell line, was cultured in RPMI 1640 medium 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) 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). Cultured cells were treated with scopolamine and incubated at 37℃ 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%.

3) Measurement of ROS

The cultured cells were pretreated with 10 μM DCFDA (6-carboxy-2',7'-dichloro-dihydrofluoresceine diacetate, dicarboxymethylester) dissolved in HCSS buffer (20 mM HEPES, 2.3 mM $CaCl_2$, 120 mM NaCl, 10 mM NaOH, 5 mM KCl, 1.6 mM $MgCl_2$, 15 mM glucose) and an anti-fouling reagent, 2% Pluronic F-127 at 37℃ for 30 min. DCF fluorescence caused by intracellular free radicals was observed by Olympus IX70 inverted microscope with mercury arc lamps (Excitation =488 nm, Emission=510 nm) at the room temperature and analyzed using NIH Image 1.65 program after capturing with a CCD camera.

4) Clinical trial (experiment on human body)

The clinical trial was conducted as a double-blind, placebo-controlled study. K-WAIS which is an officially approved tool for the objective and quantitative evaluation of cognitive function was used in this study. Among the testlets, number memorizing which evaluates short-term memory, attention and concentration was performed. Subjects were randomly assigned to the placebo group, the experimental group A and the experimental group B and participated in the trial for 3 weeks. The experimental group A had a total of 4 capsules per day (400mg), 2 capsules of BF-7 (100mg BF-7/capsule) in the morning and in the evening, and the experimental group B had a total of 4 capsules per day (200mg), 2 capsules of BF-7 (50mg BF-7/capsule) in the morning and in the evening. They were instructed not to change their usual diet during the trial.

5) Statistical analyses

All statistical analyses were performed using SPSS and p values < 0.05 were considered statistically significant. Data of the cellular experiment was presented as means ± standard deviations, and *t* test was used to examine correlations and differences between the groups. Data of the clinical trial was presented as means ± standard deviations and student's t-test or one-way ANOVA (Tukey's multiple comparison test) was used to show statistical significance for the differences depending on the items to compare.

