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원 저

Gerbil의 전뇌허혈에 대한 大黃의 신경보호효과

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Neuroprotecticve Effect of *Rhei Rhizoma* on Transient Global Ischemia in Gerbil

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목적 : 본 실험에서는 gerbil을 이용한 전뇌허혈동물모델에서 뇌허혈손상 직후 지연성 뇌손상에 대한 대황의 방어효과와 Apoptosis 과정중의 Bax와 Bcl-2 단백질에 대한 조절작용을 관찰하고, TUNEL 염색법을 통하여 대황이 gerbil hippocampus CA1 영역의 pyramidal neuron의 세포사에 미치는 영향과 PC12 세포를 이용한 세포배양 모델에서의 대황의 신경방어 효과를 관찰하였다.

방법 : Mongolian gerbil의 총경동맥을 5분간 폐색하여 가역성 전뇌허혈을 유발시킨 후 대황의 전탕액을 하루에 한번 경구 투여하였다. 대황의 신경 보호 효과는 수술 7일 후에 cresyl violet으로 염색하여, 살아있는 신경 세포의 수를 세어 측정하였다. 또, 수술 3일후에는 면역조직화학적 방법을 통하여 Bax, Bcl-2 단백질의 발현과 대황의 신경보호 효과와의 관련성을 알아보았다.

결과 : 가역적 전뇌허혈이 일어난 동물군의 경우 hippocampus의 CA1 영역에서 살아있는 신경세포의 수는 51.0±2.5개 /mm에 불과하였으나, 그에 비해 수술 후 7일간 대황을 투여한 동물군은 106.2±2.5개 /mm로 살아있는 신경세포수가 크게 증가하였다.

Apoptosis를 촉진하는 단백질인 Bax의 발현은 3일간 대황을 투여한 동물군의 경우 hippocampus의 CA1 영역에서 현저하게 저해되었고, apoptosis를 억제하는 Bcl-2 단백질의 발현은 변화가 없었다. TUNEL assay를 통하여 살펴본 결과 대황 투여군의 apoptotic 신경세포사가 감소하였으며 이는 Bax protein의 발현과 유사한 양상을 나타내었다.

결론 : 대황이 Bax 단백질의 발현을 억제하여 상대적으로 Bax/Bcl-2 자연적 세포사를 억제하여 Mogolian gerbil의 가역성 전뇌허혈 모델에서 신경보호효과를 나타내는 것으로 사료된다.

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Key Words: cerebral ishcemia, MTT assay, immunohistochemistry, *Rhei Rhizoma*, gerbil

Introduction

Stagnant blood is a kind of pathologic metabolites existed in some part of the human body[1]. It is caused by the stagnation of blood that cannot perform normal

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functions. Blood stagnation syndrome(BSS) is defined as the syndrome induced by blood stagnation[1]. Active oxygen species and free radicals react with biomolecular constituents (e.g., lipids, protein, and DNA) to cause certain clinical diseases[2,3], such as cerebral ischemia, atherosclerosis, inflammation, diabetes, and cancer, which are regarded as damage associated with BSS in Korean traditional medicine[1,4].

Rhubarb (*Rhei Rhizoma*) is commonly employed as a crude purgative drug and, in Korean herbal therapy, it is widely used in combination with other crude drugs for the treatment of many diseases[5]. It contains hydroxyanthraquinone derivatives: *Rhein*, emodin, aloe-emodin, chrysophanol, physcion and their glycosides having various pharmacological actions[2,3,5], such as purgation, anti-bacteria, anti-tumor. Rhubarbs, the rhizomes of Rheum palmatum L., R. tanguticum Maxim., R. offinale Baill., R. coreanum Nakai, and R. undulatum L., are used in remedies for BSS as well as a purgative agent in Korean, Japanese, and Chinese traditional medicines[5]. Among them, the rhizome of R. undulatum, a Korean rhubarb, is considered to have less purgative effect but more effect on BSS than other kinds of rhubarbs[2,3,6]. According to recent study, the nitric oxide (NO) production inhibitory activity in lipopolysaccharide-activated mouse macrophages[6], anti-platelet aggregation[7], and anti-allergic[3,8], anti-bacterial[9] and anti-inflammatory[10] effects in rodents were reported as its anti-blood stagnation effects.

The many diseases such as stroke, Alzheimer disease which associated with cerebral ischemia are regarded as the damage by BSS in Korean traditional medicine[1,4,11]. Therefore, the remedy for stroke could be possible by removal of blood stagnation in body.

