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The Effect of BF-7 on the Ischemia-induced Learning and Memory Deficits

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ABSTRACT Abnormal blood supply to brain, such as ischemia induce neuronal damages, possibly leading to dementia. Such damages should be attenuated by neuroprotective materials which make neurons tolerable against limited blood supply or reinforce of blood. For the animal study, transient middle cerebral artery occlusion was operated with SD rat. To check whether BF-7 attenuated the ischemic damage, BF-7 (10 mg/kg) was oral treated for 7 days once a day. To evaluate the learning and memory of the rat, 8-arm maze test was conducted. For clinical study, Rey Complex Figure Test (RCFT) was used with control group (32 person), 200 mg treated group (33 person) and 400 mg treated group (34 person). Treatment of BF-7 greatly reduced the infarct size. Also the neuronal damages in the hippocampus were significantly diminished. Furthermore, The memory impairment by ischemia was recovered. The clinical test showed that BF-7 greatly enhanced the brain function recognizing and memorizing complex two dimensional figures. Our results implicated that BF-7 plays a positive roles on protecting brain and enhancing brain function clinically.

Key words : Learning and memory, BF-7, Ischemia, 8-arm radial maze test, Rey Complex Figure Test

Introduction

Despite of continuous progress in the prevention and treatment modalities of vascular diseases, focal cerebral ischemia still remains as a major cause and death of brain diseases. Focal cerebral ischemia can be caused by cerebrovascular disease (stroke), subarachnoid hemorrhage and trauma (Sovey 2001). Central nerve system diseases caused by structural changes in cerebral vessels are appeared as stroke in 45 million people worldwide every year. Owing to the westernization of lifestyle and diet, cerebrovascular diseases such as cerebral infarct have been increasing in Korea, and considering the aging population tendency, the magnitude of the disease is steadily rising. Such brain damages occurred by cerebral ischemia continues even after reperfusion as well as during ischemia, resulting in a delay of recovery and sequelae. Therefore, the problem should be taken seriously and improvement schemes are urgently needed. Many studies have been actively investigating pathophysiological phenomena or mechanisms of neuronal cell damages induced by cerebral ischemia for the prevention, improvement and new management strategies from various angles (Koroshetz & Moskowitz, 1996; Zivin, 1998; Sobey, 2001).

Focal cerebral ischemia, resulted from the interruption of cerebral blood flow, damages neuronal cells and microvessels and also induces inflammatory response (Lipton,1999). Animal experiments and clinical trials both show that effective treatments at the early stage of ischemia reduces the extent of tissue damages, infarction and death (Koroshetz & Moskowitz, 1996; Scholler *et al*., 2004). It is known that decreased cerebral blood flow is closely associated with brain dysfunction occurred by stroke, and recent studies suggested that interruption of blood supply to the brain causes symptoms of multiple cerebral infarct (multi-infarct dementia). When cerebral

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blood flow is interrupted, not only vascular-type dementia can occur but other types of dementia such as Alzheimer's disease also can develop (Leys *et al*., 1999; Roman, 2004), resulting in cognitive dysfunction such as the disturbance and loss of memory and judgment in addition to physical and motor dysfunction. Learning and memory are unique abilities of human. Morphologically and functionally changed neurotransmitters at the synapse are transported to cholinergic systems, which plays an important role (Gray & McNaughton, 1982; Olton & Wenk, 1987). This is also found in researches with ischemia animal models reporting that memory and memory impairments are caused by the reduction of cerebral blood flow. This cognitive impairment occurs with damages and destruction of the pyramidal neuron of hippocampus which plays an important role in learning and memory (Blozovski, 1979; Chrobak *et al*., 1987) and the reduction of acetylcholine in brain (Block, 1999). Interestingly, this phenomenon is frequently found in aged population and dementia patients in human. White matter rarefaction and glial cell activation are also observed. These pathological changes are thought to be associated with pathology of multi-infarct dementia and Alzheimer's disease (Leys, 1999; Roman, 2004).

