Neuroprotection and Enhancement of Spatial Memory by Herbal Mixture HT008-1 in Rat Global Brain Ischemia Model

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Abstract: To investigate whether HT008-1, a prescription used in traditional Korean medicine to treat mental and physical weakness, has a neuroprotective effect on a rat model of global brain ischemia and an enhancing effect against memory deficit following ischemia. Global brain ischemia was induced for 10 min by using 4-vessel occlusion (4-VO). HT008-1 was orally administered at doses of 30, 100, and 300 mg/kg respectively twice at 0 and 90 min after ischemia. The effect on memory deficit was investigated by using a Y-maze neurobehavioral test 4 days after brain ischemia, and the effect on neuronal damage was measured 7 days after ischemia. The mechanism of action was studied immunohistochemically using an anti-CD11b (OX-42) antibody. The oral administration of HT008-1 at 100 and 300 mg/kg significantly reduced hippocampal neuronal cell death by 49% and 53%, respectively, compared with a vehicle-treated group, and also improved spatial memory function in the Y-maze test. The effects of HT008-1 were more pronounced than those of its individual herb components. The herbal mixture HT008-1 protects the most vulnerable CA1 pyramidal cells of the hippocampus and enhances spatial memory function against global brain ischemia; an anti-inflammatory effect may be one of the mechanisms of action.

Keywords: HT008-1; 4-Vessel Occlusion (4-VO); Brain Ischemia; Neuroprotection; Y-Maze Test.

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Introduction

HT008-1, an herbal prescription comprising the roots of *Panax ginseng*, *Scutellaria baicalensis*, *Angelica sinensis*, and *Acanthopanax senticosus*, is used in traditional Korean medicine (TKM) for the treatment of mental and physical weakness. Many pharmacological studies of the component herbs of HT008-1 have been reported having the memory-enhancing, antipyretic, antibacterial, antihypertensive, antioxidative, hypolipidemic, and anticoagulative effects (Shen *et al.*, 1991; Sui *et al.*, 1994; Zhang *et al.*, 1998; Ye *et al.*, 2001; Abebe *et al.*, 2002; Yim *et al.*, 2002; Shao *et al.*, 2004; Bao *et al.*, 2005). In particular, the component herbs have neuroprotective effects on animal stroke models attributed to antioxidative, neurotrophic factor-like, or antiinflammatory effects (Choi *et al.*, 1996; Bu *et al.*, 2005; Kang *et al.*, 2005; Zhang *et al.*, 2006). Previous clinical studies have also suggested that HT008-1 has memory-enhancing effects on healthy volunteers (Hwang, 2006; Oh, 2006).

Despite the long history of clinical use in TKM and pharmacological studies, the neuroprotective effect of HT008-1 on brain ischemia is not well established. We investigated whether HT008-1 has a neuroprotective effect on animal stroke models. We used a 4-vessel occlusion (4-VO) rat model, a widely used model introduced by Pulsinelli and Brierley (1979), to represent transient global ischemia. In 4-VO, a selective delayed neuronal damage in the CA1 region of the hippocampus and the caudate putamen were induced by a toxic biochemical cascade that occurs within several minutes or hours (Pulsinelli *et al.*, 1982). Since deficits in cognitive functions are also induced 4 or 5 days after ischemia, this model is also included in behavioral studies (Conrad *et al.*, 1996; Block and Schwarz, 1997; Mori *et al.*, 2001; Yanpallewar *et al.*, 2004).

This study was to investigate the neuroprotective effect of HT008-1 on global brain ischemia and the enhancing effect on spatial memory impairments by using a Y-maze test. The immunohistochemistry was performed by using anti-CD11b (OX-42) antibody for the mechanism study.

Materials and Methods

Sample Preparation

Each herb was identified by Dr. H. Choi at the Department of Herbal Pharmacology, College of Oriental Medicine, Kyung Hee University, Seoul, Korea. Voucher specimens (*P. ginseng*, HP085; *S. baicalensis*, HP012; *A. sinensis*, HP114; and *A. senticosus*, HP060) have been deposited at the Department of Herbal Pharmacology of the College of Oriental Medicine.