Apoptosis is associated with the activation of a genetic program in which apoptosis effector genes promote cell death[12,13], while repressor genes enhance cell survival[14,15]. Among the apoptosis repressor genes studied in mammalian cells, the proto-oncogene Bcl-2 has attracted in many cases[15,16]. Bcl-2 is expressed in high levels in neuronal tissues during embryonic development[17,18,19], but is downregulated in the adult central nervous system[20]. Bax (bcl-2 associated X protein) is a protein homologous to Bcl-2 that forms a dimer with it and prevents from the actions of Bcl-2[12]. The exact mechanism by which Bcl-2 and Bax influence cell survival is not known. However there is a possible explanation that overexpression of Bax leads to the activation of specific proteases involved in apoptosis termed caspases[21] and eventually cell destruction[12,22]. Overexpression of Bcl-2 in transgenic mice protects neurons from experimental ischemia induced by permanent middle cerebral artery(MCA) occlusion[23]. A decrease in immunoreactivity for Bcl-2, Bcl-x and an increase in immunoreactivity for Bax is observed in neurons within the ischemic cortex and thalamus, whereas all Bcl-2 family members decrease in the infarcted caudate-putamen following permanent MCA occlusion in rats[24]. These results suggest that a decline of Bcl-2 accompanied by an increase of Bax contribute to neuronal death and that Bcl-2 plays a role in cell survival[25]. In other words, the balance between neuroprotective and neurodegenerative members of the Bcl-2 family of proto-oncogenes may plays a critical role in determining cell survival following cerebral ischemia.

The purpose of this study was, therefore, to examine the protective effect of *Rhei Rhizoma* against neuronal damage following cerebral ischemia in vivo with a widely used experimental model of cerebral ischemia in Mongolian gerbils. We also immunohistochemically investigated the changes of Bax and Bcl-2 protein as well as TUNEL (terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling) reaction by treatment of *Rhei Rhizoma* following 2 vessel occlusion (VO) induced ischemia.

Materials and Methods

1. Preparation and administration of Rhei Rhizoma

Dried roots of *Rhei Rhizoma* (200 g) were purchased from Kyung Hee University Medical Center, Seoul, Korea in 2000. They were boiled with distilled water (2 L) for 2 h. The supernatant was filtered and evaporated under reduced pressure to give aqueous extracts, which was lyophilized (36.8 g). For the assessment of neuroprotective effects, powered extracts of *Rhei Rhizoma* was dissolved in distilled water to obtain a solution of 12 mg/ml.

2. Animals

Adult male Mogolian Gerbils (Meriones unguiculatus : Charles River Labs, Raleigh, NC, U.S.A) weighting 65-75 g were used for all experiments. The animals housed in a controlled environment on a 12 h light : 12 h dark cycle with free access to food and water. The animals were acclimated to the cage for a minimum of 7 days before surgery. The experimental procedures were carried out in accordance with the animal care guidelines of NIH and the Korea Academy of Medical Sciences. All chemical was obtained from Sigma Co. except mentioned.

3. Ischemic surgery

Adult male Mogolian Gerbils were subjected to transient global cerebral ischemia by occluding the carotid arteries for 5 min. The animals were anesthetized by inhalation of 5 % (v/v) isoflurane and a 70 % / 30 % (v/v) mixture of nitrous oxide/oxygen, then subsequently maintained with 1 % isoflurane. The experimental procedure for 2 vessel occlusion was similar with previously described method[16,19] except small changes. In brief, the right and left common carotid arteries were exposed through an anterior midline cervical incision and isolated from the vagus nerves and the surrounding tissues. Ischemia was induced by occluding the carotid arteries with aneurysm clamps for 5 min. The clamps were removed after 5 min and restoration of blood flow verified visually. Then the neck incision was closed with a silk suture and the animals were allowed to awaken from anesthesia and were returned to their cage. The rectal temperature, monitored with a digital thermometer inserted 5 cm into the anus, was maintained at 37℃ during the surgical procedure using heating pad and an incandescent lamp.

Animals that had been subjected to 5 min occlusion were randomly divided into two groups. A solution of *Rhei Rhizoma* (sample group, n=3, 160 mg/kg/ml) and distilled water(control group, n=7, 1 ml) were given orally once dalily for 3 or 7 days after surgery. Distilled water was used as vehicle in control group. Gerbils were sacrificed at 3 days, 7 days after surgery.

4. Tissue preparation

3 or 7 days after ischemia, animals were anesthetized with sodium pentobarbital (25 mg/kg, i.m) and then transcardially perfused for fixation with 10% formalin in 0.1 M phosphate buffer saline, pH 7.4 (PBS). Immediately afterwards, the brains were removed from the skull and fixed in the same fixative solution for 6 h. The brains were embedded in paraffin and 8-㎛-thick sections were cut and mounted on poly-L-lysine coated slides.

5. Histology

8 ㎛ deparaffinized sections were stained with cresyl violet for 10 min and the density of staining in the hippocampal CA1 area was measured using a light microscope. Cell viabilities was evaluated by counting pyramidal neurons of linear 1 ㎜ in hippocampal CA1 area.

6. Bax and Bcl-2 immunohistochemistry

For Immunohistochemical detection of Bax and Bcl-2, gerbils were sacrificed at 72 h after surgery[26].

