Neuroprotective Effect of *Citri Pericarpium* On Transient Global Ischemia in Gerbils

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The current study was carried out to evaluate neuroprotective effects of *Citri Pericarpium* after transient global ischemia in gerbils. Male Mongolian gerbils weighing 60-80g were anesthetized with 2% isoflurane mixed with 30% oxygen and 70 % nitrogen. Bilateral common carotid arteries were occluded for 5 minute with microaneurysm clips. On 3 or 7 days after ischemic surgery, the gerbils were sacrificed. The brain were removed, embedded in paraffin and sectioned at 8㎛-thickness. Gerbils that received ischemic insult for 5 min showed extensive neuronal damage in the hippocampal CA1 region, and the number of viable neuronal cell was 51.0±2.5/mm, 32.2% of normal group at 7 days after ischemic surgery. In animals that underwent the extract of *Citri Pericarpium* treatment, the number of viable neuronal cell were significantly better preserved at 110.58±3.58/mm, 72.0% of normal group than those of ischemic group ($P<0.01$). In the immunohistochemistry of Bax and Bcl-2, the *Citri Pericarpium* treated group down-regulated the expression of Bax protein at 72hr after transient global ischemia. In contrast, Bcl-2 protein level was not changed. The appearance in TUNEL assay is similar to the pattern of Bax protein. The water extract of *Citri Pericarpium* significantly reduced the number of TUNEL-positive CA1 pyramidal neurons at 72hr. The results suggest that *Citri Pericarpium* has potential neuroprotective effects in the transient global ischemia and the increase in the ratio of Bcl-2 to Bax may contribute to the anti-apoptotic effect of *Citri Pericarpium*.

Key words : ishcemia, MTT assay, immunohistochemistry, *Citri Pericarpium*, gerbil

Introduction

Transient global ischemia occurs during cardiac arrest, cardiopulmonary bypass surgery and other situations that deprive the brain of oxygen and glucose for short periods of time.[1,2] In both humans and other animals, ischemia of this type kills neurons in vulnerable regions of the brain, including the hippocampus, caudate putamen, cerebral cortex and cerebellum. Especially the pyramidal cells in hippocampal CA1 area are vulnerable, but death of these principal cells is delayed at least 3 to 4 days after a transient ischemic event.[3] The delayed neuronal cell death (DND) provides the focus of intense research of the attempt to understand mechanism underlying ischemic neuronal death and a potential therapeutic window for neuroprotective intervention after the insult.[4,5] Although it is not elucidated the exact mechanism of DND after transient global ischemia, many hypotheses have been proposed regarding the mechanism of this neuronal death. Among them, one recent hypothesis proposed that delayed degenerative neurons in the hippocampal CA1 region represented apoptosis as the evidence by DNA fragmentation and expression of caspase and low expression of Bcl-2.[2,6,7] Apoptosis is a highly regulated form of cell death that is characterized by specific morphological, biochemical, and molecular events.[4,8,9] There are distinctive differences between necrotic and apoptotic cell death that can be observed and measured. Necrosis occurs when a cell suffers lethal injury and is characterized by swelling, rupturing of the cell, and inflammation.[9] Apoptosis, however, is a normal, genetically controlled event characterized by cell shrinkage, membrane blebbing, and chromatin condensation.[10,11] Additionally, activation of endogenous endonucleases and caspases (ICE-like proteases named after interleukin-1[beta] converting enzyme) results in irreversible DNA fragmentation along with fragmentation of the cell into membrane-bound apoptotic bodies. These apoptotic bodies are subsequently phagocytosed by surrounding cells or macrophages.[9,10] An often-used marker to determine apoptosis is DNA fragmentation into oligonucleosome-sized fragments of approximately 180 base pairs, which form a DNA "ladder" upon gel electrophoresis.[6,12,13]

A variety of internal and external signals regulate the expression of genes that control the initiation of apoptosis.[7,9] Internally, genes will express proteins that initiate apoptosis (i.e, Bax, Bcl-xs)[14,15] and proteins that inhibit apoptosis (i.e.