One hundred grams of the dried roots of *P. ginseng*, *A. senticosus*, *A. sinensis*, and *S. baicalensis* were extracted separately with 70% ethanol (1,000 ml) for 6 hours at 82°C in a reflux apparatus. After reflux, the samples were evaporated using a rotary evaporator and lyophilized to yield the following amounts of extract: *P. ginseng* (PGI; 25.5 g), *A. senticosus* (ASE; 7.8 g), *A. sinensis* (ASI; 45.6 g), and *S. baicalensis* (SBA;
For the preparation of HT008-1 extract, a 3-step procedure was devised in order to improve the extraction yield. Initially, 3 herbs [A. senticosus (41.3 g), A. sinensis (33.0 g) and S. baicalensis (3.7 g)] were extracted together using the same method to produce 17.78 g of extract (yield, 22.8%). Secondly, P. ginseng (22.0 g) was extracted separately to produce 5.61 g of extract (yield, 25.5%). Finally, these 2 types of extracted powder were mixed. The mixed HT008-1 was then stored at −20°C until used.

**HPLC Analysis and Compound Profile of HT008-1 and Each Herb**

The qualitative and quantitative analyses of the compounds in HT008-1 and each herb were accomplished under the analytical conditions described below. Each dried extract was redissolved in 2 ml of high-performance liquid chromatography (HPLC)-grade methanol, filtered through a 0.45 µm membrane filter and analyzed by HPLC. HPLC with a 600 pump (Waters, Milford, MA, USA) was performed on a 250 mm × 4 mm internal diameter Hypersil™ Gold C_{18} column (ThermoElectron, Bellefonte, PA, USA) at a flow rate of 1 ml/min under the following conditions with solvent A (1% H₃PO₄) and solvent B (CH₃CN): a linear gradient from 5% to 50% of solvent B in A for 60 min and standing in 70% of solvent B in A from 61 to 85 min. The isolated compounds were monitored with a photodiode array detector (926; Waters, Milford, MA, USA). In HPLC analysis, 10 compounds were identified in HT008-1: ginsenoside Rb1 and Rg1 from P. ginseng; eleutheroside B and E and chlorogenic acid from A. senticosus; ligustilide from A. sinensis; and baicalein, baicalin, wogonin, and wogonoside from S. baicalensis. Among them, the content of one typical compound for each herb was calculated for standardization. HT008-1 was standardized to contain 0.12% ginsenoside Rb1, 0.75% baicalin, 0.04% eleutheroside E and 0.32% ligustilide. A 3-D HPLC chromatogram and the structures of the constituent compounds of HT008-1 are shown in Fig. 1.

**Animals and Drug Treatment**

All animal procedures were conducted according to the animal welfare guidelines issued by the Korean National Institute of Health (KNIH) and the Korean Academy of Medical Sciences. Male Wistar rats (SLC, Japan) weighing 180–200 g were used as study subjects. The rats were housed under controlled conditions (22 ± 2°C; lighting, 07:00–19:00), with food and water available ad libitum. Samples were dissolved in distilled water and administered orally twice at doses of 30, 100, and 300 mg/kg respectively at 0 and 90 min after ischemia (administration volume; 1.0 ml/kg). The rats in the vehicle-treated group were administered distilled water.

**4-Vessel Occlusion Rat Model**

4-VO was induced by using a method described previously (Suk et al., 2002). Briefly, the animals were anesthetized with isoflurane (initiated and maintained with 5% and 1.5%
isoflurane, respectively). After the animals were positioned in stereotaxic ear bars (Kopf; Tujunga, CA, USA), the vertebral arteries at the first cervical vertebra were coagulated by using an electrocoagulator. Both common carotid arteries (CCAs) were then isolated via a ventral, midline neck incision. All wounds were closed with surgical clips, and the rats were allowed to recover from anesthesia. On the following day, 4-VO ischemia was induced through CCA occlusion by applying aneurysm clips for 10 min. In order to minimize the variability among animals, the following criteria were strictly applied for the 10 min ischemic period and the 20 ± 5 min postischemic coma (loss of righting reflex and bilateral pupil dilation). After 10 min of 4-VO, the aneurysm clips around the CCA were removed, and the neck wound was closed with surgical clips. Body temperature was monitored and maintained at 37°C with a heating blanket (Homeothermic Blanket Control Unit; Harvard Apparatus, Edenbridge, UK) for 6 hours after ischemia. The sham-operated group underwent the same surgical procedures, except that the CCAs were not occluded.

