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뇌허혈로 인한 흰쥐에서의 기억력 및 학습효과 저해에 대한 황금의 보호효과

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Protective effects of Scutellariae Radix on impairments in learning and memory induced by brain ischemia in rats

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ABSTRACT

Objectives : It has been reported previously that the roots of *Scutellaria baicalensis* (known as Huang-Gum in Korean, henceforth referred to as *S. baicalensis*) could prevent neuronal cell death after global cerebral ischemia. In Genuine Korean medicine, *S. baicalensis* is known to relieve fever in upper body, and it was thus thought to be able to alleviate deteriorations in brain function.
Methods : The protective effects of *S. baicalensis* against post-stroke memory retardation using 4-vessel occlusion model were examined in the present study.
Results : *S. baicalensis* was shown to significantly alleviate the deficits in learning and memory by increasing the fraction of time spent in the quadrant in which the platform was initially placed (34.9 ± 3.2%, $p < 0.05$) compared to that of the ischemia group (28.0 ± 2.5%). The cytoprotective effect of *S. baicalensis* on CA1 hippocampal neurons was evaluated by measuring the neuronal cell density. Neuronal cell density in *S. baicalensis* extracts-treated ischemia group (138.0 ± 13.6 cells/mm^2) was significantly increased compared to saline-treated ischemia group (22.1 ± 9.3 cells/mm^2, $p < 0.05$). In the study of OX-42 immunohistochemistry, *S. baicalensis* could decrease the micrgial activation in hippocampus after brain ischemia.
Conclusion : These results may provide experimental support for the use of *S. baicalensis* in treating post-stroke memory impairment.

Key words : Scutellariae Radix, *Scutellaria baicalensis*, learning and memory, brain ischemia, degenerative disease..

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Introduction

The root of *Scutellaria baicalensis* Georgi (Labiatae) (known as Huang-Gum in Korean, henceforth referred to as *S. baicalensis*) is widely used to treat fever in the upper body including fever in the head, eyes, or larynx, in traditional Genuine Korean medicine[1,2]. Many scientists have noted the antipyretic, antibacterial, and antihypertensive effects of *S. baicalensis*[3,4] and have previously reported that *S. baicalensis* could prevent neuronal cell death from stroke[5].

Among hippocampal neurons, CA1 region is most vulnerable to cerebral ischemia[6]. Death of CA1 hippocampal neurons following cerebral ischemia causes a variety of neurological dysfunction. Although the exact mechanism of neurological damage caused by cerebral ischemia has yet to be elucidated, recent works suggested the involvement of excitotoxicity, activation of voltage-gated calcium channels, inflammatory cytokines, and oxidative stress6. Based on these potential neurotoxic mechanisms, various neuroprotective agents are being developed[7]. These include calcium antagonists, NMDA antagonists, glutamate release inhibitors, free radical scavengers, and leukocyte adhesion inhibitors.

It is well known that deterioration of learning and memory functions is accelerated after brain ischemia[8]. Clinical studies showed the strong evidence that the hippocampus is critical for memory function in humans[9-11]. Cerebral ischemia induced by cardiac arrest has been found to result in moderate to profound anterograde memory loss. Learning and memory impairments were found out in 20 - 40% of patients surviving a cardiac arrest. In non-human study, hippocampus have been deeply involved in memory function[12]. Hippocampal lesions could impair the spatial learning memory in rats[13,14]. Accordingly, this study was undertaken to examine the protective effects of *S. baicalensis* against impairments in learning and memory and histological damages induced by brain ischemia. Microglial activation, which is the primary immune effector cells of central nervous system[15] and plays an important role in a neurodegeneration[16], was observed in hippocampus as well.

Materials and methods

1. Plant material.

The roots of *Scutellaria baicalensis* Georgi (Labiatae) were purchased at Kyungdong Oriental drug market in Seoul, Korea in 2001. The drug was authenticated and deposited at the herbarium of Kyung Hee University (No. HP21001). Dried roots of *S. baicalensis* (1.0 kg) were extracted with 70% methanol under sonication. The extract was then filtered and vacuum-dried (yield 26.4%).

2. Surgical procedure.

Male rats of the Wistar strain weighing 160 - 180 g were used in the experiment. The experimental procedures were performed in accordance with the animal care guidelines of NIH. Animals were housed under controlled temperature (20 ± 2°C) and lighting (07:00-19:00) conditions, with food and water made available ad libitum.

