Neuroprotective effects of herbal mixture HT070 on global cerebral ischemia in rats

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ABSTRACT

Objectives : HT070 is a mixture of herbal extracts from root of *Scutellaria baicalensis* and stem bark of *Eleutherococcus senticosus*, which have long been used for stroke therapy in traditional Korean Medicine. The purpose of this study was to investigate the neuroprotective effects of HT070 on global cerebral ischemia and its potential mechanisms.

Methods : Transient global cerebral ischemia was produced by 10 min of four–vessel occlusion (4–VO) in male Wistar rats. HT070 was administered orally at a dosage of 200 mg/kg twice at 0 and 90 min after reperfusion. Hippocampal neuronal damage was measured 7 days after reperfusion. To explore the potential mechanisms, we used hydrogen peroxide (H₂O₂)–induced rat pheochromocytoma (PC12) cells as an *in vitro* model. PC12 cells were pretreated with HT070 for 1 h and then exposed to 100 μ M H₂O₂ for 6 h in the presence of HT070. Cell viability was measured by MTT assay and the mRNA expression of Bax, Bcl–2, iNOS and COX–2 were measured by quantitative RT–PCR.

Results : Oral administration of HT070 at a dose of 200 mg/kg significantly reduced neuronal death in the hippocampal CA1 region by 13.4% as compared to the vehicle–treated group. HT070 increased cell viability, reversed the down–regulated Bcl–2 mRNA level, and suppressed the up–regulated mRNA expressions of Bax, iNOS, and COX–2 in H₂O₂–treated PC12 cells.

Conclusions : HT070 protects against delayed neuronal death after global cerebral ischemia and its neuroprotection properties might be attributed to the inhibition of mitochondrial apoptosis and ROS–generating enzymes.

Key words : HT070, *Scutellaria baicalensis*, *Eleutherococcus senticosus*, global cerebral ischemia, four–vessel occlusion, neuroprotection, oxidative stress, hydrogen peroxide

I . Introduction

Global cerebral ischemia generally occurs during cardiac arrest or severe systemic hypotension that hinders the supply of oxygen and glucose to the brain for short periods of time. It causes a slow development of cell death in brain neurons, leaving patients with memory, learning and motor deficits^{1,2)}. The pathophysiology of cerebral ischemia is complex and involves numerous processes, including energy failure,

ionic imbalances, acidosis, excitotoxicity, inflammatory pathways, increase of free radicals and disturbance of protein synthesis³⁾. Numerous neuroprotective agents that antagonize the injurious events have been identified in animal studies but most of them have failed to provide protection in clinical trials⁴⁾. Recent studies have indicated that targeting a single pathway might be ineffective due to multiple pathologic processes, and thus combination therapies or neuroprotectants with pleiotropic effects have been

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suggested as an alternative^{4,5}. Medicinal plants which contain various compounds with different neuroprotective mechanisms could be a potentially rich source of neuroprotective agents.

HT070 is a mixture of herbal extracts from the root of *Scutellaria baicalensis* Georgi (Lamiaceae) and the stem bark of *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. (Araliaceae), which has been widely used in traditional Korean medicine. *Scutellariae Radix* has been reported to exert anti-inflammatory, anti-viral, anti-oxidant, anti-cancer, immunomodulating and anti-hyperlipidemic effects⁶. The active components of *Scutellariae Radix* are flavonoids; the three major flavonoids are baicalin and its aglycone baicalein, and wogonin. *Eleutherococci Senticosi Cortex*, also called 'Siberian Ginseng', has been reported to possess anti-oxidant, anti-diabetic, anti-fatigue, immunomodulating and anti-cancer effects⁷. It is rich in eleutherosides, especially eleutheroside B and E, compounds that are responsible for its diverse pharmacological activities⁸.