Immunohistochemistry was carried out following the avidin-biotin-peroxidase method (Vectastatin ABC kit, Vector Labs). In brief, 8 ㎛ deparaffinized sections were incubated with 0.3 % hydrogen peroxide and methanol for 20 min to diminish nonspecific staining and later boiled in 10 mM citrate buffer for antigen retrieval, pH 6.0 in microwave oven for 10 min. Mouse anti-Bax (B-9, Santa Cruz, CA, USA) and mouse anti-Bcl-2 (C-2, Santa Cruz, CA, USA) antibodies used at a dilution of 1:2000 and 1:100, respectively in 0.05M Tris-buffered solution, pH 7.6 (TBS) were added to the slide and incubated overnight at 4 ℃. The sections were later incubated for 1 h biotinylated and anti-mouse IgG antibody (Vector) and, finally, with the ABC complex at a dilution of 1:100 for 1h. Peroxidase was visualized with 0.05 % 3,3' -diaminobenzidine in TBS and 0.01 % hydrogen peroxide. The specificity of the immuno-reaction was tested by incubating sections without the primarily antibody.

7. Measurement of DNA fragmentation

The cleaved DNA was identified using a modified end-labeling technique originally described by Gavrieli et al. (Apoptag Kit, Intergen, U.S.A). The deparaffinized sections were permeabilized with proteinase K (20 ㎍/ml) for 15 min at room temperature and washed four times with deionized water for a total of 20 min. Endogenous peroxidase was quenched with 3 % (v/v) hydrogen peroxide in PBS for 5 min and the tissue was rinsed twice with PBS for a total of 10 min. After equilibration in buffer, the sections were incubated with terminal deoxyneucleotidyl tranferase (TdT) containing digoxigenin-dUTP (0.3 e.u./ml) at 37 ℃ for 1 h in a humidified chamber. The TdT reaction was stopped with Stop/Wash buffer for 10 min followed by three washes with PBS for 15 min. Anti-digoxigenin peroxidase was added to each tissue section and the sections were incubated for an additional 30 min at room temperature. The staining was developed with diaminobenzidine.

8. Hypoxia/reperfusion model using PC12 cell

PC12 cells (the rat pheochromocytoma cell line) were grown in DMEM (Dulbecco's modified Eagle's medium, Gibco BRL, U.S.A) supplemented with 5 % Fetal Bovine Serum (Gibco BRL, U.S.A), 10 % horse serum (Gibco BRL, U.S.A) and 1 % penicillin-streptomycin (Gibco BRL, U.S.A). The cultures were maintained in a humidified atmosphere containing 5 % CO_2 at 37 °C.

For MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] analysis, exponentially growing PC12 cells were seeded at density of 5000 cells in 90 ㎕ medium per well in 96-well plates. It was maintained in CO_2 incubator (5 % CO_2, humid atmosphere, Forma, U.S.A). After 24 h, PC12 cells were pretreated with the same medium 10 ㎕ but containing final concentrations (20 ㎍/㎖ or 2㎍/㎖) of *Rhei Rhizoma* extracts dissolved in PBS (phosphate-buffered saline) for 2 h before the exposure to hypoxic condition. Controls were treated with PBS instead of *Rhei Rhizoma* extracts. Thereafter, cell cultures were exposed to hypoxia in a humidified temperature-controlled hypoxia chamber (Forma, U.S.A), which was purged with 85 % N_2 / 10 % H_2 / 5 % CO_2 atmosphere, at 37° C. Where indicated, after exposure to hypoxia. Cultures were returned to an atmosphere with ambient oxygen levels in CO_2 incubator. Normoxic condition was induced to compare with hypoxic condition. 10 ㎕ of MTT solution(final concentration, 1 mg/ml) was added per well and incubation continued for another 1 h. Then, dimethyl sulfoxide was used to solubilize the formazan reaction product for 10 min in 60℃ incubator. It was quantitated

with an ELISA plate reader at 570, 650 nm. The absorbance directly correlates with cell number.

9. Statistical analysis

All data were presented as mean ± SEM. Statistical comparison between different treatment with P value < 0.05 were considered statistically significant.

Results

1. The morphologic features of pyramidal neurons in hippocampal CA1 area

Microphotographs of the hippocampal CA1 subfield in each group are shown in Fig. 1. Delayed cell death was observed in the CA1 area of the hippocampus on the 7 days following 5 min of forebrain ischemia in the gerbil. The CA1 neurons were almost completely destroyed in the distilled water treated group 7 days after ischemia. Only a few neurons were preserved in the pyramidal cell layer and a large number of glial cells appeared in this area (Fig. 1;D). When compared with distilled water treated group, dying cells in ischemic gerbils showed shrunken cytoplasm and degeneration of the nucleus (Fig. 1;C,D). In contrast, *Rhei Rhizoma* treated group displayed more intact pyramidal neurons in hippocampus CA1 area than distilled water treated group (Fig. 1;E,F). Although it did not return to the same staining intensity as control group, cells were quite intensely distributed and furthermore cell body preserved almost intact shape (Fig. 1;F).