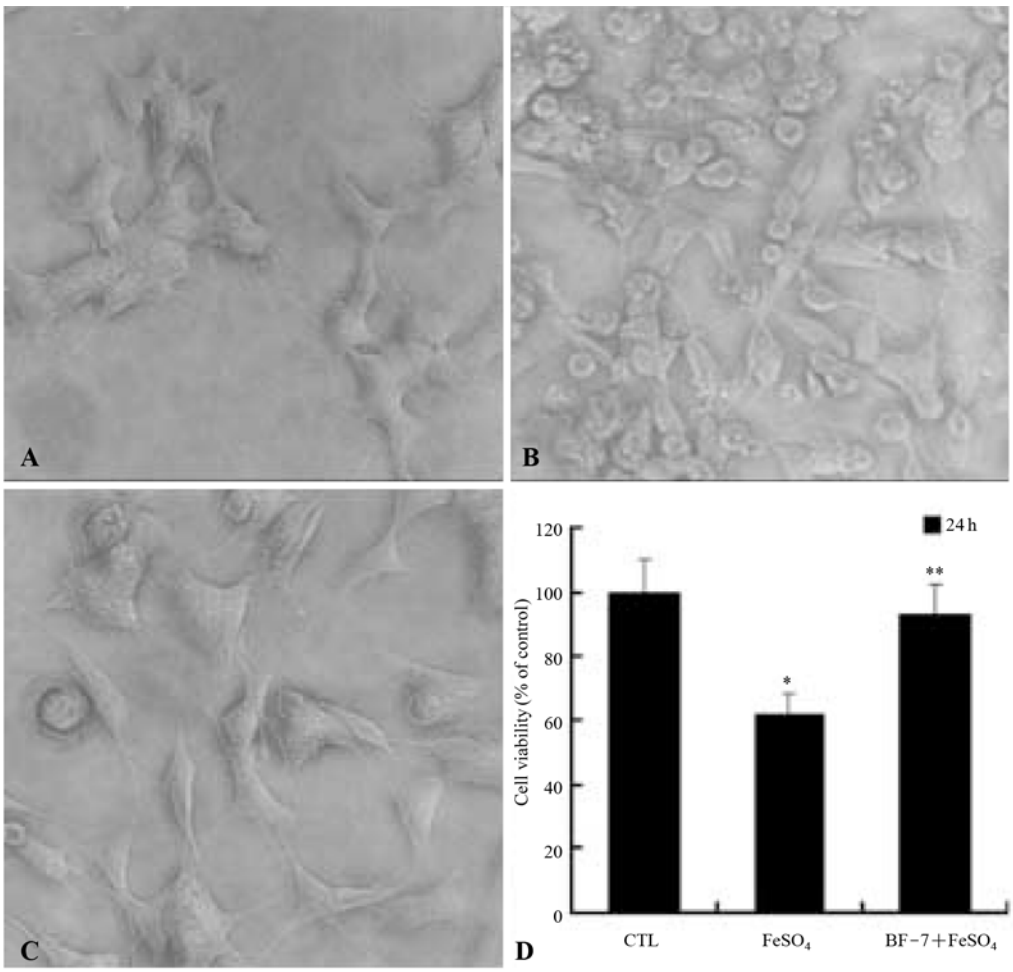

Fig. 1. Effect of BF-7 on $FeSO_4$ induced neuronal cell death in SK-N-SH neuroblastoma cells. Morphological changes associated with $FeSO_4$ toxicity and the effect of preincubation with BF-7. SK-N-SH cells were treated with no $FeSO_4$ (A) or with $FeSO_4$ (200 μM) for 24 hr (B). BF-7 (10 μM) was pretreated 2 hr before treatment with $FeSO_4$ (c).The figures photographed by phase contrast microscope are representative of three different experiments. Cell viability was determined by MTT assay at 24 h after $FeSO_4$ (200 μM) treatment (D). The effect of pretreated BF-7 (10 μM) are presented. Data are the mean value ± S.E.M. obtained from three independent experiment. *p <0.05 from control; **p < 0.05 from $FeSO_4$.

Results

1. SK-N-SH neuroblastoma cell apoptosis induced by $FeSO_4$ and protective effects of BF-7 on cellular damages

Cell death of SK-N-SH, a neuroblastoma cell line, was induced by the treatment of cytopermeable $FeSO_4$ (200 μM). Cellular morphology was analyzed to characterize the cell death pattern of SK-N-SH induced by $FeSO_4$. Unlike normal cells (Fig. 1A), the observation of the cellular morphology with a phase contrast microscope for 24 hours revealed typical apoptosis patterns such as condensation and fragmentation of the cell body, neurite loss and membrane blebbing (Fig. 1B).