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Bcl-2, Bcl-xl)[16], and the fate for the cell (death or survival) depends on the ratio of the genes expressed. For example, high levels of Bcl-2 relative to Bax promote survival, whereas the reverse ratio promotes death. These genes express their specific proteins in the nucleus (p53), mitochondria (Bcl-2, Bcl-xl, and Bax), and other subcellular organelles.[17] In this way, ischemia-induced neuronal death involves a cascade of numerous cellular events, and the neuroprotective efficacy of several different classes of drugs has been examined. These include calcium antagonists, NMDA antagonists, glutamate release inhibitors, free radical scavengers, and leukocyte adhesion inhibitors.[5,18,19] Moreover, it is started to choose new compound for neuroprotective effect from natural resource.

Most of chosen medical plants have been popularly used as therapeutic purpose in oriental medicine. *Citri Pericarpium*, which is chosen of this study, is known to have the ability to regulate and maintain the physical homeostasis in oriental medicine.[20] It has been widely used against indigestion, and digestive disorders of GI tract, and the phlegm of respiratory tract.[21] (The mature peels of Citrus unshiu are named by *Citri Pericarpium*.) It is known to contain a number of flavonoid derivatives and essential oils.[21] Recently, it have been reported to inhibit azoxymethane-induced colonic aberrant crypt foci[22] in rats and both NO and O^{2-} generation in inflamation-associated tumorigenesis.[4] The several concepts of causes for ischemia-induced brain damage like stroke have been proposed in oriental medicine.[23] One of them is a theory of Qi, which seems to maintain and regulate homeostasis in body. Many therapeutic approaches aimed at protecting the brain against ischemia are based on regulating Qi in oriental medicine.[24] *Citri Pericarpium* has also been used to treat stroke as a component of combinational medicine or single medicine.[24] Based on the usage of *Citri Pericarpium* in treatment of stroke, we hypothesized that the total extract of *Citri Pericarpium* may have neuroprotective effects against transient global ischemia. It is tested through 2-vessel occlusion model in gerbils and the 48hr hypoxia/ 6hr reperfusion model in PC12 cell.

The purpose of this study is to determine the neuroprotective effect of *Citri Pericarpium* to reduce neuronal cell death in 2-VO gerbil model of transient global ischemia. In addition, we may examine the potential mechanism of action through its effect on DND induced apoptosis.

Materials and Methods

1. Preparation and administration of *Citri Pericarpium* extracts

Dried peels of *Citri Pericarpium* (200g) was purchased at Kyunghee University Medical Center, Seoul, Korea in 2000. They were boiled with distilled water (2L) for 2h. The supernatant was filtered, evaporated under reduced pressure and lyophilized (48.74g). For the assessment of neuroprotective effects, powdered extract of *Citri Pericarpium* was dissolved in distilled water to obtain a solution of 12mg/ml. Animals that had been subjected to 5 min ischemia as described below were randomly divided into two groups. Either a solution of *Citri Pericarpium* (160mg/kg) was given orally once a day for 3 consecutive days or 7 days after surgery.

2. In vitro cell culture and MTT assay

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 at 37°C, 100% humidity in 5% CO_2. For MTT[3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] analysis, exponentially growing PC12 cells were seeded in 90$\mu\ell$ medium per well in 96-well plates. Each well contained 5000 cells. It was maintained in CO_2 incubator (5% CO2, humid atmosphere, Forma, U.S.A). After 24h, PC12 cells were pretreated with the 10$\mu\ell$ of same medium but containing final concentrations (20μg/ml or 2μg/ml) of sample extracts dissolved in PBS (phosphate-buffered saline) for 2h before the exposure to hypoxic condition. Controls were treated with PBS instead of sample 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. Cultures were returned to an atmosphere with ambient oxygen levels in CO_2 incubator. Normoxic condition was induced to compare with hypoxic condition. 10$\mu\ell$ of MTT solution (final concentration, 1mg/ml) was added per well and incubated for another 1h. Then, dimethyl sulfoxide was added to solubilize the product of formazan reaction for 10 min in 60℃ incubator. It was quantified with an ELISA plate reader at 570, 650nm. The absorbance directly correlates with cell number.

3. Induction of ischemia

Adult male Mogolian gerbils (Meriones unguiculatus: Charles River Labs, Raleigh, NC, U.S.A) weighing 65-75g were used for all experiments. The animals housed in a controlled environment on a 12hr light: 12hr dark cycle with free access to food and water. The animals were acclimated to the cage for a minimum of 7 days before surgery.

Mogolian gerbils were subjected to transient global cerebral ischemia. Anthesthesia was induced with 5% (v/v) isoflurane and a 70%/30% (v/v) mixture of nitrous oxide/ oxygen, then subsequently maintained with 1% isoflurane.

