대한한의학회지 제29권 제2호(2008년 5월)
J Korean Oriental Med 2008;29(2):21-31

Original Article

Protective Effects of *Singihwan* (腎氣丸) on Traumatic Brain Injury-induced Apoptosis in Rat Hippocampal Dentate Gyrus

Oh-Bong Kwon, Yun-kyung Song, Hyung-Ho Lim

Department of Oriental Rehabilitation Medicine, College of Oriental Medicine, Kyungwon University

Backgrounds: *Singihwan* is used "to strengthen inborn energy" and we suspected a protective effect on brain neuron cells.
Objectives: The aim of this study was to evaluate the effects of *Singihwan* (SGH) on traumatic brain injury-induced delayed apoptosis in rat hippocampal dentate gyrus.
Methods: For a surgical induction of traumatic brain injury (TBI), a 5 mm diameter stainless rod was used to make traumatic attack from the surface of the brain used by an impactor. The protective effect of the aqueous extract of SGH against TBI in the rat hippocampal dentate gyrus was investigated by using step-down avoidance task, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, Bax immunohistochemistry, and 5-bromo-2'-deoxyuridine (BrdU) immunohistochemistry.
Results: The aqueous extract of SGH suppressed the TBI-induced increase in apoptosis and cell proliferation in the hippocampal dentate gyrus.
Conclusions: It is possible that the aqueous extract of SGH has a neuroprotective effect on TBI-induced neuronal cell death.

Key Words : *Singihwan*, hippocampal dentate gyrus, apoptosis, traumatic brain injury

Introduction

Traumatic brain injury (TBI) is broadly occurred worldwide and all ages. In TBI, especially in severe case, needs long-term care, and shows significant neurological disabilities, and often leads to death[1-4]. Similar data can be found from a variety of demographic and cultures[5,6]. Also TBI patients take much of economic and social burden, especially to care of neuronal disabilities[7-9].

The neurological outcome of TBI victims depends on the extent of the primary brain insult caused by trauma itself, and on the secondary neurochemical and pathophysiological changes occurring as a consequence of the mechanical injury, which leads to additional neuronal cell loss. Although a long list of experimental studies suggest that reduction or prevention of secondary brain injury after TBI is possible, clinical trials have failed to show benefits from therapeutic strategies proven to be effective in the laboratory studies[10-12].

There are two waves of neuronal cell death in secondary brain injury after TBI. First wave

· Received Jan 29, 2008 · accepted Mar 14, 2008
· Correspondence to Hyung-Ho Lim
Department of Oriental Rehabilitation Medicine, Seoul Oriental Hospital Kyungwon University, 20-8 Songpa-Dong, Songpa-Gu, Seoul Korea
(Tel:+82-2-425-3456, Fax:+82-2-425-3560,
E-mail:von5ph@hanmail.net)

of neuronal death is induced immediately after mechanical trauma due to the impact or penetration. Neurons can die by necrosis caused by membrane disruption, irreversible metabolic disturbances and/or excitotoxicity[13]. This early application of neuroprotective protocols is critical for any possibility of reducing neuronal necrosis as an emergency. The second wave of neuronal death occurs in more delayed fashion, with morphological features of apoptosis. This second wave of neuronal cell death are targeted for therapies[14].

Apoptosis has long been identified as an evolutionarily conserved process of active cell elimination during development. It's phenotypic features include DNA fragmentation and chromatin condensation, cell shrinkage, and formation of apoptotic bodies, which are cleared by phagocytosis without initiating a systemic inflammatory response. The execution of apoptosis requires novel gene expression and protein synthesis[15-17]. Apoptosis is regulated by the B-cell lymphoma-2 (Bcl-2) family of proteins those include both pro-apoptotic and pro-survival members. They contain highly conserved Bcl-2 homology domains (BH 1-4) essential for homocomplex and heterocomplex formation. Complexes formed between proteins containing BH-3 domains such as Bax, truncated Bid, and Bad, can facilitate the release of cytochrome c from mitochondria as pro-apoptotic proteins[18].