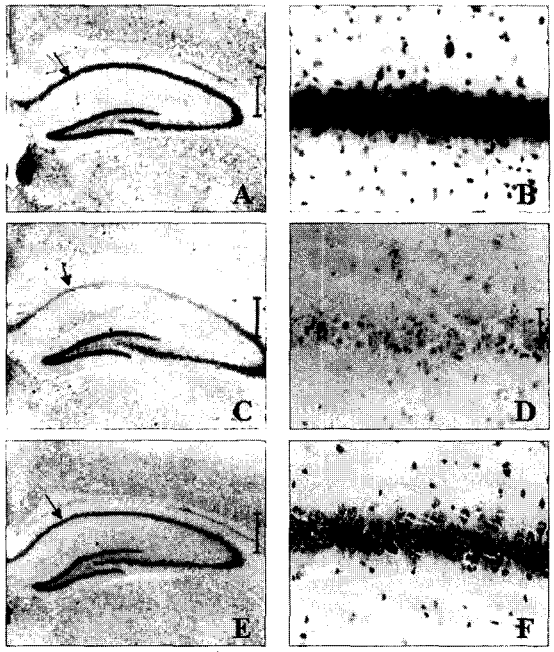

Fig. 1. Typical photomicrographs of the gerbil hippocampus 7 days after 5 min ischemia in the normal (A, B), control (C, D), sample (E, F) group. 8 ㎛ deparaffinized sections were stained with cresyl violet for 10 min. Cresyl violet staining of coronal brain sections at the level of the dorsal hippocampus show selective, delayed neuronal cell death in the hippocampal CA1 area. Arrows indicate a part of CA1 area where magnified.

Table 1. The Counts of Intact Pyramidal Cell in the CA1 Area of the Gerbil Hippocampus 7 Days after 5 Min Occlusion Induced Ischemia.

	Normal	Control	Sample
Cell counts[1)]	153.5 ± 4.9	51.0 ± 2.5	106.2* ± 2.5
n	8	7	3
Viability percent	100	33.2	69.22
Damaged percent	-	66.8	31.8

1): expressed as mean ± SEM
*; P<0.01
Normal; No treated group
Control; Distilled water treated group at 7 days after ischemia
Sample; *Rhei Rhizoma* treated group at 7 days after ischemia
The intact neurons visible within 500 ㎛ of hippocampus CA1 area were counted.

2. The cell counts of intact neurons in hippocampal CA1 area

The intact neurons visible within 500 ㎛ of hippocampus CA1 area were counted. (Table 1, Fig. 2.)

3. Immunohistochemical detection of Bax, Bcl-2

Bcl-2 immunoreactive cells, as detected with the Bcl-2 antibody, were not observed in the hippocampal CA1 area of normal group (Table 2, Fig. 3;A,B). Some Bcl-2 immunoreactivity was detected in the CA1 area on the 3

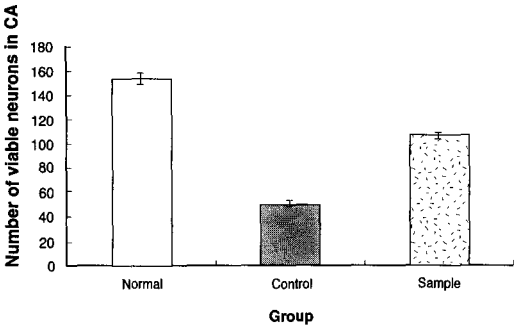

Fig. 2. The counts of intact pyramidal cell in the CA1 area of the gerbil hippocampus 7 days after 5 min occlusion induced ischemia. Normal; No treated group, Control; Distilled water treated group at 7 days after ischemia, Sample; *Rhei Rhizoma* treated group at 7 days after ischemia.

days following ischemia (Table 2, Fig. 3;C,D). In Fig. 3;D, The majority of Bcl-2 immunoreactive cells displayed the morphological features of dying cells, as revealed in cresyl violet staining (cf. Fig. 1;D). Scattered, surviving Bcl-2 immunoreactive neurons were observed in the CA1 area at 3 days after ischemia (Fig. 3;D). In CA1 nuerons of *Rhei Rhizoma* treated group, expression of Bcl-2 immunoreactivity was similar to distilled water treated group (Table 2, Fig. 3;E,F).

On other hand, Bax expression was scarcely observed in the cytoplasm of CA1 neurons in normal gerbil group (Table 2, Fig. 4;A,B). Immunoreactivity of Bax was detected very strongly in CA1 neurons at 3 day following transient forebrain ischemia (Table 2, Fig. 4;C,D). In CA1 neurons of *Rhei Rhizoma* treated group,

Table 2. Change in Bax, Bcl-2 in the CA1 Sector following Transient Ischemia after 72 hrs.