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 sutured with a silk. The animals were allowed to awaken from anesthesia and returned to their cage. The rectal temperature, monitored with a digital thermometer inserted 5cm into the anus, was maintained at 37℃ during the surgical procedure using heating pad and an incandescent lamp. Gerbils were sacrificed at 3 days for immunohistochemistry and at 7 days after surgery for cell counting.

4. Tissue preparation

Three or seven days after ischemic surgery, animals were anesthetized with sodium pentobarbital (25mg/kg, i.m) and perfused transcardially with 10% formalin in 0.1M phosphate buffered saline, pH 7.4 (PBS). Immediately afterwards, the brains were removed from the skull and fixed in the same fixative solution for 6hr. The brains were embedded in paraffin and sectioned at 8-㎛-thickness and mounted on poly-L-lysine coated slide.

5. TUNEL assay

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 15min at room temperature and washed four times with deionized water for a total of 20 min. Endogenous peroxidease 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 1h in a humidified chamber. The TdT reaction was stopped with Stop/Wash buffer for 10min followed by three washes with PBS for 15min. 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.

6. Immunohistochemistry for Bcl-2, Bax

Immunohistochemistry was carried out following the avidin-biotin- peroxidase method (Vectastatin ABC kit, Vector Labs). 8㎛ deparaffinized sections were incubated with 0.3% hydrogen peroxide and methanol for 20 min to diminish nonspecific staining and later boiled in 10mM citrate buffer, 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 and with anti-mouse biotinylated IgG antibody (Vector, U.S.A) for 1h and, then 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 immunoreac -tion was tested by incubating sections without the primarily antibody.

7. Cresyl violet staining and cell counting

8㎛ deparaffinized sections were stained with cresyl violet for 10min and the neuronal density in the CA1 subfield of the hippocampus was measured using a light microscope. The neuronal density was expressed as number of viable cells per ㎟ CA1 hippocampus region.

8. Statistical analysis

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

Results

1. Cytoprotective effect of *Citri Pericarpium* against 48hr hypoxia/ 6hr reperfusion injury in PC12 cell.

Our initial purpose of this study was to investigate the neuroprotective action of *Citri Pericarpium* in cell culture system. Active mitochondria of living cells can cleave MTT to produce fromazan, the amount of which is directly to the living cell number. MTT is the earliest identified marker for cell viability. Through cell viability data as determined by MTT reduction, it indicated that pretreatment of PC12 cells with the water extract of *Citri Pericarpium* significantly protected the cells from 48hr hypoxia/ 6hr reperfusion induced injury (Fig. 1, Table 1).

In Table 1, the cell viability of *Citri Pericarpium* treated PC12 cell represents 115.23% at 2㎍/ml and 133.23% at 20㎍ /ml in comparison with non-treated PC12 cell. Viability of untreated cells in 48hr hypoxia/ 6hr reperfusion state was set to 100%. In this way, *Citri Pericarpium* treatment exerted significantly cytoprotective effects in PC12 cell exposed to 48hr hypoxia/ 6hr reperfusion. Also, the effective ratio of *Citri Pericarpium* extract on PC12 cell increased in a dose-dependent manner.

Table 1. The Effective Ratio(%) of *Citri Pericarpium* on 48hr Hypoxia/6hr Reperfusion Model with PC12 Cell.

Dose of *Citri Pericarpium*	2 ㎍/ml	20㎍/ml
Effective Ratio(%)	115.04	133.23

Effective Ratio(%): hypoxia/ normoxia (The ratio of control is 100%)

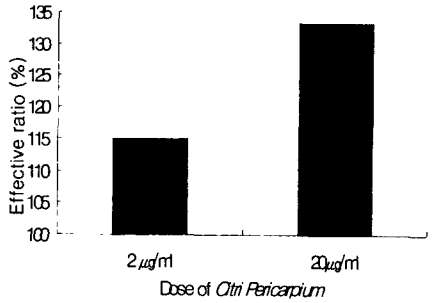

Fig. 1. Cytoprotective effect of *Citri Pericarpium* against 48hr hypoxia/ 6hr reperfusion injury. After the pretreatment of PC12 cells with the water extract of *Citri Pericarpium* (2, 20㎍/ml) for 2hr, the cells were exposed to 48hr hypoxia/ 6hr reperfusion condition. Then cell viability measured by MTT assay. Viability of untreated cells in 48hr hypoxia/ 6hr reperfusion state was set to 100%. *Citri Pericarpium* treatment exerted significant cytoprotective effects in PC12 cell exposed to 48hr hypoxia/ 6hr reperfusion.