Scutellariae Radix and *Eleutherococci Senticosi Cortex* have a long history of use in stroke prevention and therapy⁹ and have been intensively studied as neuroprotective agents in a variety of experimental brain injury models^{7,8,10}. Previously, our group found that *Scutellariae Radix* and *Eleutherococci Senticosi Cortex* had protective effects against hippocampal cell death on global cerebral ischemia in rats^{11,12}. Baicalin, a major flavonoid in *Scutellariae Radix*, attenuates global cerebral ischemia/reperfusion injury in gerbils by increasing the activities of anti-oxidative enzymes, such as superoxide dismutase and glutathione peroxidase, and non-enzymatic scavenger glutathione in hippocampus¹³. Baicalein, the most effective antioxidant among major flavonoids of *Scutellariae Radix* in *in vitro* neuroprotection assay¹⁴, increases the activities of antioxidant enzymes in chronic cerebral hypoperfused rats^{15,16}. Its anti-oxidative properties may render this compound to improve ATP synthesis, reduce mitochondrial ROS production, regulate expression of Bcl-2 family proteins, and inhibit cytochrome c release against chronic cerebral hypoperfusion¹⁶. The fruits of *E. senticosus* has been reported to induce the expression of HO-1, a crucial enzymatic intermediate in antioxidant defense mechanisms through Nrf2 and p38-CREB pathways,

and thereby reduces ROS production and neuroinflammation in hippocampal and microglial cells¹⁷. Polysaccharides of *Eleutherococci Senticosi Cortex* protect hippocampal neurons from oxidative stress by inducing the expression of OGG1, which plays important roles in redox regulation against oxidative stress¹⁸. These previous findings led us to hypothesize that *Scutellariae Radix* and *Eleutherococci Senticosi Cortex* in combination may simultaneously act on different steps of pathways involved in cerebral ischemia and thus serve as a potent neuroprotective agent.

In the present study, we investigated the neuroprotective effects of HT070 in global cerebral ischemia using a four-vessel occlusion (4-VO) rat model. To gain further insight into the possible mechanisms of HT070, we used hydrogen peroxide (H₂O₂)-induced rat pheochromocytoma (PC12) cells as an *in vitro* model. Cytotoxicity and the mRNA expression of Bax, Bcl-2, iNOS and COX-2 were measured by MTT assay and quantitative RT-PCR, respectively.

II. Materials and Methods

1. Plant materials

The root of *S. baicalensis* and the stem bark of *E. senticosus* were purchased from Tulaoba Zhongyao Yinpian Co., Ltd. (Anhui, China). The origin of plant materials was Neimenggu, China. *Scutellariae Radix* and *Eleutherococci Senticosi Cortex* were authenticated by Professor Hocheol Kim of the Department of Herbal Pharmacology, Kyung Hee University, where voucher specimens were deposited.

2. Sample preparation and HPLC analysis

The root of *S. baicalensis* and the stem bark of *E. senticosus* were extracted separately with water for 2 h at 100 °C in a reflux apparatus for two times. The filtrate was concentrated under reduced pressure and then the concentrate was spray-dried to yield a powder. The powders of *Scutellariae Radix* and *Eleutherococci Senticosi Cortex* were mixed in a ratio of 1:2 (w/w). Samples were stored at -4 °C until use. The quantitative authentication of individual powders and their mixture HT070 was performed on a Waters

instrument (Waters Corp., USA) equipped with a Waters 1525 binary pump, a Waters 2707 autosampler, and a Waters 2998 PDA detector. Separation was achieved on Sunfire™ C¹⁸ column (5 μ m; 250 $\times$ 4.6 mm i.d.; WatersCorp., USA) at 40 °C. The mobile phase composition was 0.1% phosphoric acid (A) and acetonitrile (B) eluted for separation as following: 0–35 min, 5%–37%; 35–37 min, 37%–65%; 37–38 min, 65%–5%; 38–45 min, 5%–5% solvent B. The flow rate was 1.0 mL/min. The injection volume was 10 μ L and effluent was monitored at 205 nm. Baicalin and Eleutheroside E were chosen as marker compounds for *Scutellariae Radix* and *Eleutherococci Senticosi Cortex*, respectively. The extracts were analyzed in triplicate.