	Bax	Bcl-2
Normal	+	-
Control	++	+
Sample	+	+

The results of immunohistochemistry(Bax, Bcl-2) are summarized. Gerbils are treated *Rhei Rhizoma* for 72 h after 5 min ischemia. The staining intensity are devided into three groups (-; negative, +; positive, ++; strongly positive). Normal; No treated group, Control; Distilled water treated group at 7 days after ischemia, Sample.; *Rhei Rhizoma* treated group at 7 days after ischemia

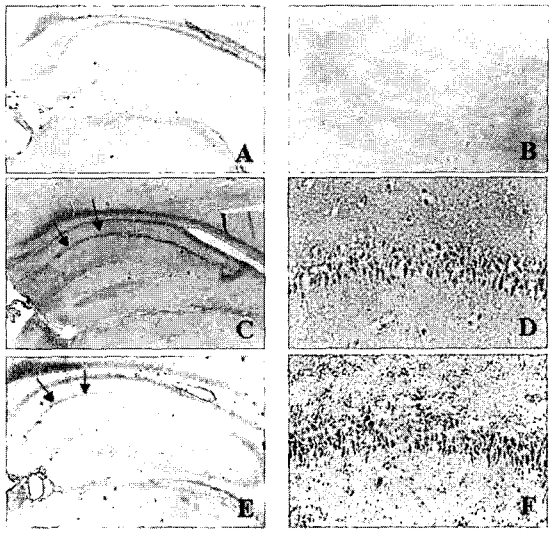

Fig. 3. Immunohistochemical analysis of Bcl-2 protein in sections from the normal (A, B), control (C, D), sample (E, F) group following transient forebrain ischemia. B, D, F shows magnified CA1 area in C, E (arrows). Normal; No treated group, Control; Distilled water treated group at 7 days after ischemia, Sample; *Rhei Rhizoma* treated group at 7 days after ischemia. A, C, E; ×20, B, D, F; ×200.

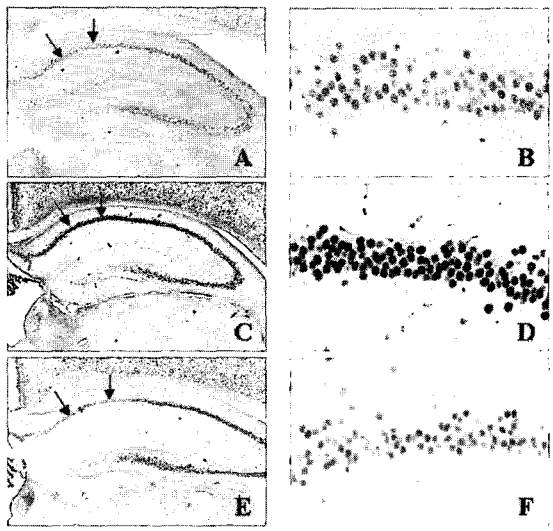

Fig. 4. Immunohistochemical analysis of Bax protein in sections from the normal (A, B), control (C, D), sample (E, F) group following transient forebrain ischemia. B, D, F shows magnified CA1 area in A, C, E (arrows). Normal; No treated group, Control; Distilled water treated group at 7 days after ischemia, Sample; *Rhei Rhizoma* treated group at 7 days after ischemia. A, C, E; ×20, B, D, F; ×200.

immunoreactivity of Bax was decrease obviously compare to distilled water treated group (Table 2, Fig. 4;E,F).

4. TUNEL staining

The ischemia-induced apoptotic reaction, visualized by TUNEL, was observed almost exclusively in the pyramidal neurons of the CA1 region of gerbil hippocampus at 72 h after 5 min transient cerebral ischemia.

No TUNEL reaction-positive CA1 pyramidal neurons were present in normal group (Table 3, Fig. 5;A,B). Positive staining for TUNEL reaction in CA1 pyramidal neurons of 2VO-induced ischemia group were detected at 72 h after the ischemic insult (Table 3, Fig. 5;C,D). Positively stained neurons were localized not only in the nuclei but also in the cell bodies and dendrites.

Rhei Rhizoma treated group also showed TUNEL reaction-positive CA1 pyramidal neurons at 72 h (Table 3, Fig. 5;E,F). The morphological features of TUNEL-positive hippocampal CA1 pyramidal neurons of *Rhei Rhizoma* treated animals were similar to those of 2VO induced ischemia group, but TUNEL-reactivity of *Rhei Rhizoma* treated group was more decreased in comparison with distilled water treated group (Fig. 5;F).

5. The effect of Rhei Rhizoma on PC12 cell

In hypoxia/reperfusion model induced by cultured in normoxic chamber at 6 h after cultured in hypoxic chamber at 48 h, the cell viability of *Rhei Rhizoma* treated PC12 cell represents 113% at 2 ㎍/ml and 118% at 20 ㎍/ml in comparison with non-treated PC12 cell. The effectiveness ratio of *Rhei Rhizoma* on PC12 cell increased with dose-increasing (Fig. 6.).