A brain is the sea of a bone marrow, which is stored in a bone, and kidney can control the bone. Namely, we can control the condition of a bone and a brain by the treatment of kidney. *Singihwan*(SGH), an Oriental herbal medicine formulation, can be traditionally used for strengthen a 'Yang' and 'Yin' of kidney[19].

SGH has been reported that it is used for delayed mental and physical development in children, complications of diabetes and treatment of glomerulonephritis patients[20,21]. However, the effects of the aqueous extract of SGH on TBI have not been reported yet. In the present study, the neuroprotective effects of the aqueous extract of SGH on TBI in rat hippocampal dentate gyrus were investigated. For this study, step-down avoidance task, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, Bax immunohistochemistry, and 5-bromo-2'-deoxyuridine (BrdU) immunohistochemistry were performed.

Materials and Methods

1. Preparation of the aqueous extract of SGH

Rehmanniae Radix	16 g
Dioscorae Radix	8 g
Corni Fructus	8 g
Alimatis Rhizoma	6 g
Moutan Cortex Radicis	6 g
Hoelen	6 g
Schizandrae Fructus	8 g
Cervi Cornu	4 g

The ingredients of SGH are as follows:

All ingredients were obtained from the pharmacy of oriental medical hospital Kyung Won university (Seoul, Korea). After washing, to obtain the aqueous extract of SGH, the ingredients were added to distilled water, heat-extracted, pressure-filtered, concentrated with rotary evaporator and lyophilized (EYELA, Tokyo, Japan). The resulting powder, weighing 15.48 g (a yield of 24.97%) was diluted to the concentrations needed with D.D.W. The animals in the SGH treated groups orally received the

aqueous extract of SGH at the respective dose of groups for 10 consecutive days, and those in the sham-operation group and in the TBI-induction group received an equivalent amount of water once a day for the same duration of time. Following their respective treatment, all the animals were injected intraperitoneally with 50 mg/kg BrdU (Sigma Chemical Co., St. Louis, MO, USA).

2. Animals and treatments

Adult male Sprague-Dawley rats weighing 320 ± 10 g were used in this study. The experimental procedures were performed in accordance with the animal care guidelines of National Institutes of Health and the Korean Academy of Medical Sciences. The animals were housed under controlled temperature (20 ± 2℃) and lighting (07.00-19.00 hours) conditions and were supplied with food and water ad libitum. Total number of the rats was 34. The rats were divided five groups: the sham-operation group (N=7), the TBI induction group(N=8), the TBI induction with 50 mg/kg SGH-treated group (N=8), the TBI induction with 100 mg/kg SGH-treated group(N=8), and the TBI induction with 200 mg/kg SGH-treated group(N=7).

3. Surgical induction of TBI

For induction of TBI, the rats were anesthetized with Zoletil 50 (10 mg/kg, i.p.; Vibac Laboratories, Carros, France), and they were placed in a stereotaxic frame. Through a hole that center placed 2.7 mm lateral to the midline, 2.7 mm anterior to the coronal suture in the rat skull made about 5.5 to 6 mm diameter, diameter 5 mm stainless rod was used to make traumatic attack as velocity 5 m/s, duration time 0.2 sec, 2.5 mm depth from the surface of the brain used by Benchmark Stereotaxic Impactor (myNeuroLab. Co., St. Louis, MO, USA).

4. Step-down avoidance task

In order to evaluate the short-term memory ability, we determined the latency time of the step-down avoidance task before surgical induction of TBI. On the 9th day from the beginning of SGH treatment, the rats were trained on a step-down avoidance task. The rats were placed on the 7 ± 25 cm platform, 2.5 cm in height and allowed to rest on the platform for 2 min. The platform faced a 42 ± 25 cm grid of parallel 0.1 cm-caliber stainless steel bars placed 1 cm apart. In training sessions, the animals received 0.3 mA scramble foot shock for 1sec immediately upon stepping down. Retention time was determined 24 h after training. The interval of gerbils stepping down and placing all four paws on the grid was defined as the latency of step-down avoidance task.