2. Neuroprotective effect by *Citri Pericarpium*

1) Changes in hippocampal CA1 pyramidal cells by light microscopic investigation.

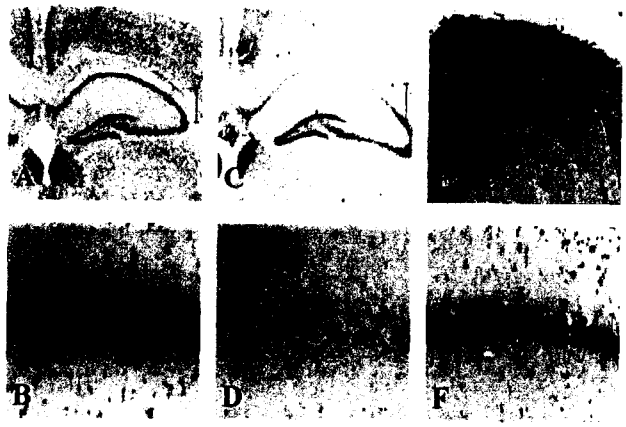

Fig. 2. Representative photomicrographs of pyramidal cells in the hippocampal CA1 region in ischemic gerbils at 7 days after 5-minute ischemia in the Normal(A,B), ischemia(C,D), Citri Percarpium treated groups (E,F,oral injection, 160mg/kg). Cresyl violet staining shows selective, delayed neuronal cell death induced by transient global ischemia in the hippocampal CA1 region. After ischemic insult, only a few cells are seen normal with round cell bodies and clear nuclei and nucleoli. Damaged cells are shrunken and distorted with small dense nuclear segments. (A,C,E=×20 ; B,D,F=×200)

The cytoprotective effect of *Citri Pericarpium* extracts was evaluated by measuring the neuronal cell density in hippocampal CA1 region at 7 days after ischemia. The viable neuronal cell number of *Citri Pericarpium* extracts-treated ischemic animals was significantly increased compared to ischemic animals(Table 2). The dosage of treatment (160mg/kg) is determined by clinical dosage of human in oriental medicinal therapy. Representative photomicrographs of Cresyl violet-stained hippocampal neurons in each experimental group are shown in Fig. 2. These histological examination of the nervous system demonstrated remarkable cell damage in the hippocampal CA1 region in the ischemic gerbils when compared to the normal group. CA1 pyramidal neurons showed pyknosis, eosinophilia, karyorrhexia, and chromosome condensation in ischemic group (Fig. 2: C, D). This neuronal cell damage was suppressed by the extract of *Citri Pericarpium*. The normal morphology of pyramidal cell (Fig. 3: A, B) is maintained in *Citri Pericarpium* treated group (Fig. 3: E, F).

2) Neuroprotective ratio of *Citri Pericarpium* on ischemic neuronal damage

The results of histological examination of hippocampal CA1 region are summarized in Table 2. Normal group alone caused no neuronal death, and the neuronal density of normal animals was 153.5±4.9 per 1-mm length of the CA1 pyramidal cell layer (Fig. 2: A, B). Gerbils that received ischemic insult for 5 min showed extensive neuronal damage in the hippocampal CA1 region, and the density of the area was 51.0±2.5/mm, 32.2% of normal(Fig. 2: C, D). In animals that underwent the extract of *Citri Pericarpium* pretreatment, pyramidal cells were significantly better preserved at 110.5± 3.58/mm, 72.0% of normal group(Fig. 2: E, F) than those of ischemic group ($P<0.01$).

Table 2. The Neuroprotective Effects of Citri Percarpium's Oral Administration the Neuronal Density in the Hippocampal CA1 Region in Gerbils at 7 Days after 5-minute Occlusion.