Male Wistar rats weighing between 160 and 180 g were used. Before the experiment, food was withheld overnight, but water was freely available. The animals were anesthetized with isoflurane (initiated with 5% and maintained with 1.5% of isoflurane). Anesthetized animals were surgically prepared for 4-vessel occlusion (4-VO) as originally described by Pulsinelli17). In brief, after the animals were positioned in stereotaxic ear bars (Kopf; Tujunga, CA) with the head tilted down at 30° to the horizontal, incision of 1 cm in length was made behind the occipital bone directly overlying the first two cervical vertebrae. The paraspinal muscles were separated from the midline, and the right and left alar foramina of the first cervical vertebrae were exposed with the use of an operating microscope. A 0.5-mm diameter electrocautery needle was inserted through each alar foramen and both vertebral arteries electrocauterized and permanently occluded. Next,

both common carotid arteries were isolated via a ventral, midline cervical incision. An atraumatic arterial clasp was loosely placed around each common carotid artery without interrupting carotid blood flow and the incision was closed with a single suture. On the following day, 10 min of 4-VO ischemia was induced by tightening the clasp around the common carotid arteries. Carotid clasps were then removed following 10 min to restore carotid blood flow. To minimize variability among animals, following criteria were strictly applied for the 10 min ischemic period and 20 ± 5 min postischemic coma: loss of righting reflex and bilateral pupil dilation. Body temperature was monitored and maintained at 37 ± 0.5°C with a rectal thermistor coupled to a heating blanket (Homeothermic Blanket Control Unit, Harvard apparatus; Edenbridge, UK). Sham-operated animals that underwent surgery were used for non-ischemic control.

3. Learning and memory tests.

Three groups of animals were used - one group of sham-operated animals (Sham group, n = 6), one group of 4-VO rats which was administrated intraperitoneally with saline (Control group, n = 6) and one group of 4-VO rats which was administrated intraperitoneally with 10 mg/kg of *S. baicalensis* (*S. baicalensis* group, n = 6). The dose of *S. baicalensis* was chosen as it has been shown to be neuroprotective5. The substances were applied intraperitoneally 0 and 90 min after induction of ischemia. Impairments in learning and memory were studied with Morris water maze as previously described8. One week after surgery, spatial learning was tested in a water maze13: a black, circular pool (diameter 100 cm, height 40 cm) was filled to 22 cm with water (26°C). It was placed in a dimly lit and large testroom, and surrounded by various extramaze visual cues. Six days after occlusion the rats received a habituation trial (1 min) in which there was no platform present. Over three subsequent days each rat performed 20 escape trials (five blocks of four trials). During a particular trial, the rats were able to escape from the water only by climbing on an invisible, submerged platform (diameter 6 cm). For each rat, the location of the hidden platform remained unchanged during the whole experiment. Beginning in the Northern quadrant the start locations were semi-randomly varied across subsequent trials. A trial was terminated as soon as the rat found the platform; or if it did not succeed within 120 s, it was placed on the platform by hand. The animal was allowed to stay on the platform for 20 s before the next trial started. Immediately after the final escape trial, each rat was subjected to a probe trial (60 s) in which there was no platform present. The time spent in the quadrant of the former platform position was obtained as a measure for spatial memory. All trials were recorded on videotape for analysis of escape latency, swim distance, percentage of swimming path along the sidewall and swim speed of the escape trials and for analysis of time spent in the quadrant of the former platform location within the probe trial using a computer-assisted video tracking system (Smart 2.0 Software, Panlab, Spain).

4. Histology

Two weeks after surgery, the animals were deeply anaesthetized with pentobarbital and intracardially perfused with Ringer and then with formaldehyde. Fixed brains were cut into 30 μm sections on a sliding microtome and the sections stained with cresyl violet. Neuronal cell density was measured by counting viable cells in the total six frames (1.0 × 1.0 mm^2) of left and right CA1 regions of three coronal sections (about 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 done by three technicians blinded to the experimental conditions. For the detection of microglial activation, OX-42 immunohistochemistry was performed. Frozen sections of rat brain tissues were incubated with anti-OX-42 antibody (1 : 500

dilution; Serotec Co., USA) after goat serum blocking. Thereafter, incubation with biotinylated anti-mouse IgG antibody, and then with avidin-biotin-peroxidase complex (Vector, Burlingame, USA) followed. Diaminobenzidine was used as a color substrate.