5. Tissue preparation

The animals were anesthetized using Zoletil 50 (10 mg/kg, i.p.), transcardially perfused with 50 mM phosphate-buffered saline (PBS), and fixed with a freshly prepared solution consisting of 4% paraformaldehyde in 100 mM phosphate buffer (PB, pH 7.4). The brains were dissected and postfixed in the same fixative overnight and transferred into a 30% sucrose solution for cryoprotection. Coronal sections of 40 m thickness were made with a freezing microtome (Leica, Nussloch, Germany).

6. TUNEL assay

For visualization of DNA fragmentation, a marker of apoptotic cell death, TUNEL staining was performed using an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. To begin the procedure, the sections were post-fixed in ethanol-acetic acid (2:1) and rinsed. Then the sections were incubated with 100 g/ml proteinase K, rinsed, incubated in 3% H2O2, permeabilized with 0.5% Triton X-100, rinsed again, and incubated in the TUNEL reaction mixture. The sections were rinsed and visualized using Converter-POD with 0.02% 3,3'-diaminobenzidine (DAB) and 40 mg/ml nickel chloride. The slides were air dried overnight at room temperature, and the coverslips were mounted using Permount.

7. Bax immunohistochemistry

For visualization of Bax expression, Bax immunohistochemistry was perfomed. The sections were drawn from each brain and incubated overnight with mouse anti Bax antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were then washed three times with PBS and incubated for 1 h with a biotinylated mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Bound secondary antibody was then amplified with Vector Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.02% 3,3-diamonobenzidine (DAB) and the sections were finally mounted onto gelatin-coated slides. The slides were air dried overnight at room temperature, and coverslips were mounted using Permount (Fisher Scientific, Fair Lawn, NJ, USA).

8. BrdU Immunohistochemistry

For detection of newly generated cells in hippocampal dentate gyrus, 5-bromo-2'-deoxyuridine (BrdU) incorporation was visualized via immunohistochemical. The sections were first permeabilized by incubating them in 0.5% Triton X-100 in PBS for 20 min. They were then incubated in 50% formamide-2 x standard saline citrate (SSC) at 65℃ for 2 h, denaturated in 2 N HCl at 37℃ for 30 min, and rinsed twice in 100 mM sodium borate (pH 8.5). Afterwards, the sections were incubated overnight at 4C with a BrdU-specific mouse monoclonal antibody (1:600; Boehringer Mannheim, Germany). The sections were then washed three times with PBS and incubated for 1 h with a biotinylated mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Then, the sections were incubated for another 1 h with avidin-peroxidase complex (1:100; Vector Laboratories). For visualization, the sections were incubated in 50 mM Tris-HCl (pH 7.6) containing 0.02% DAB, 40 mg/ml nickel chloride, and 0.03% hydrogen peroxide for 5 min. The slides were air dried overnight at room temperature, and the coverslips were mounted using Permount.

9. Data Analysis

The numbers of TUNEL-positive, Bax-positive, and BrdU-positive cells in the subgranular layer of hippocampal dentate gyrus were counted hemilaterally in every eighth section throughout the entire extent of the dentate gyrus at 400 magnification. The area of the granular layer of dentate gyrus was traced

using Image-ProPlus image analyzer (Media Cybernetics Inc., Silver Spring, MD, USA) at 40 magnification. The numbers of TUNEL-positive, caspase-3-positive, and BrdU-positive cells were expressed as mean number of cells per mm2 of the cross sectional area of the granular layer of the dentate gyrus. Data were expressed as mean ± standard error of the mean (S.E.M). For comparisons between groups, one-way ANOVA and Duncan's post-hoc test were performed with $P < 0.05$ as an indication of statistical significance.