	Normal	ischemia	*Citri Pericarpium* treated
mean	153.50	51.00	110.58**
S.E.M	4.92	2.50	3.58
n	8	7	3
Viability %	100	33.22	72.04
Damaged %	-	66.78	27.96

1): mean and SEM. were expressed as viable cells, 2): neuroprotective ratio **, P<0.01

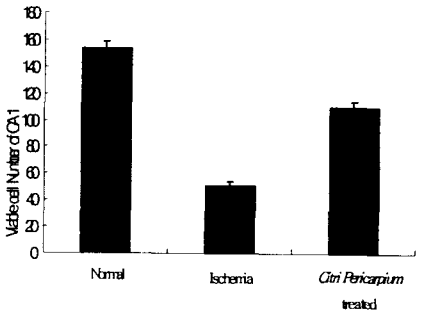

Fig. 3. Numbers of viable neuronal cell in the hippocampal CA1 region at 7 days after transient global ischemia. *Citri Pericarpium* (160mg/kg) was oraliy administered into the animals following 5 min ischemia. *Citri Pericarpium* treated group significantly protected the neurons of hippocampal CA1 region($P<0.01$). Statistically ischemic group are significantly different from *Citri Pericarpium* treated group($P<0.01$). The latter gave 72.04% neuroprotection, respectively. Normal group (n=8), ischemic group(n=7), *Citri Pericarpium*-treated group following transient global ischemia (n=3 for 160 mg/kg, oral)

3. Effect of *Citri Pericarpium* through Immunohistochemical analysis of apoptosis-related protein

1) Bax

The immunoreactivity for Bax increased following transient global ischemia with a peak in staining intensity occurring 72hr after ischemia. Thereafter, the immunoreactivity for Bax disappeared by 96hr following ischemia. The normal animals, granular immunostaining for Bax was observed in cytoplasm of the neuron of hippocampal CA1 region. The staining pattern and intensity of immunostaining were uniformed in the hippocampal CA1 region (Fig. 4: A, B). After 72hr following ischemia, much increased intensity of immunostaining was observed in ischemic animals and many neurons showed strong immunoreactivity (Fig. 4: C, D). In *Citri Pericarpium* treated animals, however, the intensity of immunostaining for Bax was relatively weaker than that of ischemic animals after 72hr (Fig. 4: E, F).

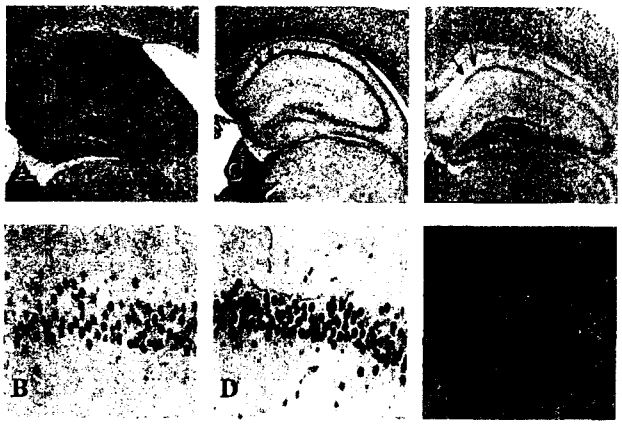

Fig. 4. Immunohistochemical analysis of Bax protein in brain sections from the normal(A, B), ischemia (C, D), *Citri Pericarpium* treated group(E, F) following transient global ischemia after 72hr. Much greater increase of immunostaining was observed in ischemic animals and the hippocampal CA1 region especially showed strong immunoreactivity (C, D). In *Citri Pericarpium* treated animals, the intensity of immunostaining for Bax was relatively weaker than that of ischemic animals after 72hr (E, F). (A, C, E : ×20, B, D, F: ×200)

2) Bcl-2

To investigate the relation of Bax & Bcl-2, we tested the immunoreactivity of Bcl-2 at 72hr after ischemic surgery.

In the normal animals, the immunostaining for Bcl-2 was not observed in all the section (Fig. 5: A, B).

After 72hr following ischemia in ischemic animals, some sign of increased immunostaining was observed and many neurons showed very weak but detectable immunoreactivity of Bcl-2 in the hippocampal CA1 region (Fig. 5: C, D).

In *Citri Pericarpium*-treated animals, markedly, the intensity of immunostaining for Bcl-2 was relatively stronger than that of normal animals after 72hr. But, it was not different from animals in the ischmic condition (Fig. 5: E, F).