**Y-Maze Test**

A Y-maze test was performed 4 days after ischemia using the protocol described by Mori et al. (2001), with a slight modification. Spontaneous alternation behavior in the Y-maze was assessed as a measure of short-term spatial memory. The maze was constructed with...
wood painted black, and each arm was 40 cm long, 20 cm high and 10 cm wide. The rats were placed in the Y-maze for 8 min, 4 days after brain ischemia. Entry into an arm was defined as when the hind paws of the rats were completely within the arm. Spontaneous alternation was defined as rats entering all 3 arms in the overlapping triplet sets. The percentage of alternation was calculated as \[ \text{successive triplet sets/(total number of arm entries – 2)} \times 100. \]

**Histology**

Seven days after ischemia, the animals were anesthetized, and their brains were fixed with 4% paraformaldehyde (PFA) after transcardial wash-out with heparinized 5% sodium nitrite saline. The fixed brains were cut into 30 µm sections on a sliding microtome (HM440; Carl Zeiss, Heidelberg, Germany) and the sections, stained with cresyl violet. Neuronal cell density was measured by counting viable cells in a total of 6 frames (1.0 mm × 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) for each animal. Neuronal cell density is equivalent to the average number of viable cells in one frame. Cell counting was performed by 3 technicians blinded to the experimental conditions.

**Immunohistochemistry**

Seven days after ischemia, the brains were fixed with 4% PFA. Free-floating (40-µm) sections were incubated with a mouse polyclonal antibody against OX-42 (against the CD11b antigen, diluted 1:100; Serotec, Oxford, UK) for 60 min at room temperature. After incubation, the sections were reacted with a fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (diluted 1:100; Jackson ImmunoResearch Lab, West Grove, PA, USA) for 60 min at room temperature. The samples were observed by using a confocal laser scanning microscope (LSM5 PASCAL; Carl Zeiss, Heidelberg, Germany). Fluorescence intensities were analyzed using a computer-based image analysis system (Optimas 6.0; Media Cybernetics, Silver Spring, MD, USA).

**Statistical Analysis**

All data are presented as the mean ± SEM. The effects of different treatments were compared by using the student’s t-test in GraphPad Prism 4 (GraphPad Software Inc., USA). \( p < 0.05 \) were considered to be statistically significant.

**Results**

**Neuronal Cell Density in the CA1 Region of the Hippocampus**

The neuroprotective effects of HT008-1 were evaluated by measuring the neuronal cell density in the CA1 hippocampal region 7 days after ischemia. Representative
photomicrographs of cresyl violet-stained hippocampal neurons in each experimental group are shown in Fig. 2. In the sham-operated group, the neuronal density in the CA1 region was normal (Fig. 2A), and most of the pyramidal neurons in the CA1 region exhibited an intact morphology and no cell damage (Fig. 2A, 2a). In the vehicle-treated group, the neuronal density was reduced, and the neurons exhibited a shrunken morphology (Fig. 2B, 2b). Compared to the vehicle-treated group, the HT008-1-treated animals exhibited dose-dependent increases in neuronal density (Fig. 2).

The neuronal density in the CA1 regions was measured using a blind test as shown in Fig. 3. In the sham-operated group, the neuronal density in the CA1 region was normal (Fig. 2A), and most of the pyramidal neurons in the CA1 region exhibited an intact morphology and no cell damage (Fig. 2A, 2a). In the vehicle-treated group, the neuronal density was reduced, and the neurons exhibited a shrunken morphology (Fig. 2B, 2b). Compared to the vehicle-treated group, the HT008-1-treated animals exhibited dose-dependent increases in neuronal density (Fig. 2).

The neuronal density in the CA1 regions was measured using a blind test as shown in Fig. 3. The neuronal density of the vehicle-treated group was reduced as compared with that of the sham-operated group.
of the sham-operated group (98.3 ± 12.4 vs 374.1 ± 34.8 cells/mm²). Oral administration of HT008-1 at doses of 100 and 300 mg/kg resulted in neuronal densities of 234.4 ± 20.5 and 245.5 ± 46.4 cells/mm², respectively (p < 0.01 for the 100 mg/kg-treated group and p < 0.05 for the 300 mg/kg-treated group vs the vehicle-treated group; Fig. 3).