Results

1. Effect of SGH on step-down avoidance task

The latency time of the step-down avoidance task was 229.85 ± 37.45 sec in the sham-operation group, and this time was decreased to 69.88 ± 33.68 sec in the TBI induction group. It was respectively 201.63 ± 44.81 sec by pre-treatment with SGH at 50 mg/kg, 201.38 ± 39.64 sec by pre-treatment with SGH at 100 mg/kg, and 196.88 ± 32.69 sec by pre-treatment with SGH at 200 mg/kg.

The latency in the TBI group was shorter than control group. However, treatment with the aqueous extract of SGH increased the latency time. The present results showed that the aqueous extract of SGH improved TBI-induced short-term memory impairment (Fig. 1).

2. Effect of SGH on the number of TUNEL-positive cells

Photomicrographs of TUNEL-positive cells in the hippocampal dentate gyrus are presented in Fig. 2. The number of TUNEL-positive cells was 6.57 ± 4.24 /mm2 in the sham-operation group, 288.92 ± 46.15 /mm2 in the TBI induction group, 289.09 ± 26.88 /mm2 by pre-treatment with SGH at 50 mg/kg, 155.09 ± 20.97 /mm2 by pre-treatment with SGH at 100 mg/kg, and 139.10 ± 27.04 /mm2 by pre-treatment with SGH at 200 mg/kg.

The present results showed that TBI-induced apoptotic neuronal cell death increased in the hippocampal dentate gyrus and treatments with the aqueous extract of SGH at 100 mg/kg and

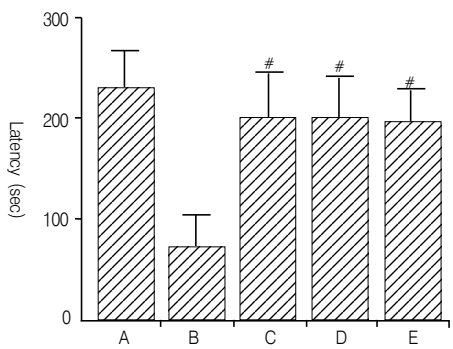

Fig. 1. Effect of SGH on the latency time of the step-down avoidance task.
(A) Sham-operation group; (B) TBI-induction group; (C) TBI-induction with 50 mg/kg SGH-treated group; (D) TBI-induction with 100 mg/kg SGH-treated group; (E) TBI-induction with 200 mg/kg SGH-treated group. * represents $p < 0.05$ compared to the sham-operation group. # represents $p < 0.05$ compared to the TBI-induction group.

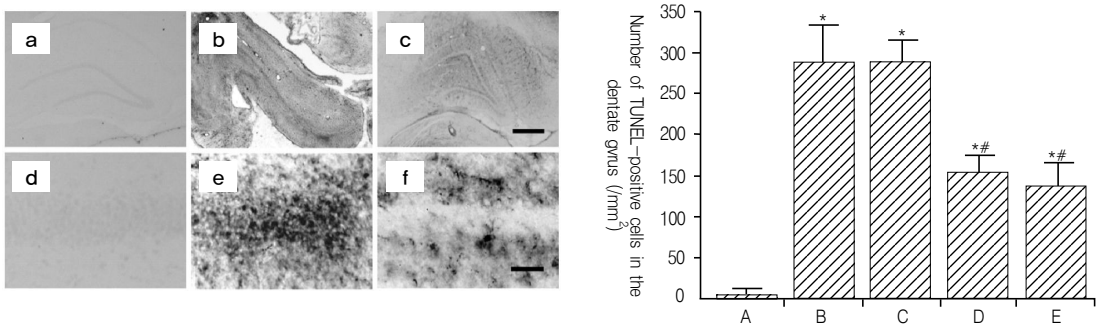

Fig. 2. Effect of SGH on DNA fragmentation in the hippocampal dentate gyrus.
Upper: Photomicrographs showing immunostaining for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells. (a),(d) Sham-operation group; (b),(e) TBI-induction group; (c),(f) TBI induction with 200 mg/kg SGH-treated group. (a),(b),(c) magnified 40. The scale bar of (c) represents 500 μm. (d), (e), (f) magnified 400. The scale bar of (f) represents 50 μm. Lower: Number of TUNEL-positive cells in each group. * represents $p < 0.05$ compared to the sham-operation group. # represents $p < 0.05$ compared to the TBI-induction group. (A) Sham-operation group; (B) TBI-induction group; (C) TBI-induction with 50 mg/kg SGH-treated group; (D) TBI-induction with 100 mg/kg SGH-treated group; (E) TBI-induction with 200 mg/kg SGH-treated group.