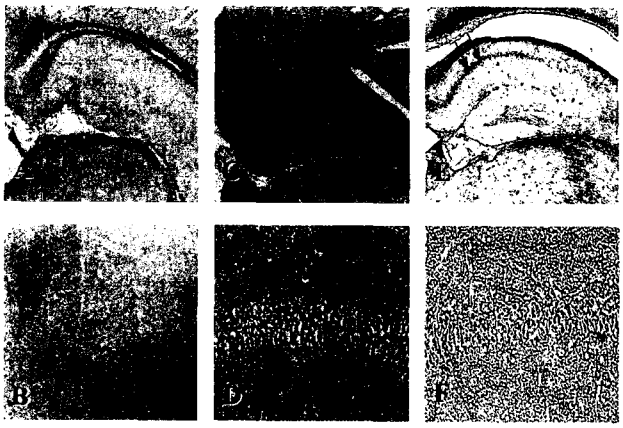

Fig. 5. Immunohistochemical analysis of Bcl-2 protein in brain sections from the normal(A, B), ischemia (C, D), *Citri Pericarpium* treated group(E, F) following transient global ischemia after 72hr. Moderate increase of immunostaining was observed in the hippocampal CA1 region of ischemic condition (C, D). In *Citri Pericarpium* treated animals, the intensity of immunostaining for Bcl-2 was relatively stronger than that of normal animals after 72hr (E, F). But, it was not different from animals in the ischmic condition. (A, C, E : 20×, B, D, F: 200×)

3) TUNEL Assay

The terminal deoxynucleotidyl transferase (TdT) mediated dUTP- biotin nick end labeling (TUNEL) reaction was used to detect DNA fragmentation, a typical characteristics of apoptosis, in cell nuclei. In ischemic condition, DND occurred at 3 days after ischemic insult and CA1 pyramidal neurons completely disappeared at 7 days. Three days after ischemia, when significant cell death occurred, varying numbers of CA1 pyramidal cells exhibited a positive TUNEL reaction. Most of gerbils in ischemic condition exhibited a positive TUNEL reaction throughout the entire cell body layer although not all pyknotic cells were TUNEL positive (Fig. 6: C, D). The water extract of *Citri Pericarpium* significantly reduced the number of TUNEL-positive CA1 pyramidal neurons at 72hr (Fig. 6: E, F). No TUNEL-positive CA1 pyramidal neurons were present in normal animals (Fig. 6: A, B).

Positively stained fragments for TUNEL were localized not only in the nuclei but also in cell bodies and dendrites in both ischemic animals and *Citri Pericarpium* treated animals (Fig. 6: D, F).

Table 3. Summary of Immunohistochemical Bax, Bcl-2 Expression and TUNEL-positive Cell in the CA1 Region following Transient Ischemia after 72 hr.

	Bax	Bcl-2	TUNEL
Normal	+	-	+
Ischemia	+ + +	+	+ + +
Citri Pericarpium treated group	+ +	+	+ +

* The results of immunohistochemistry (Bax, Bcl-2, TUNEL) are summarized. The staining intensity are devided into three groups: -: negative, +: mild positive , + +: positive, + + +: strongly positive.

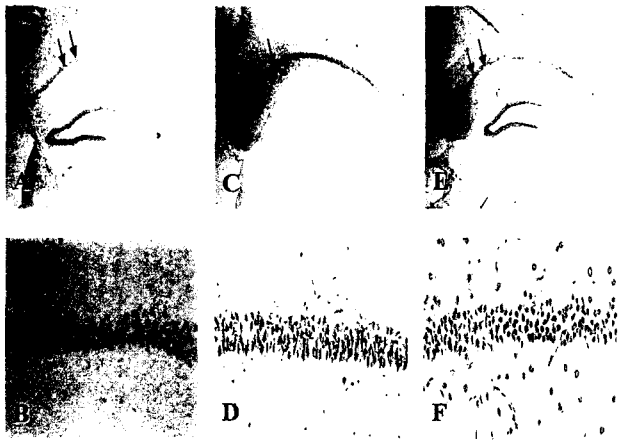

Fig. 6. Immunohistochemical analysis of TUNEL assay in brain sections from the normal (A, B), ischemia (C, D), *Citri Pericarpium* treated group (E, F) following transient global ischemia after 72hr. No TUNEL-positive CA1 pyramidal neurons were present in normal animals (Fig. 6: A, B). Most of gerbils in ischemic condition exhibited a positive TUNEL reaction (Fig. 6: C, D). The water extract of *Citri Pericarpium* significantly reduced the number of TUNEL-positive CA1 pyramidal neurons at 72hr (Fig. 6: E, F). (A, C, E : ×20, B, D, F: ×200)

Discussion

Citri Pericarpium has been used for the treatment of stroke in traditional Oriental medicine. We evaluated potential neuroprotective effects of *Citri Pericarpium* after transient global ischemia in gerbils. Our results indicated that it conferred a significant neuroprotection against 5 min transient global ischemia induced in gerbils. Moreover, our studies showed that it protected PC12 cell from 48hr hypoxia/ 6hr reperfusion in dose-dependent manner.