3. Animals

Male Wistar rats weighing 170–190 g were obtained from Samtako Co. (Osan, Korea) and allowed access to water and food *ad libitum*. Rats were acclimatized and maintained under a constant temperature (23 $\pm$ 1 °C), humidity (60 $\pm$ 10%), and a 12 h light/dark cycle (light on 07:00–19:00 h). Animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care (NIH Publication No. 85–23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University.

4. Induction of transient global cerebral ischemia and sample treatment

Transient global cerebral ischemia was induced using the four-vessel occlusion (4-VO) model described by Pulsinelli and Brierly¹⁹. Briefly, under anesthesia with 2% isoflurane in 70% N₂O/30% O₂, the vertebral arteries were electrocauterized and both common carotid arteries were isolated using a loop of thread. On the following day, both common carotid arteries were occluded with aneurysm clips to induce global cerebral ischemia. After 10 min of occlusion, the aneurysm clips were removed to allow reperfusion. Rats displaying loss of righting reflex and with bilateral pupil dilation were included in the study. Rats which developed seizure activity during or after ischemia were excluded from the study. Rectal temperature was maintained at 37 $\pm$ 0.5 °C until 6 h after ischemia. Rats were randomly allocated to three groups: sham-operated, control (rats with 4-VO and

vehicle treatment) and HT070 group (rats with 4-VO and HT070 treatment). HT070 was dissolved in normal saline and administered orally at a dosage of 200 mg/kg twice at 0 and 90 min after reperfusion. Rats in the control or sham-operated group were administered normal saline in the same manner. The sham-operated group received the same surgical procedures except for the occlusion of common carotid arteries.

5. Histology

Seven days after global cerebral ischemia, rats were anesthetized and perfused transcardially with heparinized 0.5% sodium nitrite saline, followed by 4% paraformaldehyde. Brains were removed, fixed and cut into 30 μ m sections on a sliding microtome (Microm HM 440E, Walldorf, Germany) and the sections were stained with cresyl violet (CV). Neuronal density in hippocampal CA1 region was measured according to the method described in our previous study¹². Viable cells in the total of six frames (1.0 mm $\times$ 1.0 mm) of the left and right CA1 regions of 3 coronal sections (approximately 3.3, 3.5, and 3.7 mm caudal to the bregma) were measured at a magnification of $\times$ 400 for each rat. Neuronal density was expressed as the mean number of viable cells per one frame. Cell counting was performed by an investigator blinded to the experimental groups.

6. Cell culture

Rat pheochromocytoma cells (PC12) purchased from Korean cell line bank (Seoul, Korea) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin (all Gibco; Thermo Fisher Scientific, Inc., USA) in a humidified atmosphere of 95% air and 5% CO₂ at 37° C. All experiments were carried out 24h after the cells were seeded.

7. Analysis of cell viability

PC12 cells were seeded at a density of 2×10^4 cells/well in 96-well plates and cell viability was measured using MTT assay. To investigate the cytotoxicity of HT070 on PC12 cells, the cells were incubated with HT070 for 24 h at 37°C. For the H₂O₂

injury model, cells were pre-incubated with HT070 for 1 h and then treated with H₂O₂ in the presence of HT070 for 6 h at 37°C (final H₂O₂ concentration: 100 μ M). At the indicated time after treatment, 100 μ L of MTT solution (0.5 mg/mL) was added to each well followed by incubation for 1 h at 37 °C. MTT solution was removed and the formazan was dissolved in 100 μ L of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm and 630 nm using Epoch 2 Microplate Spectrophotometer (Biotek Instruments, Inc., USA). Cell viability was expressed as the percentage of control cells.