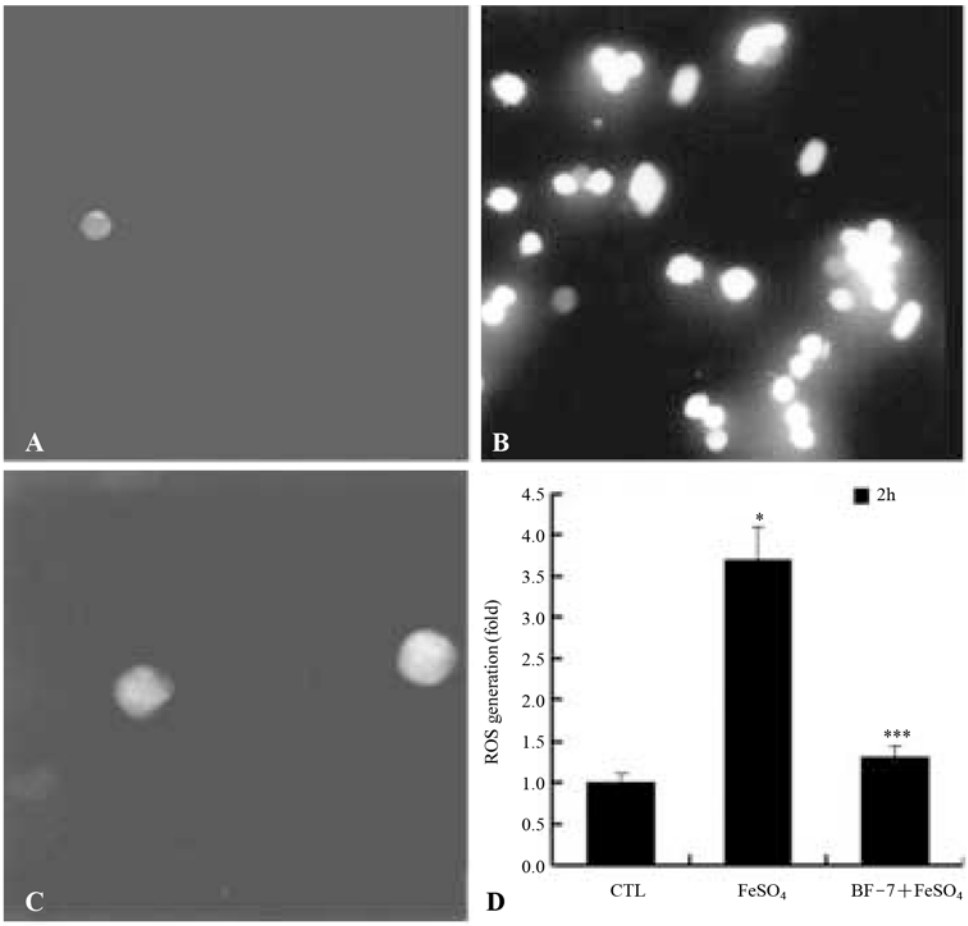

Fig. 2. The level of ROS generated by $FeSO_4$ after pre-treatment with BF-7. SK-N-SH cells were untreated control (A) or treated (B) with $FeSO_4$ (200 μM) for 2h. SK-N-SH cells were pretreated with BF-7 (10 μM) for 2h before treatment with $FeSO_4$ (C). Hydrogen peroxide generated by $FeSO_4$ was measured by incubation with fluorescent probe 6-carboxy-2′, 7′-dichloro-dihydrofluoresceine diacetate, dicarboxym-ethylester (DCF-DA). Quantification of fluorescent level was estimated using flow cytometry at 2 h after 200 μM $FeSO_4$ treatment (D). Fluorescent levels are expressed as fold of relative value compared with control. The figures are representative for three different experiments. *$p < 0.05$ from control; **$p < 0.05$ from $FeSO_4$.

On the other hand, apoptosis was effectively suppressed in the cells treated with 10 μM BF-7 two hours prior to the observation, remaining their appearance similar to that of the control group (Fig. 1C).

For the quantitative analysis of the cell death induced by $FeSO_4$, MTT reduction assay was performed. The result showed that the viability of the cells treated with $FeSO_4$ (200 μM) was approximately 60% comparing to that of the control group and that of the cells pre-treated with 10 μM BF-7 two hours prior to the observation was increased up to 90% (Fig. 1D).

2. The production of ROS by $FeSO_4$ and the suppression effects of BF-7

To examine whether BF-7 effectively removes ROS gene rated by $FeSO_4$, cells were observed by a fluorescence microscope using 10 μM DCF-DA. Whereas fluorescence that reflects the production

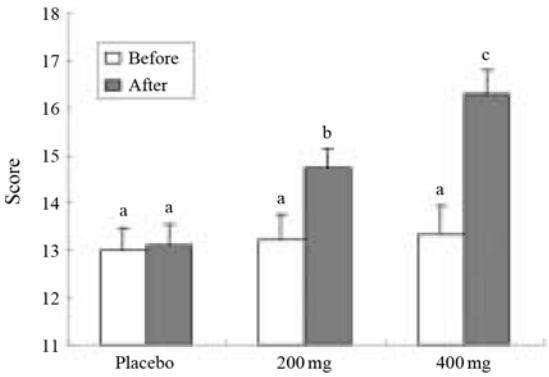

Fig. 3. Improvement of cognitive function by BF−7 in clinical settings. Volunteers were randomly divided into placebo and two treated groups (200 mg and 400 mg). Improvement effects were evaluated by comparison of scores of K−WAIS test before and after intake of BF−7. Indication was two capsules b.i.d., p.o., three weeks. The values are reported as mean±S.E.M. Student's t−test was used to analyze the relationship between the scores of before and after intake. The dose−dependent relationship among placebo, 200 mg, and 400 mg groups was analyzed by using oneway ANOVA (Tukey's multiple comparison test). a,b,c: different characters indicate statistically significant and same character means statistically non−significant ($p < 0.05$).

of ROS was not observed in the control group (Fig. 2A), ROS was significantly increased within 2 hours in the cells treated with 200 μM $FeSO_4$, showing fluorescent light (Fig. 2B). However, color-development in the cells pre-treated with 10 μM BF-7 two hours prior to the observation was similar to that in the negative control group (Fig. 2C). A quantitative analysis showed that the production of ROS was 3.8 times increased with $FeSO_4$ treatment and suppressed with BF-7 treatment (Fig. 2D).