In the present study, we focused on showing the relation of Bcl-2 and Bax after transient global ischemia. The possible involvement of these genes in the anti-apoptotic mechanism of *Citri Pericarpium* was particularly investigated. 5 minutes of global ischemia induced an up-regulation of Bax in hippocampal CA1 region at 72hr.[25] It markedly reduced the intensity of Bax proteins in the hippocampal CA1 region of *Citri Pericarpium*-treated group. These neurons showed moderate immunoreactivity. In *Citri Pericarpium*-treated animals, however, the intensity of immunoreactivity for Bcl-2 was not changed. These result point out an involvement of the ratio of Bcl-2 to Bax in the anti-apoptotic effect of *Citri Pericarpium*. The present study suggests that *Citri Pericarpium* may effectively modulate the expression of Bax proteins after global ischemia. Comparing the effect of *Citri Pericarpium* on the expression of Bcl-2 and Bax proteins after ischemic insult, we could suggest that the expression of these protein by *Citri Pericarpium* is faster than that caused by ischemia. Moreover DNA fragmentation and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-positive cells in the hippocampus following transient global ischemia were considerably diminished by *Citri Pericarpium*, which suggests an anti-apoptotic potency of this herb. Apoptosis or programmed cell death is an active process of cell death requiring protein synthesis.[9] It is reasonable to consider modulating apoptosis-associated gene expression by which drugs could inhibit (in neuronal degeneration) or promote (in tumor) apoptosis.

The pro-apoptotic gene, Bax, was up-regulated after ischemia.[14] Bax was the first member of the Bcl-2 family to be shown to actively promote apoptosis. Over-expression of Bax can counteract the anti-apoptotic activity of Bcl-2, unless it is bound either by Bcl-2 or Bcl-xl.[7,15,26] It has been suggested that direct binding of Bcl-2 to Bax to form heterodimers is essential for the anti-apoptotic activity of Bcl-2.[5] Bcl-xl has been demonstrated as a powerful regulator of neuronal apoptosis in the postnatal CNS.[26,28] Unlike Bcl-2, Bcl-xl could suppress apoptosis without binding to Bax. The up-regulation of pre-apoptotic genes, Bcl-2, Bcl-xl and the heterodimerized Bcl-2 and Bax in the ischemic brain could be suggested as a protective mechanism produced by injured cells or as a response to stress.[5] However, such up-regulation may occur too late to protect neurons against the damage caused by ischemia. It is beneficial for neuronal survival to shift the up-regulation of pre-apoptotic gene expression by *Citri Pericarpium* to an earlier time-point after the ischemic insult. In addition, the marked down-regulation of Bax expression induced by *Citri Pericarpium* could also potentially contribute to its anti-apoptotic effect. It has been proposed that an increase in the ratio of Bcl-2 to Bax can prevent apoptotic cell death.[29,30] It may be able to cause an increase in this ratio, and therefore may support the neurons against apoptosis induced by transient global ischemia. In any events, it is evident that total water extracts of *Citri Pericarpium* have an ability to protect neuronal cells from ischemic damage, and this cytoprotective effect together with inhibitory effects on apoptotic cell death may partly explain neuroprotective mechanism of *Citri Pericarpium* against cerebral ischemia.

Our work suggests the possibility of clinical applications of *Citri Pericarpium* based on its neuroprotective effects. Therapeutic agents from herbal sources are usually perceived as being natural and devoid of side effects. However, herbal remedies may contain toxic heavy metals, and their indications and dosage are not defined as well as other drugs, since the concentrations of the active components for most herbal medicines are not precisely known. Thus, with a better understanding of the active components of *Citri Pericarpium* that exert neuroprotective activities, *Citri Pericarpium* could be an invaluable herbal source for the development of novel neuroprotective drugs.

Acknowledgments

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