Table 3. Change of TUNEL-reactivity in the CA1 Area following Transient Ischemia after 72 hrs.

Group	TUNEL
Normal	-
Control	++
Sample	+

Gerbils are treated *Rhei Rhizoma* for 72 h after 5 min ischemia. The staining intensity are devided into three groups (-; negative, +; positive, ++; strongly positive) Normal; No treated group, Control; Distilled water treated group at 7 days after ischemia, Sample.; *Rhei Rhizoma* treated group at 7 days after ischemia

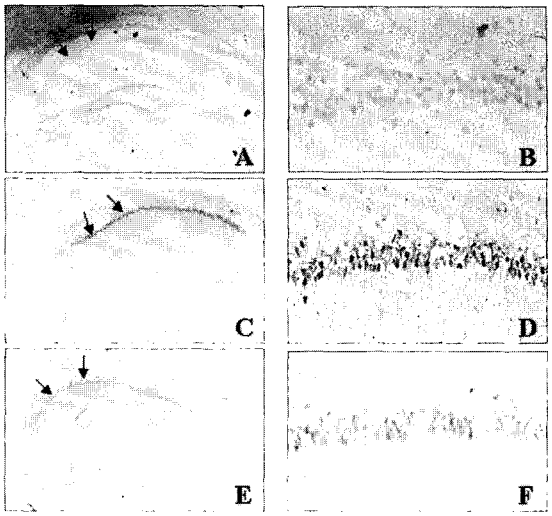

Fig. 5. Terminal transferase dUTP nick-end labelling (TUNEL) staining of hippocampal CA1 pyramidal neurons at 72 h after ischemic insult in normal (A, B), control (C, D) and sample (E, F) groups. Arrows show that TUNEL staining is exclusively localized to CA1 area. Normal; No treated group, Control; Distilled water treated group at 7 days after ischemia, Sample; *Rhei Rhizoma* treated group at 7 days after ischemia. A, C, E; ×20, B, D, F; ×200.

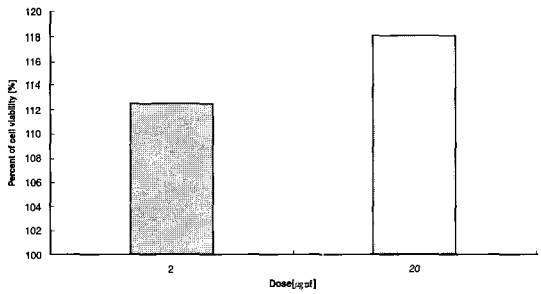

Fig. 6. The effect of *Rhei Rhizoma* on hypoxia/reperfusion model using PC12 cell. PC12 cells were pretreated with Rhei Rhizoma 2 h before the exposure to hypoxic condition. Reperfusion was induced by cultured in normoxic chamber at 6 h after cultured in hypoxic chamber at 48 h. The effective ratio was evaluated by MTT assay.

Discussion

The intracellular processes leading to neuronal death are markedly different in different types of cerebral ischemia in regard to morphology, cytoskeletal integrity and gene expression[27,28]. After severe ischemia, as often seen in the ischemic core of focal ischemia, neurons show typical necrosis with cell swelling and membrane disruption[27]. On the other hand, process of apoptosis is showed in neuronal death after milder ischemic insult, such as in delayed neuronal death (DND) after transient global ischemia[29] or in neuronal death in the ischemic penumbra[30,31].

Although there are many hypotheses proposed regarding the mechanism of delayed neuronal death (DND) after transient forebrain ischemia, it is influential hypothesis that mitochondrial damage plays a key role in DND[32]. Bcl-2 family genes play a important role in regulating mitochondrial permeability. Increase of mitochondrial permeability result from down-regulation of Bcl-2 or other antiapoptotic Bcl-2 family members, or from overexpression and translocation of proapoptotic Bcl-2 family members, such as Bax, to the mitochondrial membrane[32]. Increased mitochondrial permeability results in release of cytochrome c from the mitochondria to the cytosol.

The present results showed that Bcl-2 is expressed at low levels, whereas Bax is moderately expressed in the normal hippocampus of the adult gerbil. Similar findings have been reported in adults of other species of rodents[26,33]. Our results have also shown moderate expression of Bcl-2 and very strong expression of Bax in the CA1 area of the hippocampus following transient forebrain ischemia. On the other hand, it is shown that expressions of both Bcl-2 and Bax are moderate in the *Rhei Rhizoma* treated group. In present study, the results showed that the treatment of *Rhei Rhizoma* significantly reduced the delayed neuronal damage induced by transient global ischemia in the gerbils when administered immediately after ischemic insults. It seems that the treatment of *Rhei Rhizoma* inhibits the expression of Bax in hippocampal CA1 area resulting in promoting Bcl-2 expression relatively and leads to promoting cell survival in apoptosis following global ischemia. It could also be possible the neuroprotective mechanism of *Rhei Rhizoma* prevents cytocrome c release from mitochondria to cytosol by regulating Bax and Bcl-2 protein.