200 mg/kg significantly suppressed the ischemia-induced apoptosis of neuronal cells (Fig. 2).

3. Effect of SGH on the number of Bax-positive cells

Photomicrograph of TUNEL-positive cells in the hippocampal dentate gyrus are presented in Fig. 3. The number of TUNEL-positive cells was respectively 36.84 ± 15.69 /mm2 in the sham-operation group, 459.59 ± 73.25 /mm2 in the TBI induction group, 346.71 ± 63.97 /mm2 by pre-treatment with SGH at 50 mg/kg, 266.50 ± 56.51 /mm2 by pre-treatment with SGH at 100 mg/kg, and 184.02 ± 19.06 /mm2 by pre-treatment

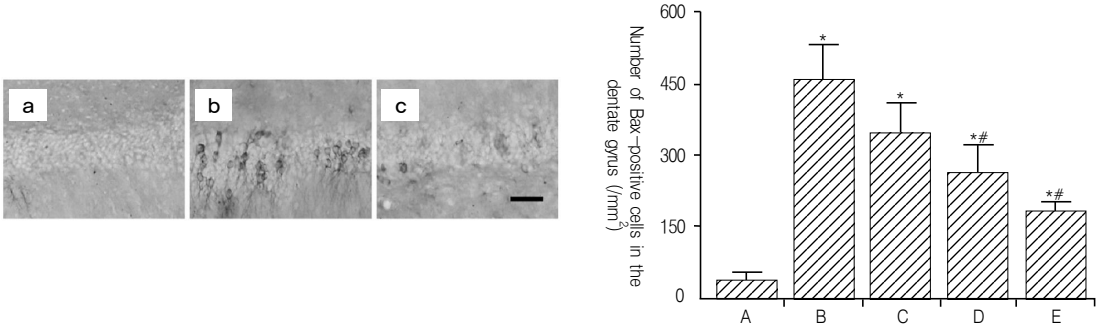

Fig. 3. Effect of SGH on Bax expression in the hippocampal dentate gyrus.
Upper: Photomicrographs showing immunostaining for Bax-positive cells. (a) Sham-operation group; (b) TBI-induction group; (c) TBI induction with 200 mg/kg SGH-treated group. The scale bar represents 50 μm. Lower: Mean number of Bax-positive cells in each group. * represents $p < 0.05$ compared to the sham-operation group. # represents $p < 0.05$ compared to the TBI-induction group. (A) Sham-operation group; (B) TBI-induction group; (C) TBI-induction with 50 mg/kg SGH-treated group; (D) TBI-induction with 100 mg/kg SGH-treated group; (E) TBI-induction with 200 mg/kg SGH-treated group.

with SGH at 200 mg/kg.

The present results showed that TBI increased Bax expression in the hippocampal dentate gyrus and treatment with the aqueous extract of SGH significantly suppressed the TBI-induced Bax expression as dose-dependent manner (Fig. 3).

4. Effect of SGH on the number of cell proliferation

Photomicrograph of BrdU-positive cells in the hippocampal dentate gyrus are presented in Fig. 4. The number of BrdU-positive cells was 92.03 ± 11.82 /mm2 in the sham-operation group, 661.11 ± 106.67 /mm2 in the TBI induction group, 606.55 ± 86.35 /mm2 by pre-treatment with SGH at 50 mg/kg, 310.61 ± 56.26 /mm2 by pre-treatment with SGH at 100 mg/kg, and 316.78 ± 50.31 /mm2 by pre-treatment with SGH at 200 mg/kg.