There are 3 management methods for cerebral ischemia known to date; first, rapid restoration of oxygen supply to ischemic tissues; second, the prevention of inflammatory changes in affected areas; third, the protection of neuronal cells and effective functional recovery after focal cerebral ischemia. Currently used and developing agents for cerebral ischemia include anti-thrombic agents, immunizing agents, anti-inflammatory agents, free radical scavengers that scavenge toxic free radicals, Ca^{2+} channel antagonists and NMDA/AMPA antagonists (Zivin, 1998; Sobey, 2001; Scholler *et al*., 2004). However, use of these drugs is controversial because their effects are not only temporary and unsatisfactory but there are also serious side effects and toxicities.

Thus, efforts to develop effective cerebral function improving agents and therapeutic agents with minimal side effects for radical treatments are desperately needed. Thus, this study examined the neuroprotective effects of BF-7, the extraction of *Bombyx mori* which is known to enhance learning and memory to develop prophylactic and therapeutic agents with low toxicity that protect and improve brain functions by improving learning and memory impairments induced by cerebral ischemia (Chae *et al*., 2004). In this study, animal models of cerebral ischemia were developed by the temporary occlusion of middle cerebral artery followed by reperfusion to induce neuronal cell damage and memory impairment and the suppression of brain cell death and tissue damages resulted from transient cerebral ischemia were evaluated. Improvements in the deteriorated memory and cognitive function of animal models were examined by a cognitive function test using 8-arm radial maze. Furthermore, the effects of BF-7 on the improvement in the cognitive function of the human brain were evaluated by a clinical trial that included general population with various backgrounds using a standardized memory test, Rey complex figure test.

Materials and Methods

1. 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. A total of 40 rats, containing 10 rats for each group were used.

2. Preparation of materials

BF-7 was prepared by the method reported by Joo-Hong Yeo *et al.* (Yeo *et al*. 2004). Cocoons of *Bombyx mori* were boiled in 0.03% sodium carbonate and 0.05% marseilles soap solution for 30 min and dried. It was dissolved with $CaCl_2$ 5M solution at 98°C for 1 hr. The salt was completely removed by dialysis against distilled water and mixed with mixed protease solution. Then, BF-7 was separated and purified using Sephadex G-25 column chromatography. All materials for this experiment were purchase from Sigma Co.

3. Preparation of animal models of cerebral ischemia

Ketamine 30-40mg/kg was given by intramuscular injection to Sprague-Dawley rats weighing 200-250g and skin incision was made to the neck in the supine position. The sternocleidomastoid muscle was retracted laterally to expose the carotid artery, the internal carotid artery and the external carotid artery, and the arteries were split from adjacent tissues. Electrocautery was first applied to the superior thyroid artery branched from external carotid artery, larynx and arteries, and then to the pterygopalatine branched from the internal carotid artery. After the external carotid artery was dissected and 4-0 nylon was inserted to the internal carotid artery via the external carotid artery, the nylon was place in the 16-18mm from the carotid bifurcation. The incision was closed and nylon was removed an hour after the recovery from anesthesia. After reperfusion of the blood, BF-7 was administered and passive avoidance test, rota-rod test, 8-arm maze test and histopathologic staining were performed.

4. Histopathologic staining

The affected areas of cerebral infarct were measured 2 weeks after behavioural experiment. Rats were decapitated and the brain was removed. Confirming the origin of the middle cerebral artery in the internal carotid artery, bleeding and the location of nylon were assessed. After nylon in the vessel was removed, the whole brain was placed on the rodent brain matrix (RBM-4000C, ASI, U.S.A) and coronal sections were made at 2 mm interval from the end of frontal lobe. The slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride for 30 min, and then fixed with 10% neural buffer formalin. The fixed slices were photographed using the color video printer attached to a stereoscopic microscope and the affected areas of cerebral infarct were measured by an areameter. To examine the extent of cellular damages in the hippocampus, ketamine and Xylazine were administered and normal saline was run through the left ventricle. Then, rats were fixed with 4% buffed paraformaldehyde and their brain was removed. After paraffin embedding, serial coronal sections were made and hematoxylin and eosine (H&E) staining was performed.