8. RNA extraction and Quantitative RT-PCR

PC12 cells were seeded in 60 mm dishes at a density of 2×10⁵cells/dish. After 24 h, cells were pretreated with HT070 12.5 and 25 ug/mL for 1 h and then exposed to 100 μ M H₂O₂ for 6 h in the presence of HT070. Total cellular RNA was isolated from PC12 cells using QIAzol lysis reagent (Qiagen, USA). The concentration of total RNA was assessed using the Take3™ Multi-volume Plate (BioTek, USA) and cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA). After cDNA synthesis, quantitative RT-PCR was performed in ABI 7500 Real Time PCR system (Applied Biosystems, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, USA). Relative gene expression was determined using the comparative Ct method and normalized to housekeeping gene GAPDH. The sequences of the primers are shown in Table 1.

Table 1. Sequences of the primers used in the quantitative RT-PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Bax	TGTTTGCTGATGGCAACTTC	GATCAGCTCGGGCACTTTAG
Bcl-2	GGGATGCCTTTGTGGAAC TA	CTCACTTGTGGCCCAAGGTAT
iNOS	AGCGGCCCATGACTCTCA	CTGCACCCAAACACCAAGGT
COX-2	CCGTGGTGAATGTATGAGCA	GATTGACAGCCCCACCACTT
GAPDH	TGGCCTCCAAGGAGTAAGAAAC	CAGCAACTGAGGGCCTCTCT

9. Statistical analysis

All data were expressed as mean ± SD. One-way ANOVA followed by Tukey's test was used to compare the differences between multiple groups, and Student's *t*-test was used for two groups. Statistical significance

was set at *P* < 0.05.

III. Results

1. HPLC Analysis

Three dimensional HPLC chromatograms and the structures of the constituent compounds are shown in Figure 1. HPLC analysis showed that extract of *Scutellariae Radix* contained 170.80 mg/g of baicalin, extract of *Eleutherococci Senticosi Cortex* contained 8.94 mg/g of eleutheroside E, and their mixture HT070 contained 63.36 mg/g of baicalin and 6.10 mg/g of eleutheroside E.

Figure 1. Three dimensional HPLC chromatograms of *Scutellariae Radix* extract (A), *Eleutherococci Senticosi Cortex* extract (B) and HT070 (C). X-axis is retention time; Y-axis is wavelength, and Z-axis is absorbance unit.

2. Effects of HT070 on neuronal density in rat hippocampal CA1 region

A typical feature of 4-VO rat model is delayed and selective loss of pyramidal neurons in hippocampal CA1. As expected, the number of viable neurons sharply decreased in the hippocampal CA1 region at 7 days following reperfusion. Under magnification, sham-

operated rats showed viable neurons with round nuclei and intact morphology, whereas control rats displayed neurons with shrunken cell bodies and pyknotic nuclei (Figure 2D vs. E). To quantify the extent of the neuronal damage, we counted the number of surviving pyramidal neurons in the hippocampal CA1 subfield (Figure 3). A marked reduction in neuronal density was observed in the control group (67.2 ± 12.3 cells/mm²) in comparison to the sham-operated group. In contrast, oral administration of HT070 at a dose of 200 mg/kg significantly increased the neuronal density (105.0 ± 33.1 cells/mm², 13.4%, $p < 0.05$) compared to control group.

Figure 2. Representative photomicrographs of cresyl violet-stained hippocampus of sham-operated (A, D), control (B, E), and HT070-treated rats (C, F). Transient global cerebral ischemia resulted in delayed neuronal cell death in the hippocampal CA1 region (B, E). HT070 200 mg/kg-treated group showed a reduction of the number of irreversibly damaged pyramidal cells in the CA1 subfield (C, F). Scale bars: 1 mm in A–C; 100 μ m in D–F.

Figure 3. Neuroprotective effects of HT070 in a rat model of global cerebral ischemia. Either a vehicle or HT070 200 mg/kg was orally administered at 0 and 90 min after reperfusion. Seven days after reperfusion, neuronal density in CA1 region was measured by counting viable cells. Treatment with HT070 significantly protected CA1 neurons from global cerebral ischemia ($p < 0.05$). The values are mean $\pm$ SD. * $p < 0.05$ vs. control group. Sham, sham-operated group ($n=1$); Control, 4-VO rats treated with vehicle ($n=6$); HT070 200 mg/kg, 4-VO rats treated with HT070 200 mg/kg ($n=7$).