The present results showed that TBI induced the cell proliferation in the hippocampal dentate gyrus and treatments with the aqueous extract of SGH at 100 mg/kg and 200 mg/kg significantly suppressed the ischemia-induced cell proliferation (Fig. 4).

Discussion

In the present results, TUNEL-positive cells was increased in by induction of TBI and treatment with the aqueous extract of SGH suppressed TBI-induced number of TUNEL-positive cells in the hippocampal dentate gyrus. TUNEL staining has been used to identify DNA fragmentation, however, TUNEL-staining labels both apoptotic and necrotic cells[22]. As it is known that TBI does not affect direct mechanical damage to hippocampal dentate gyrus, TUNEL-positive cells presented in this study represent apoptosis.

In broad terms, neuronal apoptosis can be divided into two pathways. One involves the activation of a family of cysteine proteases, ‘caspases’, and the another involves the caspase-

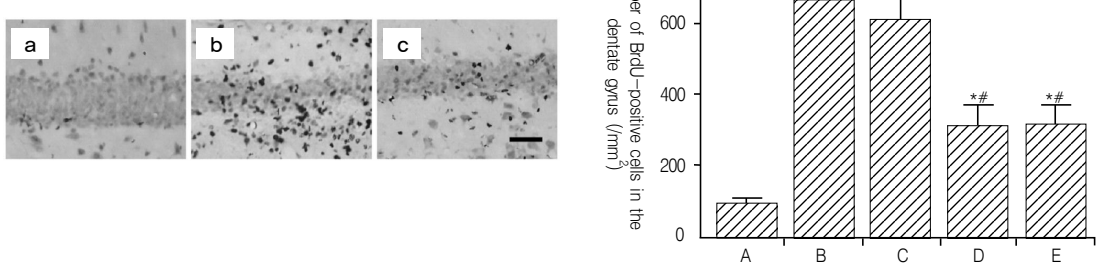

Fig. 4. Effect of SGH on cell proliferation in the hippocampal dentate gyrus.
Upper: Photomicrographs of 5-bromo-2′-deoxyuridine (BrdU)-positive cells. Sections were stained for BrdU (black) and neuronalnuclei (NeuN; brown). (a) Sham-operation group; (b) ischemia-induction group; (c) TBI induction with 200 mg/kg SGH-treated group. The scale bar represents 50 μm. Lower: Number of BrdU-positive cells in each group. * represents $p < 0.05$ compared to the sham-operation group. # represents $p < 0.05$ compared to the TBI-induction group. (A) Sham-operation group; (B) TBI-induction group; (C) TBI-induction with 50 mg/kg SGH-treated group; (D) TBI-induction with 100 mg/kg SGH-treated group; (E) TBI-induction with 200 mg/kg SGH-treated group.

independent release of apoptotic factors from mitochondria[23]. Apoptosis is known to be regulated by Bcl-2 protein family and mitogen-activated protein kinase (MAPK) signal-transduction pathways. In this study, we focused capases-dependant pathway and Bcl-2 family. In caspases-dependant pathway, targeted caspase-3 that activates the process of apoptosis is irreversible[24]. Kaya et al[25]. reported that capase-3 expression is induced mainly 2-3 day after TBI. Over 1 week after TBI insult like in this study, caspase-3 is not appropriate for the detection of apoptosis. Otherwise, Bax, an apoptotic protein in Bcl-2 family, is known that Bax expression starts from 6 days after TBI insult[26]. In this study, Bax expression was presented as the same manner of TUNEL-positive cells.

In the present results, markedly increased cell proliferation was observed in the hippocampal dentate gyrus following TBI insult. Increased cell proliferation in the hippocampal dentate gyrus and cerebral cortex after ischemia has been previously documented[27-29]. Liu et al[27]. reported that cell proliferation in the dentate gyrus of gerbils was significantly increased after neuronal apoptosis and that it reaches the maximal level on the 11th day after the neuronal damage episode. It is generally believed that increment in cell proliferation after neuronal damage is a compensatory response to excessive apoptotic cell death[27,29].