Morphological characteristics of apoptosis include cell shrinkage, cytoplasmic condensation, chromatin segregation and condensation, membrane blebbing, and the formation of membrane-bound apoptotic bodies[28] whereas the biochemical hallmark of apoptosis is internucleosomal DNA cleavage[34] into oligonucleosome- length fragments that can be directly visualized by end-labeling with biotinylated dUTP in a reaction that employs terminal deoxynucleotide transferase TUNEL[27,35]. In present study, we demonstrated that the treatment of *Rhei Rhizoma* decreased the number of TUNEL-positive CA1 pyramidal neurons and inhibited the appearance of TUNEL-positive neurons. Thus, these results suggest that *Rhei Rhizoma* may inhibit the delayed neuronal damage, by preventing from the DNA damage. This effect is probably due to regulation of Bax and Bcl-2 system. Therefore, *Rhei Rhizoma* may be considered as a potentially effective intervention in cerebral ischemic diasease.

It could be possible that some agents have neuroprotective effects with their hypothermic effect during or after ischemia. However, the neuroprotective effect by hypothermia is possible only within 3 h after ischemia[36]. We excluded a possibility of neuroprotective effect by hypothermia. Therefore, we considered that the neuroprotective effect of *Rhei Rhizoma* was not

based on the hypothermia.

It is generally believed that ischemia/reperfusion-mediated brain injury results from the oxidation of cellular macromolecules[16]. It is found that formation of lipids and DNA oxidation products cause cellular dysfunction and cell death, which leads to further impairment of the central nervous system[37]. The formation of these oxidation products in the human brain increases with age and ischemia/reperfusion animal model[20]. Therefore, the effects of antioxidants, free radical scavengers or trapping agents are studied with interest for use as potential neuroprotective agents of various brain injuries. Resently, *Rhei Rhizoma* has been reported to have antioxidant properties[2]. In present study, we demonstrated that the treatment of *Rhei Rhizoma* increased cell viability in hypoxia/reperfusion model using PC12 cell. It seemed that the treatment of *Rhei Rhizoma* had neuroprotective effect as antioxidant.

In conclusion, these findings clearly demonstrate that *Rhei Rizoma* has a protective effect on ischemia-induced brain injury. This study suggests that *Rhei Rhizoma* may be a useful agent for the prevention of cerebral ischemic damage. Recently, it was also found that *Rhei Rhizoma* has anti-platelet aggregation activity[7] in the human platelet. This may have an important influence on the re-establishment and maintenance of cerebral blood flow after ischemia. The roles and mechanisms of the anti-platelet activities of *Rhei Rhizoma* on ischemia-induced brain injury, and other biological functions of *Rhei Rhizoma*, are currently under investigation.

References

1. 安圭錫. 東醫病理學. 서울. 高文社. 1990:74-76.
2. Matsuda H, Morikawa T, Toguchida I, Park JY, Harima S, Yoshikawa M. Antioxidant constituents from rhubarb: structural requirements of stilbenes for the activity and structures of two new anthraquinone glucosides. Bioorganic & Medicinal Chemistry. 2001;9(1):41-50.
3. Matsuda H, Tomohiro N, Hiraba K, Harima S, Ko S, Matsuo K, Yoshikawa M, Kubo M. Study on anti-Oketsu activity of rhubarb II. Anti-allergic effects of stilbene components from *Rhei* undulati *Rhizoma* (dried rhizome of Rheum undulatum cultivated in Korea). Biol Pharm Bull. 2001;24(3):264-267.
4. 金永錫. 臨床中風學. 서울:書苑堂. 1997:343.
5. 金讚哲. 韓藥藥理學. 서울:集文堂. 2001:174-147.
6. Matsuda H, Kageura T, Morikawa T, Toguchida I, Harima S, Yoshikawa M. Effects of stilbene constituents from rhubarb on nitric oxide production in lipopolysaccharide-activated macrophages. Bioorganic & Medicinal Chemistry Letters. 2000;10(4):323-327.
7. Ko SK, Lee SM, Whang WK. Anti-platelet aggregation activity of stilbene derivatives from Rheum undulatum. Arch Pharm Res. 1999;22(4):401-403.
8. Kim DH, Park EK, Bae EA, Han MJ. Metabolism of rhaponticin and chrysophanol 8-o-beta-D-glucopyranoside from the rhizome of rheum undulatum by human intestinal bacteria and their anti-allergic actions. Biol Pharm Bull. 2000;23(7):830-833.
9. Jiang WJ. Effect of processing on the antibacterial action and constituents of Rhubarb. Zhong Yao Tong Bao. 1983;8(3):18-20.
10. Wu LY. Effect of rhubarb processing on its anti-inflammatory action. Zhong Yao Tong Bao. 1985; 10(6):18-21.
11. 權純琮. 臨床韓醫學講座. 서울:不二學會出版. 1997:99.
12. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell. 1993; 27;74(4):609-619.
13. Vekrellis K, McCarthy MJ, Watson A, Whitfield J,

Rubin LL, Ham J. Bax promotes neuronal cell death and is downregulated during the development of the nervous system. Development. 1997;124(6):1239-1249.