In order to investigate the effects of the constituent herbs of HT008-1 on neuronal death in the 4-VO rats, each herb was orally administered at a dosage of 300 mg/kg. Representative photomicrographs of cresyl violet-stained hippocampal neurons in each experimental group are shown in Fig. 4. The numbers of viable CA1 neurons in the treated groups were also counted and compared with that in the vehicle-treated group. Among

![Figure 4. Representative photomicrographs of cresyl violet-stained hippocampal regions treated with each component herb of HT008-1 in 4-VO rats. Sham-operated group (A, a), vehicle-treated group (B, b), or HT008-1 (C, c), PGI (D, d), SBA (E, e), ASI (F, f), and ASE (G, g)-treated groups. a–g are boxed regions in A–G, respectively. Scale bar = 100 µm.](image)

![Figure 5. Neuronal density of the hippocampal CA1 region after treatment with each component herb of HT008-1. HT008-1 and individual herbs were orally administered at a dosage of 300 mg/kg 0 min and 90 min after of brain ischemia. Neuronal cell density was equivalent to the average number of viable cells in the frames (1.0 × 1.0 mm) of the CA1 regions. The protective effects of HT008-1 were more pronounced than those of the individual components in the 4-VO rats. Each group comprised 4–5 animals. The values are means ± SEM (°p < 0.05, °°p < 0.01, °°°p < 0.001 vs. vehicle-treated group).](image)
the animals treated with the component herbs, the PGI- and SBA-treated groups exhibited a significant number of intact neurons — 180.4 ± 13.6 and 182.5 ± 24.6 cells/mm², respectively — compared with the vehicle-treated group (p < 0.05 for the PGI-treated group and p < 0.01 in SBA-treated group; Fig. 5). The protective effects of HT008-1 were more pronounced than those of each component in the 4-VO rat model (Fig. 5).

**Y-Maze Test**

In order to evaluate the effects of HT008-1 on the spatial memory function after ischemia, we performed a Y-maze test. The percentage of spontaneous alternation over an 8 min period was recorded and used as an index of spatial memory. Among all groups, there were no significant differences in the total number of entries into the arms during the 8 min observation period (Fig. 6A). However, the vehicle-treated group exhibited a significant memory deficit compared to the sham-operated group (45.8 ± 2.4% vs 74.5 ± 5.6%), and the HT008-1-treated group at a dosage of 100 mg/kg exhibited a significant improvement in the 4-VO-induced impairment of spontaneous alternation behavior (74.4 ± 10.7% vs 45.8 ± 2.4%, p < 0.05; Fig. 6B).

**Immunohistochemistry**

In the vehicle-treated group, the histochemical marker of microglia, OX-42, was upregulated in the CA1 region after ischemia compared to the sham-operated group (Figs. 7a, 7b, 7d, and 7e). In the HT008-1-treated group, the expression was downregulated compared to the vehicle-treated group (Figs. 7b, 7c, 7e, and 7f).

![Figure 6](image-url)
Discussion

In the present study, we tested the effects of HT008-1 on hippocampal injury and spatial memory deficits in rats subjected to a 4-VO-induced transient global brain ischemia. At doses of 100 and 300 mg/kg, the oral administration of HT008-1 significantly attenuated hippocampal neuronal cell death and significantly improved the spatial memory function in a Y-maze test. At the same dosage (300 mg/kg), HT008-1 potentiated the neuroprotective effects of ischemia-evoked neuronal cell damage more effectively than its component herbs.

Figure 7. Inhibitory effects of HT008-1 on the expression of OX-42 in the hippocampal regions after 4-VO. The samples were stained with the OX-42 antibody activated by fluorescence-conjugated secondary antibodies. The expression of OX-42 was reduced in the HT008-1-treated group compared to the vehicle-treated group. Sham-operated group (a, d), vehicle-treated control group (b, e), HT008-1-treated group (300 mg/kg p.o.; c, f). Scale bars are 100 µm (c) and 25 µm (f). Data shown is typical result from 3 independent experiments. (g) Statistical analysis of data was obtained from observed fluorescence intensities. The values are means ± SEM (**p < 0.001 vs. vehicle-treated group).
Our study has demonstrated the neuroprotective effects of HT008-1 on the most vulnerable population of CA1 pyramidal cells 10 min after ischemia and 7 days of reperfusion. It can be concluded that the administration of HT008-1 following ischemia has a beneficial effect on the surviving neurons that are destined to die. Among its component herbs, PGI and SBA (at 300 mg/kg) reduced the neuronal density in transient global ischemic rats by 29% and 30%, respectively. In contrast, in the present study, ASE and ASI failed to exhibit any significant effects, although both herbs have previously been reported to have neuroprotective effects (Liu et al., 2004; Bu et al., 2005; Kuang et al., 2006).