It is well documented that the hippocampus affects memory formation[30]. Injury to the hippocampus is known to induce memory impairment[31]. In step-down avoidance task presented in this study, TBI induced short-term memory impairment. Treatment with the aqueous extract of SGH improved TBI-induced impairment of short-term memory in this study. The present results provide direct evidence that the improvement of short-term memory by SGH is associated with the reduction of apoptotic neuronal cell death by it.

For the first time SGH is introduced by Geumgueyoryak[32]. Since then, it was quoted from several documents, in which the ingredients of SGHs are different each other, but they have a common effect that strengthens ‘Yang’ and ‘Yin’ of kidney. A brain is the sea of a bone marrow, which is stored in a bone, and kidney can control the bone[19]. Namely, we can control the condition of a bone and a brain by the treatment of kidney. There are records that SGH is comprised of Yukmijihwang-pill added with Aconiti Tuber, Cinnamomum Loureirii and Schizandrae Fructus, by which we can treat a deficiency of kidney's ‘yang’ and ‘yin’[19]. And a document reported that the SGH with Cervi Cornu can be used for a deficiency of kidney's ‘qi’[33].

In this study, the SGH, made by Yukmijihwang-pill added with Schizandrae Fructus and Cervi Cornu was experimented. Lee et al[34]. reported that H2O2 has toxic effect, and herb extract, Schizandrae Fructus is very effective against H2O2-induced neurotoxicity in cultured cerebral neurons of mouse. Park et al[35] reported that Schizandrae fructus can increase the regional cerebral blood flow and decrease the blood pressure, that is related to guanylyl cyclase activity. Kim et al[36] suggested that the Cervus Elaphus-herbal acupuncture therapy could be used as a medication for controlling the stroke induced by deficiency. Kim et al[37] concluded that Cervus elaphus oral administration and

Cervus elaphus acupuncture on acupoint G39 showed effects on growth and intellectual development of animals.

SGH can be used for strengthen an inborn energy and it is expected that we can have a protective effect of brain neuron cells by it. Shin et al[38]. reported that SGH has neuroprotective effect on H2O2-induced damage in HiB5 cell line and they also showed that this neuroprotective effect of SGH on exitotoxicity was induced by suppressing glutamate-activated and NMDA-activated ion currents in rat hippocampal CA1 neurons. Also Yang et al[39]. reported that Yukmijihwang-tang decoction, similar to SGH, suppressed K+ and Ca2+ ion currents in cultured rat hippocampal neurons.

Here in this study, we have shown that SGH overcomes the TBI-induced apoptotic neuronal cell death and thus facilitates the recovery of short-term memory impairment induced by TBI. Additional studies on the ingredient herbs of SGH and their mechanisms may yield novel ideas with possible implications for further therapeutic approaches.