14. Hockenberry D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature. 1990;348:334-336.
15. Shimazaki K, Ishida A, Kawai N. Increase in Bcl-2 oncoprotein and the tolerance to ischemia-induced neuronal death in the gerbil hippocampus. Neurosci Res. 1994;20:95-99.
16. Siesjo BK. A new perspective on ischemic brain damage?. Prog Brain Res. 1993;96:1-9.
17. Ferrer I, Tortosa A, Condom E, Blanco R, Macaya A, Planas AM. Increased expression of Bcl-2 immunoreactivity in the developing cerebral cortex of the rat. Neurosci Lett. 1994;179:13-16.
18. LeBrun DP, Warnke RA, Cleary ML. Expression of bcl-2 in fetal tissues suggests a role in morphogenesis. Am J Pathol. 1993;142(3):743-753.
19. Merry DE, Veis DJ, Hickey WF, Korsmeyer SJ. bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. Development. 1994;120(2):301-311.
20. Mecocci P, MacGarvey U, Kaufman AE, Koontz D, Shoffner JM, Wallace DC, Beal MF. Oxidative damage to mitochondrial DNA shows marked age-dependent increases in human brain. Ann Neurol. 1993;34:609-616.
21. Villa P, Kaufmann SH, Earnshaw WC. Caspases and caspase inhibitors. Trends Biochem Sci. 1997;22(10): 388-393.
22. Antonsson BC. Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim the mitochondrion. Cell Tissue Res. 2001;306(3):347-361.
23. Martinou J-C, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C, Huarte J. Overexpression of bcl-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron. 1994;13:1017-1030.
24. Guillardon F, Lenz C, Waschke KF, Krajewski S, Reed JC, Zimmermann M, Kuschinsky W. Altered expression of Bcl-2, Bcl-x, Bax, and c-Fos colocalizes with DNA fragmentation and ischemic cell damage following middle cerebral artery occlusion in rats. Brain Res Mol Brain Res. 1996;40:254-260.
25. Vaux DL, Cory S, Adams JM. Bcl-2 promotes the survival of hematopoietic cells and cooperates with c-myc to immortalize pre-B cells. Nature. 1988;335:440-442.
26. Hara A, Iwai T, Niwa M, Uematsu T, Yoshimi N, Tanaka T, Mori H. Immunohistochemical detection of Bax and Bcl-2 proteins in gerbil hippocampus following transient forebrain ischemia. Brain Res. 1996;711(1-2):249-253.
27. Charriaut-Marlangue C, Margaill I, Represa A, Popovici T, Plotkine M, Ben-Ari Y. Apoptosis and necrosis after reversible focal ischemia: an in situ DNA fragmentation analysis. J Cerebr Blood Flow Metab. 1996;16:186-194.
28. Walker NI, Harmon BV, Gobe GC, Kerr JF. Patterns of cell death. Methods Achiev Exp Pathol. 1988;13:18-54.
29. Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res. 1982;239:57-69.
30. Du C, Hu R, Csernansky CA, Hsu CY, Choi DW. Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? J cerebr Blood Flow Metab. 1996;16:195-201.
31. Linnik MD, Zobrist RH, Hatfield MD. Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke. 1993;24:2002-2009.
32. Reed JC, Jurgensmeier JM, Matsuyama S, Bcl-2

family proteins and mitochondria. Biochim Biophys Acta. 1998;1366:127-137.

33. Ferrer I, Lopez E, Blanco R, Rivera R, Ballabriga J, Pozas E, Marti E. Bcl-2, Bax, and Bcl-x expression in the CA1 area of the hippocampus following transient forebrain ischemia in the adult gerbil. Exp Brain Res. 1998 Jul;121(2):167-173.
34. Collins JA, Schandi CA, Young KK, Vesely J, Willingham MC. Major DNA fragmentation is a late event in apoptosis. J Histochem Cytochem. 1997; 45:923-934.
35. MacManus JP, Buchan AM, Hill IE, Rasquinha I, Preston E. Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. Neurosci Lett. 1993;164:89-92.
36. Carroll M, and Beek O. Protection against hippocampal CA1 cell loss by post-ischemic hypothermia is dependent on delay of initiation and duration. Metab Brain Dis. 1992;7:45-50.
37. Islekel H, Islekel S, Guner G, Ozdamar N. Evaluation of lipid peroxidation, cathepsin L and acid phosphatase activities in experimental brain ischemia reperfusion. Brain Res. 1999;843:18-24.