5. 8-arm radial maze test

8-arm radial maze stands 4 cm from the floor consisting of eight equidistantly spaced arms (60 cm long and 12cm wide) which are all radiating from an octogonal central platform with a radius of 34cm radius and 40cm wall. At the end of each arm, there is a food cup and sunflower seeds were given as a reward. The light was dimmed and rats were monitored with a video camera under a lamp (50W). To train the rats, food supply was reduced down to 80% from a week prior to the experiment. Rats put in the maze for 30 min 3 times a day for 3 days to allow adaptation. The number of visit to each arm and time taken until sunflower seeds are all eaten were checked and rats were trained until the number of error was less than 2 times within 2 min. The number of error was defined as the number of visit to the arms where rats had already visited. Animal models of cerebral ischemia were prepared using trained rats, and BF-7 (10 mg/kg) was orally administered daily for 7 days. Then, the numbers of error and latency time were monitored for the following 5 days and compared with the control group.

6. Subject for clinical trial

Healthy Korean adults aged from 19 to 65 years with various backgrounds including university students, housewives, employees, people who visit religious organizations were recruited and randomized to each group regardless their jobs, the level of educations and residence to organize universally representative groups of Korea. 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 99 subjects including 26 men and 73 women were enrolled for the clinical trial and their mean age was 41.9 year (range 19 ~ 64 years). Initially, a total of 119 volunteers were enrolled; 39 volunteers were randomized into the placebo group; 40 volunteers were randomized into the BF-7 200mg group; 40 volunteers were randomized into the BF-7 400mg group. But, some of them were excluded and dropped during the trial and 99 volunteers who remained at the end of the trial were included for analyses. The overall participation rate was 83.5% including 32 volunteers from the placebo group (participation rate 82%), 33 volunteers from the BF-7 200mg group (participation rate 83.5%) and 34 volunteers from the BF-7 400mg (participation rate 85%). The numbers of subjects in each group were sufficient enough to prove statistical validity. The subjects were instructed to keep their diet and smoking habits but alcohol was prohibited.

7. Rey Complex Figure Test (RCFT)

Rey Complex Figure Test is a domestically and internationally approved neuropsychological test that is especially useful to assess memory and ability of organization among the functions of frontal lobe where memory, analysis, calculation, thought and judgment are controlled. The total score is 36 and memory is assessed using age graded scores.

8. Statistical analyses

All statistical analyses were performed using SPSS program and p <0.05 was considered statistically significant. Data of cellular and tissue experiments was presented as means ± standard deviations, and t test was used to examine correlations and differences between the groups. In the clinical trial, paired t-test was used to compare the differences among the BF-7 200mg group, BF-7 400mg group and placebo group, student's t-test or one-way ANOVA (Tukey's multiple comparison test) was used to show statistical significance for the differences.

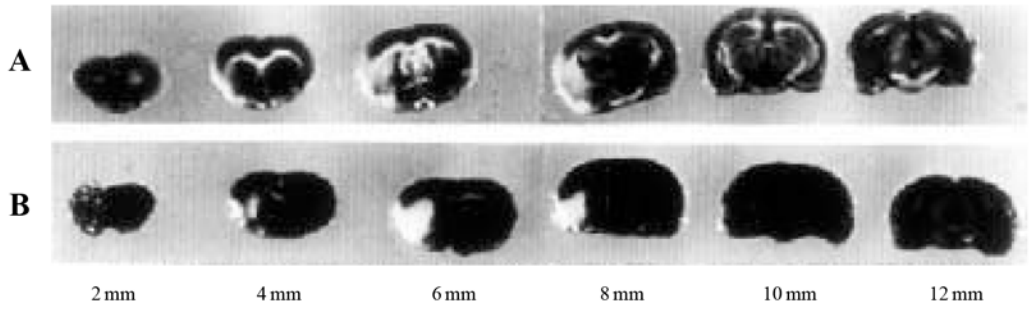

Fig. 1. The effect of BF-7 on the infarction by transient brain ischemia was examined using TTC staining. A. The brain sections from the rat treated with PBS alone. B. The brain sections from the rat treated with 10 mg/kg BF-7 once a day for 7 days. White area represented infarcted lesion.