3. Effects of HT070 on PC12 cell viability

To establish the non-cytotoxic concentration range, PC12 cells were treated with different concentrations

of HT070 for 24 h. As shown in Figure 4A, in the range of concentration 12.5–100 μ g/mL, HT070 did not affect cell viability as compared to control cells. The protective effects of HT070 against oxidative stress-induced neuronal injury were assessed in H₂O₂-treated PC12 cells. The results shown in Figure 4B suggest that exposure to H₂O₂ at 100 μ M for 6 h caused significant cytotoxicity compared to control cells. Treatment with HT070 over the range of concentrations from 12.5 to 100 μ g/mL significantly increased the percentage of viable cells as compared to cells treated with only H₂O₂. Since a concentration of 25 μ g/mL was more effective than a higher concentration (50–100 μ g/mL), it was decided to use the concentrations of 12.5 and 25 μ g/mL for further mechanism studies.

Figure 4. Effect of HT070 on H₂O₂-induced cytotoxicity in PC12 cells. (A) PC12 cells were treated with HT070 for 24 h. (B) PC12 cells were treated with 100 μ M H₂O₂ for 6 h after pretreatment with HT070 for 1 h. Cell viability was measured using MTT assay and were expressed as a percentage of the control. Values are given as the mean $\pm$ SD. ### $p < 0.001$ vs. control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. H₂O₂ only-treated group.

4. Effect of HT070 on the mRNA levels of Bax and Bcl-2 in H₂O₂-induced PC12 cells

As shown in Figure 5, after treatment with 100 μ M H₂O₂ for 6 h, quantitative RT-PCR analysis showed that mRNA levels of Bax increased (1.6-fold), while those of Bcl-2 decreased (0.2-fold). HT070 at the

concentrations of 12.5 and 25 μ g/mL significantly reduced Bax and raised Bcl-2 expressions in PC12 cells treated with H_2O_2 .

Figure 5. Effect of HT070 on the mRNA levels of Bax and Bcl-2 in H_2O_2 -induced PC12 cells. PC12 cells were pretreated with HT070 12.5 and 25 μ g/mL for 1 h and then exposed to 100 μ M H_2O_2 for 6 h. The mRNA expressions of Bax (A) and Bcl-2 (B) were measured by quantitative RT-PCR and normalized to GAPDH mRNA. Results are expressed as mean $\pm$ SD. ^{##}p < 0.01, ^{###}p < 0.001 vs. control group. ^{**}p < 0.01, ^{***}p < 0.001 vs. H_2O_2 only-treated group.

5. Effect of HT070 on the mRNA levels of iNOS and COX-2 in H_2O_2 -induced PC12 cells.

The effects of HT070 on H_2O_2 -induced up-regulation of iNOS and COX-2 mRNA were measured by quantitative RT-PCR. As shown in Figure 6, increases in iNOS and COX-2 mRNA expressions were observed when cells were treated with 100 μ M H_2O_2 for 6 h (5.3 and 8.0-folds). HT070 at the concentrations of 12.5 and 25 μ g/mL markedly inhibited H_2O_2 -induced increase in iNOS and COX-2 mRNA expressions compared to H_2O_2 -treated cells. Especially, COX-2 mRNA levels in HT070-treated cells were similar to that in control (normal) cells.

Figure 6. Effect of HT070 on the mRNA levels of iNOS and COX-2 in H_2O_2 -induced PC12 cells. PC12 cells were pretreated with HT070 12.5 and 25 μ g/mL for 1 h and then exposed to 100 μ M H_2O_2 for 6 h. The mRNA expressions of iNOS (A) and COX-2 (B) were measured by quantitative RT-PCR, which were normalized to GAPDH mRNA. Results are expressed as mean $\pm$ SD. ^{##}p < 0.001 vs. control group. ^{**}p < 0.01, ^{***}p < 0.001 vs. H_2O_2 only-treated group.