Improvement of cognitive function in general population (clinical trial)

In K-WAIS, a set of items evaluating short-term memory, attention and concentration was implemented to evaluate the improvement of cognitive function by BF-7. The scores after BF-7 intake were significantly improved comparing to those before BF-7 intake, demonstrating improvement of cognitive function. As shown in figure 3, whereas there is no significant statistical difference in the placebo group between the scores prior to the BF-7 intake and post to the BF-7 intake, 11.3% and 22.2% of improvement were shown in the BF-7 200 mg intake group and the BF-7 400 mg intake group in a dose-dependent manner, respectively. The subjects of the experiment groups were subdivided according to sex and age to examine if there were any differences between sex and age but there was no difference. Furthermore, it was found that the level of improvement in the cognitive function of the BF-7 400mg group was comparable to that reported in the previous study conducted with high school students.

Discussion

ROS, the secondary product of free radicals, produces harmful oxidative products such as lipoperoxide by attacking intracelluar DNA, protein and membrane and damages tissues, developing various diseases and symptoms.

This oxidative stress constantly occurs during energy metabolism and the brain, in particular, is much more sensitive to oxidative stress, negatively affecting brain activities in healthy population. It is expected that the effective removal of routinely occurred oxidative stress will protect the brain and its function. Thus, developing substances to enable this is considered to be a meaningful task.

Recent studies reported that BF-7 extracted from *Bombyx mori* has neuroprotective effects and suppressed the toxicity of beta amyloid which is a

causative substance of dementia. This study aimed to clinically examine whether BF-7 protects neurons from oxidative stress and BF-7 intake has positive effects on brain function such as learning and memory in general population. The results showed that BF-7 effectively removed oxidative stress caused by external influences due to $FeSO_4$, resulting in a reduction of neuronal damages. The exact mechanisms how BF-7 reduces oxidative stress have not yet been established but it is thought that the chemical properties of BF-7 themselves contribute to the removal of oxidative stress or BF-7 triggers cellular defense mechanisms to remove oxidative stress. Further studies are needed to establish clear mechanisms.

Brain function was tested using K-WAIS which is the most widely used intelligence test. This test consists of several testlets for verbal and non-verbal functions to evaluate memory, concentration, thinking skill, judgment, spatial perception, organization and visual-motor integration. Among the testlets, number memorizing that evaluates short-term memory, attention and concentration was implemented in this study. Trial monitoring staff monitored changes in subjects' weight, alcohol intake, changes in diet, the presence of side effects, enthusiasm and compliance on weekly basis. Brain function was not improved in the placebo group but was improved in the BF-7 group (both 200mg intake group and 400mg intake group) in a dose dependent manner.

This study results imply that BF-7 effectively improves cognitive function regardless age and sex and the improvement is dose-dependent. Although the sample sizes of male and female populations were different, there was no difference in the improvement, implying that it was equally effective in both men and women. It is thought that the removal of ROS by BF-7 also contributed to the improvement of brain function. However, it is believed that there are many other protective mechanisms involved in the improvement of brain function. Further studies on the neuroprotective effects of BF-7 at the molecular level will be significantly valuable in terms of neuroprotective effects and function activity and it will also provide important information for the prevention and treatment of dementia.

References

Andersen JK : Oxidative stress in neurodegeneration :Cause or consequence? *Nature Neurosci.* **1** : 518-523, 2004.

Beal MF : Does impairment of energy metabolism results in excitotoxic neuronal death in neurodegenerative illnesses, *Ann. Neurol.* **31** : 119-30,1992.

Chae HS, Kang YK, Shin YK, Lee HJ, Yu JI, Lee KG, Yeo JH, Kim YS, Shon DS, Kim KY, Lee WB, Lee SH Kim SS : The role of BF-7 on neuroprotection and enhancement of cognitive function, *Korean J Physiol Pharmacol* **8** : 173-179, 2004.

Chu CT, Levinthal DJ, kulich SM, Chalovich EM, CeFranco DB : Oxidative neuronal injury, *Eur J Biochem* **271** : 2060-2066, 2004.

Emerit J, Edeas M, Bricaire F : Neurodegenerative disease and oxidative stress, *Biomedicine and Pharmacotherapy* **58** : 39-46, 2003.

Mattson MP, Daun W, Pedersen WA, Culmsee C : Neurodegenerative disorders and ischemic brain disease, *Apoptosis* **6** : 69-81, 2001.

Scandalios JG : Oxidative stress response-What genome scale studies taught us? *Genome Biol,* **3** : 1-6, 2002.

Yeo JH, Lee KG, Kweon HY, Woo SO, Han SM, Lee YW, lim JI, KimSS, Demura M, *Koean J. Seric Sci.* **46** : 23-27, 2004.

Yun HY, Dawson VL, Dawson TM : eurobiology of nitric oxide. *Crit Rev Neurobiol* **10** : 291-316, 1996.

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Abstract

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