Results

1. Histopathological staining

TTC staining enables to confirm the typical morphology of brain damages from transient focal ischemia induced by the artificial occlusion of middle cerebral artery (MCA) and the division between the affected areas and the normal tissue. Six coronal sections of frontal lobe were made at 2 mm interval and TTC staining was performed. The comparative analysis of the affected areas of cerebral infarct, infarction did not occur in the sham operated group and the overall extent of infarct was reduced in the BF-7 group compared to the vehicle control group as expected. Especially, cerebral infarct was significantly reduced in the most frequently damaged area by ischemic insult, 6 mm from the frontal pole (Fig. 1, 2). Moreover, the extent of neuronal cell damage was evaluated by H&E staining. The number of red (eosinophilic) neuron that appears during neuronal cell death was significantly reduced in the BF-7 group compared with the control group. In addition, cells with pyknosis were rarely appeared in the BF-7 (Fig. 3). This shows that BF-7 effectively defended damages of neural cells and brain tissues induced by transient focal cerebral ischemia. In this study, 10 rats were assigned for each group and none of rats showed abnormal symptoms or died.

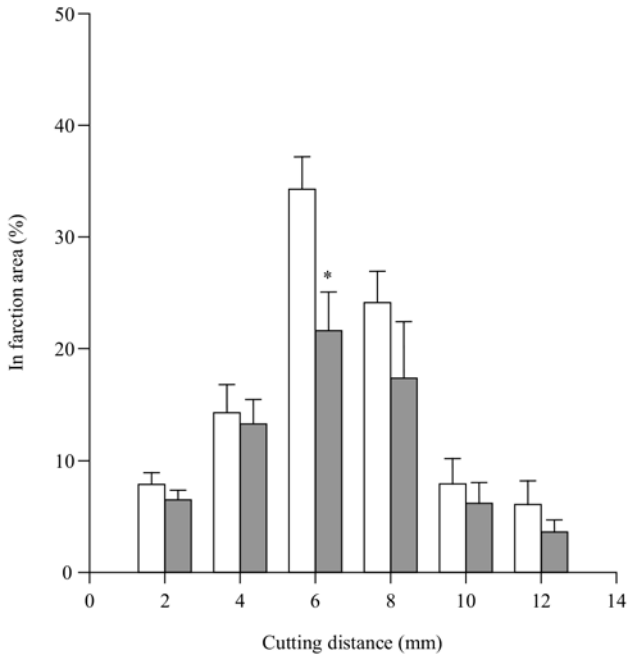

Fig. 2. Each bar represents the size of infarcted area examined by TTC staining. Slashed bar represents the BF-7 treated group for 7 days with 10 mg/kg, once a day. Empty bar indicated no BF-7 treated group. *: $p < 0.05$ vs. vehicle control.

2. 8-arm maze test

To evaluate protective effects of BF-7 on spatial memory impairment induced by cerebral ischemia, animal models of cerebral ischemia that were developed by the temporary occlusion of middle cerebral artery and changes in learning performance and memory were monitored using 8-arm maze test after 2 weeks of recovery. 8-arm maze test was conducted for 5 days after a week of BF-7 administration and the result showed that the number of error of the sham control group was not significantly different from that prior to the operation between the 1st day and the 5th day of the administration (Fig. 4). The number of error was reduced in the BF-7 group from the 3rd day, compared with the control group that received dissolvent and the difference was statistically significant. The number of error of the BF-7 was reduced down to that of the sham control group but the number of the error of the control group that received