IV. Discussion

Findings from *in vivo* studies showed that oral administration of HT070 at a dose of 200 mg/kg significantly attenuated neuronal cell death in the hippocampal CA1 region after transient global cerebral ischemia induced by 4-VO. Our *in vitro* studies showed that HT070 exerted protective effects against H_2O_2 -induced cytotoxicity and restored the downregulated Bcl-2 mRNA level and up-regulated Bax, iNOS, and COX-2 mRNA expression in PC12 cells.

A brief period of global cerebral ischemia causes selective neuronal death in vulnerable brain areas, including the hippocampal CA1 region. Hippocampal neuronal death usually occurs at day 3 or 4 and peaks at 7 days after an initial ischemic insult, the so-called delayed neuronal death²⁰. The four-vessel occlusion (4-VO) model used in this study is a well-established and widely used technique for induction of global cerebral ischemia¹⁹. In the present study, 4-VO rats showed a significant decrease in neuronal densities in the CA1 region compared to sham-operated group at 7 days after reperfusion. Oral administration of HT070 at a dose of 200 mg/kg at 0 and 90 min after reperfusion significantly increased neuronal density in the hippocampal CA1 region, suggesting that HT070 has neuroprotective effects against delayed neuronal death induced by global cerebral ischemia. Considering the potential of using HT070 as a dietary ingredient in dairy products, we additionally tested the effects of HT070-added milk in 4-VO model. HT070-added milk sterilized by ultra-high temperature processing (132 $^{\circ}$ C for 3 sec) also provided significant neuroprotection against delayed neuronal damage (data not shown).

To investigate the mechanisms underlying the neuroprotective effects of HT070, H_2O_2 was used to develop an oxidative stress in PC12 cells. Oxidative stress has been implicated as a major cause of cellular injuries in cerebral ischemia. Accumulating evidences suggest that delayed neuronal cell death induced by global cerebral ischemia/reperfusion is directly or indirectly linked to oxidative stress, which is mediated by reactive oxygen species²¹. During ischemia-reperfusion, superoxide anions ($O_2^{\cdot-}$) are produced in the brain and further detoxified to H_2O_2 by superoxide dismutase (SOD). Hydroxyl radicals ($\cdot$ OH) are then produced from H_2O_2 through the Fenton and Haber-Weiss reactions or by peroxynitrite, and they irreversibly oxidize macromolecules including DNA, lipid and

protein, and eventually cause severe cell injury. Thus, H_2O_2 has been used extensively as an inducer of oxidative stress in *in vitro* neuroprotection assay. The PC12 cells, derived from a rat adrenal medullary pheochromocytoma, provide an established neuron-like system²²⁾, and thereby are commonly used as a model for investigating neuroprotection against oxidative stress. In the present study, treatment with HT070 at concentrations $\geq 12.5 \mu\text{g/mL}$ significantly reduced H_2O_2 -induced cytotoxicity in PC12 cells, suggesting neuroprotection against oxidative stress.

After confirming the protective effects of HT070 on H_2O_2 -induced cytotoxicity, we focused on the mitochondria-dependent apoptotic pathway. The mitochondria-dependent apoptotic pathway is known to be involved in H_2O_2 -induced cytotoxicity in PC12 cells, and the Bcl-2 family plays pivotal roles in this pathway²³⁾. Bcl-2 family proteins, mainly located on the mitochondrial membrane, regulate cell survival and apoptosis, which may be either anti-apoptotic (e.g. Bcl-2 and Bcl-XL) or pro-apoptotic (e.g. Bax, Bak, Bid, Bad and Bik)²⁴⁾. Lowered Bcl-2 and increased Bax expression, both of which have been demonstrated as early events in the process of apoptosis, alter mitochondrial membrane permeability, induce the release of cytochrome c or trigger caspase cascade activation in neurons²⁵⁾. In accordance with a previous study²⁶⁾, our current study showed a remarkable decrease of Bcl-2 and increase of the Bax mRNA levels in PC12 cells following H_2O_2 exposure. HT070 significantly attenuated H_2O_2 -induced upregulation of Bax and rehabilitated the Bcl-2 level at the 6 h time point. These results suggest that the modulation of apoptosis-related gene expression might at least partly contribute to the protective effect of HT070 against neuronal injury. Baicalin and baicalein, the major active flavonoids in *Scutellariae Radix*, have been reported to modulate the expression of Bcl-2 family proteins and block the caspase cascade in H_2O_2 -induced PC12 cells^{27,28)}. These studies support our suggestion and indicate that flavonoids in HT070 are responsible for the modulation of apoptosis-related gene expression.