The oral administration of 300 mg/kg of HT008-1 was more effective than the oral administration of the same dosage of any one of its component herbs. Three hundred milligrams of HT008-1 contains 66.0 mg of P. ginseng, 123.9 mg of A. senticosus, 99.0 mg of A. sinensis, and 11.1 mg of S. baicalensis. Although we did not compare the effect of HT008-1 with the proportional content dosage of each component herb in HT008-1, none of them was as efficacious as HT008-1, even at the accepted maximum dosage. This suggests that the constituent herbs may exert an additive neuroprotective effect when mixed together. A. senticosus and A. sinensis did not exhibit a significant neuroprotective effect in this experiment; however, their contribution to the effect cannot be excluded. The results may explain the “harmonizing theory” of herbs empirically used in TKM; when they are administered together, some herbs are synergistic, whereas others are antagonistic.

Immunochemical analysis demonstrated that HT008-1 inhibited postischemic OX-42 expression. This result suggests that HT008-1 may inhibit microglial activation following ischemia. Microglia are activated in response to a pathological state in the brain, such as ischemia. Activation of microglia precedes the manifestation of tissue injury and may exert a cytotoxic effect by producing proinflammatory cytokines such as IL-1β, which is a major cytokine produced following ischemia (Bhat et al., 1996). Interference with microglial activation may be the mechanism of HT008-1 action.

Similar to the inhibitory effect on microglial activation, the neuroprotective mechanisms of HT008-1 against ischemic injury are still not completely understood. However, other antiinflammatory and antioxidative activities of the component herbs have previously been considered. P. ginseng, for example, has been demonstrated in animal and human studies to exhibit neuroprotective effects. The major effective compounds in this herb were identified as ginsenoside Rb1 and Rg1, and the mechanisms of action are antioxidative and neurotrophic factor-like effects (Chu and Chen, 1990; Wen et al., 1996; Lim et al., 1997; Xuejiang et al., 1999). S. baicalensis has also been proved to have neuroprotective effects in global brain ischemia. Its major effective compounds are flavonoids, such as baicalein, baicalin, wogonin, and wogonoside, and the mechanisms of action are antioxidative and antiinflammatory effects (Kim et al., 2001; Lee et al., 2003). Although A. senticosus and A. sinensis did not exhibit significant effects in the present study, they have previously been reported to have neuroprotective effects attributed to antioxidative and antiinflammatory mechanisms (Liu et al., 2004; Bu et al., 2005). Based on these previous studies on the neuroprotective effects of each constituent herb, we suggest that antioxidative and antiinflammatory effects might constitute the underlying mechanism of action of the observed effect (Kim et al., 1999; Siddique et al., 2000; Kim et al., 2001).
In the present study, the brains of the experimental animals were histologically processed only at the end of behavioral testing, and only intact-appearing pyramidal neurons were counted. Although, ischemia-surviving neurons may appear intact, they may have been dysfunctional at the time of behavioral testing. Here, we demonstrated that 100 mg/kg of HT008-1 reduced ischemia-induced cognitive dysfunction as measured in the Y-maze test, a test used to assess spatial recognition memory caused by brain damage (Conrad et al., 1996; Wright and Conrad, 2005). One of the characteristics of the 4-VO rat model is memory deficit resulting from delayed neuronal death in the region of the hippocampus, which is closely related to memory function (Nunn and Hodges, 1994; Olsen et al., 1994; Squire and Zola, 1996; Milani et al., 1998). This behavioral effect was concomitant with the sustained reduction in hippocampal neuro-degeneration induced by ischemia. These results support the premise that HT008-1 increases spatial memory in addition to protecting against hippocampal neuronal death in transient global ischemia.

In conclusion, our results suggest that the herbal mixture HT008-1 protects the most vulnerable CA1 pyramidal cells and enhances the spatial memory function against global brain ischemia.

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