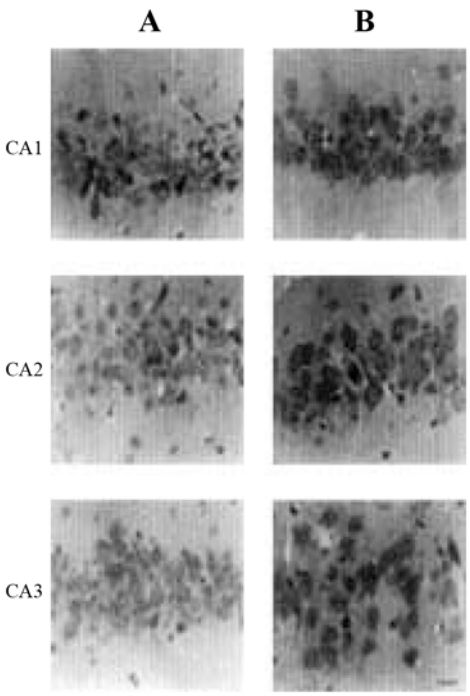

Fig. 3. Neuronal cell death in hippocampus A. Control group (no BF-7 treated) B. BF-7 treated group. The number of eosionophilic neurons in the BF-7 treated case was significantly reduced compared with that of no treated case.

only dissolvent failed to recover. This result showed that spatial memory deteriorated by the temporary occlusion of MCA but the deterioration was suppressed by the repetitive administration of BF-7, demonstrating that BF-7 effectively suppresses learning and memory impairment induced by cerebral ischemia and enhances spatial learning and memory.

3. Improvement in Rey Complex Figure Test

RCFT is a tool to evaluate the correlation between the ability of organization and memory by examining how effectively subjects remember and draw complex figures. A higher percentile means improvement in memory. The result showed that there was no significant difference in the placebo group (P>0.05) but, memory significantly improved from 36.8% to 56.5% in the BF-7 200mg group and from 24.7% to 65.2% in the BF-7 400mg group **(Fig. 5)**. These results suggest that administration of BF-7 effectively improved memory and the ability of complex figures and the improvement was statistically significant and dose-dependent.

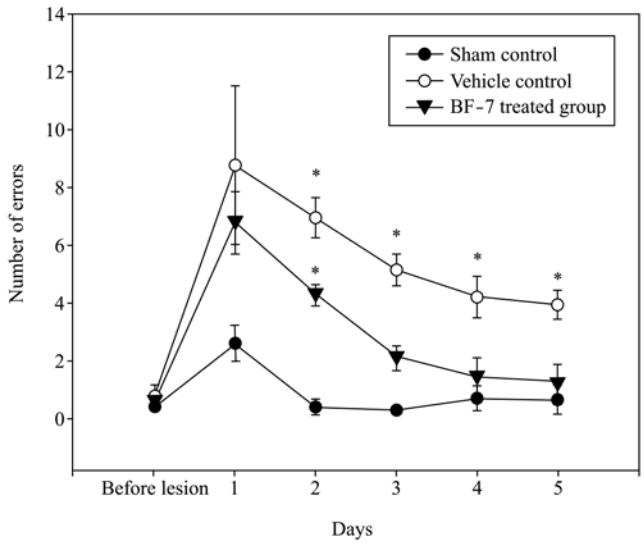

Fig. 4. The protective role of BF-7 on learning and memory impairment induced by brain ischemia. *: $p<0.05$ vs. vehicle control and sham control group.

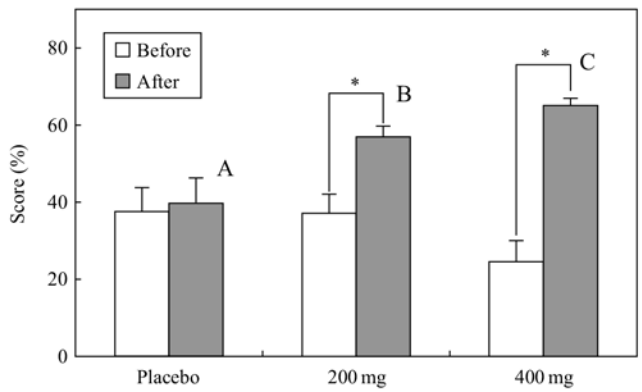

Fig. 5. Improvement effects of BF-7 on memory of complex figure. RCFT test was performed as described (Kim, H.G). The values are reported as a mean S.E.M. The scores before and after administration of BF-7 were compared with t-tests. And significant differences are shown as * ($p<0.05$). Relationship among placebo, 200 mg BF-7, and 400 mg BF-7 group were analyzed by one-way ANOVA (Turkey's multiple comparison test), and their statistical significant difference are shown as different characters (A, B, and C) ($p<0.05$).