iNOS and COX-2 are known to play pivotal roles in the generation of free radicals in ischemic stroke²⁹⁾ and free radicals themselves can increase and/or induce the expression of iNOS and COX-2³⁰⁻³²⁾. Increased iNOS after cerebral ischemia serves as the major source of nitric oxide (NO) production and NO reacts

with most free radicals with one unpaired electron³³⁾. Reaction of NO with superoxide anion leads to the production of peroxynitrite ($ONOO^-$), a highly reactive oxidant with neurotoxic actions³⁴⁾. COX-2, possessing two active sites, catalyzes two key steps in the conversion of arachidonic acid (AA) to prostaglandin H_2 (PGH_2). The first active site utilizes two oxygen molecules to form the hydroxy endoperoxide prostaglandin G_2 (PGG_2) from AA. Subsequently, PGG_2 is converted to PGH_2 by peroxidative reduction in the second active site. This peroxidase activity of COX reduces PGG_2 to PGH_2 by removal of oxygen, which may be a source of oxygen radicals^{35,36)}. It has been demonstrated in PC12 cells that iNOS and COX-2 mRNA levels are increased subsequent to H_2O_2 treatment³⁷⁻³⁹⁾ and iNOS-specific inhibitor blocks H_2O_2 -mediated apoptosis in PC12 cells⁴⁰⁾. In addition, it has been demonstrated that COX-dependent neuronal death proceeds via superoxide anion generation⁴¹⁾. Results of the present study showed that H_2O_2 increased the expression of iNOS and COX-2, a finding in agreement with previous studies, and that pretreatment with HT047 inhibited this effect. These results suggest that the modulation of enzyme expression responsible for secondary generation of ROS may be related to the protective effect of HT070 against oxidative stress-induced neuronal injury.

Our *in vitro* data suggest that the neuroprotective effect of HT070 against global cerebral ischemia might be partly associated with inhibition of oxidative stress by modulation of Bax and Bcl-2 expression and blocking iNOS and COX-2 expression. Findings from previous studies support our suggestion: Baicalin, the major active compounds in *Scutellariae Radix*, attenuates neuronal apoptosis in global cerebral ischemia by inhibition of the caspase-3, known to act downstream of Bax/Bcl-2, and by reduction of COX-2 expression in the hippocampal CA1 region^{13,42)}. *E. seticosus* protects delayed neuronal death and reduces COX-2 expression in the CA1 region of the hippocampus after global cerebral ischemia in rats¹²⁾.

V. Conclusion

This study addressed the neuroprotective effects of HT070 after global cerebral ischemia and the possible mechanisms underlying neuroprotection. The summary

of results and conclusion are as follows:

1. Oral administration of HT070 significantly increased neuronal density in rat hippocampal CA1 region following four-vessel occlusion.
2. HT070 exerted protective effects against H_2O_2 -induced cytotoxicity in PC12 cells.
3. HT070 restored the down-regulated Bcl-2 mRNA level and up-regulated Bax, iNOS, and COX-2 mRNA expression in H_2O_2 -treated PC12 cells.

Taken together, HT070 protects against delayed neuronal death after global cerebral ischemia and its neuroprotective effects might be achieved at least in part by the inhibition of mitochondrial apoptosis and reduction of the expression of ROS-generating enzymes. With a long history of human use, HT070 has potential for neuroprotection in global cerebral ischemia.

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