5. Statistical Analysis

The results were expressed as means ± standard error of the mean (SEM). One-way ANOVA with Tukey's multiple comparison test was used to compare the differences between the groups. Values of $p < 0.05$ were considered significant.

Results

1. Learning and memory tests.

All animals were able to swim normally during the habituation trial. As illustrated in Figure 1A, all rats were able to locate the hidden platform during the escape trials. After three days, 4-VO induced control group required more time than sham or *S. baicalensis* groups (Figure 1A). The swim distance and swim speed of control group did not differ from that of sham or *S. baicalensis* groups (Figure 1B and C). Although there is no significant difference for comparing the three groups with respect to the percentage of the swimming path along the sidewall, sham and S. baicalensis groups showed the tendency to reduce the ratio (Figure 1D).

Analysis of the swimming performance during the probe trial revealed that control group (28.0 ± 2.5%) spent significantly less time in the quadrant of the former platform position than sham group (41.6 ± 1.6%, $p < 0.05$) and *S. baicalensis* group (35.9 ± 2.2%, $p < 0.05$; Figure 2B). Control group (3.8 ± 1.0) performed less crossings over the former platform

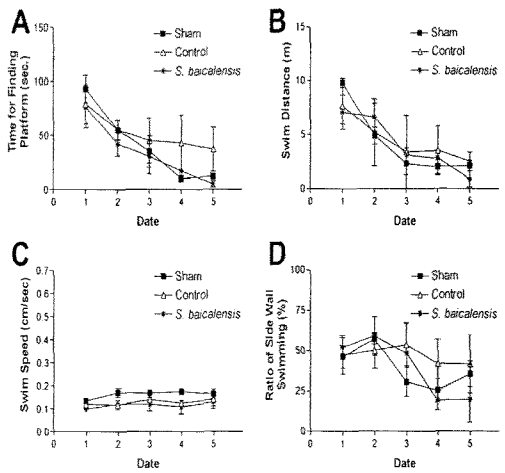

Figure 1: Escape latency in sec (A), swim distance in m (B), swim speed in cm/sec (C) and percentage of swimming path along the sidewall (D) by sham-operated controls (n = 6, sham group), brain ischemia induced rats treated with saline (n = 6, control group), or brain ischemia induced rats treated with *Scutellaria baicalensis* extract, 10 mg/kg (n = 6, *S. baicalensis* group) for each block of four escape trials throughout the experiment. Values are expressed as means ± standard error of the mean.

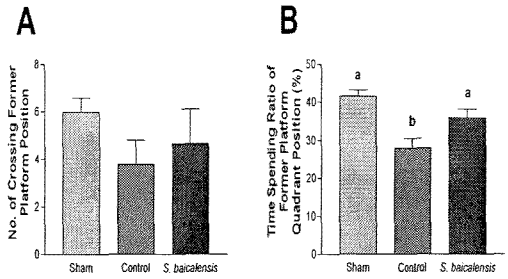

Figure 2: Performance during the probe trial as measured by the number of platform crossings over the former platform position (A) and time spent in the quadrant of the former platform position (B). Values are expressed as means ± standard error of the mean. Significance between a and b is $p < 0.05$.

2. Histological results.

The cytoprotective effect of *S. baicalensis* extracts on CA1 hippocampal neurons was evaluated by measuring the neuronal cell density in CA1 hippocampal region at two weeks after

ischemia. Neuronal cell density in *S. baicalensis* extracts-treated ischemic animals (138.0 ± 13.6 cells/mm^2) was significantly increased compared to saline-treated control group (22.1 ± 9.3 cells/mm^2, $p < 0.05$; Figure 3). Body temperature of animals exposed to ischemia was monitored for 6 h of cerebral reperfusion. There was no significant difference in body temperature between saline-treated groups and *S. baicalensis* extracts-treated groups at any time point recorded (data not shown), indicating that neuroprotective effects of *S. baicalensis* extracts were not due to a decreases in body temperature. Representative photomicrographs of Cresyl violet-stained hippocampal neurons in each experimental group are shown in Figure 4.

In the hippocampus of sham-operated normal rat, immunoreactive microglial cells showing fine branches were sparsely distributed (Figure 4D, d). A large numbers of OX-42-positive microglial cells exhibiting intense immunoreactivity and appearing hypertrophied with short and thick processes were confined to all the areas in the hippocampus, where degenerating neurons were observed (Figure 4E, e). In S. baicalensis group, the immunoreactivity of microglial cells with OX-42 was still intense in CA1 region compared with sham group (Figure 4F, f). However, OX-42 immunoreactivity of other regions were effectively diminished in *S. baicalensis* group (Figure 4F, f).