Discussion

This study was to investigate whether BF-7 improves the cellular and tissue damages and deteriorated cognitive function and learning ability in animal models using Sprague-Dawley rats and in human; animal models of transient cerebral ischemia was developed by occluding.

the middle cerebral artery (MCA) for an hour to block blood supply to the brain followed by reperfusion; subjects with various background and age groups were recruited for the clinical trial. With animal models of cerebral ischemia, cellular and tissue damages were assessed by histopathologic examination and the improvement of cognitive function and learning ability was evaluated using 8-arm maze test. In the clinical trial, the improvement of memory was evaluated using RCFT.

TTC staining confirmed that focal cerebral infarct occurred by the occlusion of MCA and the affected areas of cerebral infarct was significantly smaller in the BF-7 group than in the control group. This implies that BF-7 has an excellent protective effect on the damages and destruction of neuronal cells and brain tissues induced by cerebral ischemia. Previous studies with rat models of cerebral ischemia reported that learning and memory impairments resulted from cerebral ischemia are closely associated with damages in the hippocampus.[12, 14] In this study, ischemia was induced by 1 hour of temporary MCA occlusion and learning and spatial cognitive memory were evaluated using 8-arm radial maze after 7 days. Having observed the numbers of errors in learning and memory-based judgment, it was apparent that cognitive impairment was induced by cerebral ischemia. With repetitive administration of BF-7 (7 times), memory failure induced by cerebral ischemia significantly improved. This is the first study demonstrating that BF-7 improves memory failure induced by cerebral ischemia. Eight-arm radial maze is a useful tool to evaluate spatial memory and learning ability and widely used to examine memory.[15-17] According to this study results, serious learning disability occurred during radial arm maze task when cerebral ischemia was induced by MCA occlusion in animal models and deficits in cognitive function and disability were improved after administration of BF-7. There are several agents that proved to be effective for cerebral ischemia and some of them are currently used. Despite of that, there are no therapeutic agents or management modalities that proved to have a clear effect for cognitive dysfunction and disability. Although a number of studies are actively conducted to develop therapeutic agents for cognitive dysfunction and disability from various angles, there is a dearth of researches on restoration of memory failure and learning ability.

Thus, this study aimed to examine the effects of BF-7 through an experiment on animal models of cerebral infarct and a clinical trial and we found that BF-7 improves cognitive functions such as learning and memory. To evaluate the effects of BF-7 on memory, ability of organization and learning in general populations, Rey Complex Figure Test (RCFT) was used. RCFT is an internationally approved and representative cognitive function test tool to examine memory and ability of organization. To organize groups that represent general Korean population, various age group and populations with various backgrounds were recruited, and to exclude subjectivities of investigators and subjects, this study was conducted as a double blind, placebo-controlled study. With RCFT, memory of complex figures and correlations between short-term and long-term memories of the figures can be evaluated. The results showed that there is no statistically significant difference in the placebo group but, memory was improved in the BF-7 groups (from 36.8% to 55.6% and from 24.7% to 65.2%, respectively) and the improvements were dose-dependent. This result suggests that BF-7 enhances spatial/three-dimensional memory and ability of organization for complex figures and has an excellent effect on improvement of cognitive functions such as learning and memory.

In conclusion, BF-7 is a raw material that effectively enhances brain function such as memory and cognitive function. This study results suggest that BF-7 has an protective effect on overall brain functions by defending neuronal cell damages and necrosis induced by cerebral ischemia and improving learning and memory impairments although clear mechanisms should be established via further studies in the future. Therefore, it is worth conducting further study to investigate pharmacological effects of BF-7 for the development of new therapeutic agents.

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