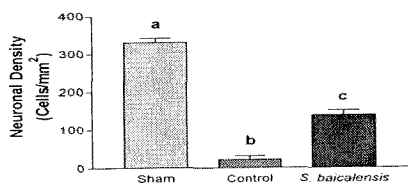

Figure 3: Neuroprotective effects of *S. baicalensis*. Either saline or S. baicalensis (10 mg/kg) was intraperitoneally administered into the rats following 10 min ischemia. Two weeks later, neuronal cell density in CA1 region was measured. *S. baicalensis* significantly protected CA1 neurons compared with ischemia induced control group ($p < 0.05$). Statistically significant differences between a, b and c are $p < 0.05$.

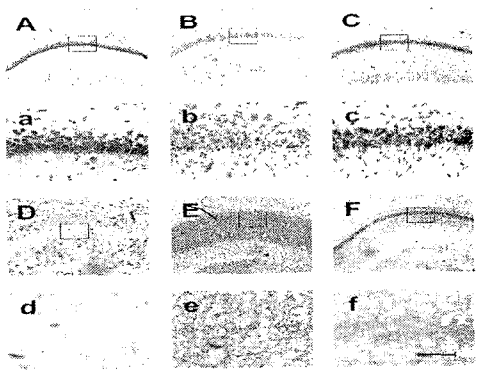

Figure 4: Representative photomicrographs of Cresyl violet-stained hippocampal regions of either sham-operated animals (A, a; sham group) or animals that had been subjected to 10 min ischemia followed by the treatment with either saline (B, b; control group) or 10 mg/kg of *S. baicalensis* (C, c). OX-42-positive microglial cells in the CA1 region of hippocampus in sham group (D, d), control group (E, e), and *S. baicalensis* group (F, f). Boxed regions in A, B, C, D, E, and F are shown in a, b, c, d, e, and f, respectively. The 10 min ischemia caused selective and delayed neuronal cell loss in the hippocampal CA1 region (B, b). In contrast, *S. baicalensis* treatment conferred neuroprotection by markedly reducing the number of damaged pyramidal cells in the CA1 subfield (C, c). The OX-42-positive microglial cells in the CA1 and other regions of control group (E, e) are hypertrophied and show intense immunoreactivity at 2 weeks after brain ischemia. *S. baicalensis* treatment could reduced the intense immunoreactivity in the CA1 and other regions of hippocampus (F, f). Scale bars for A, B, C, D, E, and F are 300 μm and for a, b, c, d, e, and f are 60 μm.

Discussion

A consistent deficit in spatial learning following global cerebral ischemia has been demonstrated using the water maze[18 21]. In the present study 4-VO rats also displayed an increased escape latency in comparison to sham-operated controls. It has been demonstrated that several substances which interact with the pathophysiological processes leading to ischemic cell death attenuated the deficit in spatial learning induced by ischemia[22 27]. In this study, brain ischemia induced the significant memory impairment (28.0 ± 2.5%, $p < 0.05$) compared with sham group (41.6 ± 1.6%) and *S. baicalensis* could significantly protect the

memory impairment induced by brain ischemia (35.9 ± 2.2%, $p < 0.05$). Many other papers about the protective effects of *S. baicalensis* were published. Flavones from *S. baicalensis* were known to attenuate apoptosis and protein oxidation in neuronal cell lines[28]. Natural polyphenols from *S. baicalensis* protected neuronal death in primary cultured central neurons induced by against glutamate- and glucose deprivation[29] and in hippocampal slice culture exposed to oxygen and glucose deprivation[30]. Moreover, some studies showed the potent inhibitory effects of flavonoids in *S. baicalensis* on amyloid beta protein-induced neurotoxicity[31] and on inflammation-mediated degeneration of dopaminergic neurons by the inhibition of microglial activation[32]. These studies are in agreement with our observations. In this study, we studied the protective effects on the memory impairments and neuronal cell death induced by brain ischemia. The protective effects were found out to be related in the inhibition of microglial activation.

In conclusion, *S. baicalensis* effectively alleviated the impairments in learning and memory after brain ischemia. Further study would be necessary, but these data would provide experimental support of the utility of *S. baicalensis* in treating memory impairments after stroke.

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