References

1. Adekoya N, Thurman DJ, White DD, Webb KW. Surveillance for traumatic brain injury deaths - United States, 1989-1998. Morb Mort Weekly Rept Surveill Summ. 2002;51:1-14.
2. Firsching R, Woischneck D. Present status of neurosurgical trauma in Germany. World J Surg. 2001;25:1221-1223.
3. Engberg A, Teasdale TW. Traumatic brain injury in children in Denmark: a national 15-year study. Eur J Epidemiol. 1998;14:165-173.
4. Masson F, Thicoipe M, Aye P, Mokni T, Senjean P, Schmitt V, Dessalles PH, Cazaugade M, Labadens P; Aquitaine Group for Severe Brain Injuries Study. Epidemiology ofsevere brain injuries: a prospective population-based study. J Trauma-Inj Infect Crit Care. 2001;51:481-489.
5. Song SH, Kim SH, Kim KT, Kim Y.Outcome of pediatric patients with severe brain injury in Korea: a comparison with reports in the west. Childs Nervous System. 1997;13:82-86.
6. Gururaj G: Epidemiology of traumatic brain injuries: Indian scenario. Neurol Res. 2002;24: 24-28.
7. Hawley CA, Ward AB, Magnay AR, Long J.Parental stress and burden following traumatic brain injury amongst children and adolescents. Brain Inj. 2003;17:1-23.
8. McGarry LJ, Thompson D, Millham FH, Cowell L, Snyder PJ, Lenderking WR, Weinstein MC. Outcomes and costs of acute treatment of traumatic brain injury. J Trauma-Inj Infect Crit Care. 2002;53:1152-1159.
9. Sosin DM, Sniezek JE, Waxweiler RJ.Trends in death associated with traumatic brain injury, 1979 through 1992. Success and failure. JAMA. 1995;273:1778-1780.
10. Clifton GL, Miller ER, Choi SC, Levin HS, McCauley S, Smith KR Jr, Muizelaar JP, Wagner FC Jr, Marion DW, Luerssen TG, Chesnut RM, Schwartz M. Lack of effect of induction of hypothermia after acute brain injury. N Engl J Med. 2001;344(8):556-563.
11. Ikonomidou C, Turski L. Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? Lancet Neurol. 2002;1:383-386.

12. Narayan RK, Michel ME, Ansell B, Baethmann A, Biegon A, Bracken MB, Bullock MR, Choi SC, Clifton GL, Contant CF, Coplin WM, Dietrich WD, Ghajar J, Grady SM, Grossman RG, Hall ED, Heetderks W, Hovda DA, Jallo J, Katz RL, Knoller N, Kochanek PM, Maas AI, Majde J, Marion DW, Marmarou A, Marshall LF, McIntosh TK, Miller E, Mohberg N, Muizelaar JP, Pitts LH, Quinn P, Riesenfeld G, Robertson CS, Strauss KI, Teasdale G, Temkin N, Tuma R, Wade C, Walker MD, Weinrich M, Whyte J, Wilberger J, Young AB, Yurkewicz L. Clinical trials in head injury. J Neurotrauma. 2002;19(5):503-557.
13. Lenzlinger PM, Morganti-Kossmann MC, Laurer HL, McIntosh TK. The duality of the inflammatory response to traumatic brain injury. Mol Neurobiol. 2001;24:169-181.
14. Liou AK, Clark RS, Henshall DC, Yin XM, Chen J.To die or not to die for neurons in ischemia, traumatic brain injury and epilepsy: a review on the stress-activated signaling pathways and apoptotic pathways. Prog Neurobiol. 2003;69:103-142.
15. Lockshin RA. Programmed cell death. Activation of lysis by a mechanism involving the synthesis of protein. J Insect Physiol. 1969;15:1505-1516.
16. Marovitz WF, Shugar JM, Khan KM. The role of cellular degeneration in the normal development of (rat) otocyst. Laryngoscope. 1976;86: 1413-1425.
17. Webster DA, Gross J. Studies on possible mechanisms of programmed cell death in the chick embryo. Dev Biol. 1970;22:157-184.
18. Graham SH, Chen J, Clark RS. Bcl-2 family gene products in cerebral ischemia and traumatic brain injury. J Neurotrauma. 2000;17: 831-841.
19. Heojun. Dongyibogam. Yeogang Publisher. Seoul. 2005;352, 353, 608, 1066.
20. Chen Y, Wei L, Ma M, Wu G, Zhang G and Wei Z. Effect of jiawei shenqi dihuang tang on the content of urinary protein in patients with diabetic nephrothy. J Tradit Chin Med. 1997;17:184-186.
21. Wang, J and DS Zhang. Effect of Shenqi-wan on immunological function in the nephrotic syndrome of chronic glomerulonephritis patients. Zhong Xi Yi Jie Za Zhi. 1987;7:731-733.
22. Charriaut-Marlangue C, Ben-Ari Y. A cautionary note on the use of the TUNEL stain to determine apoptosis. Neuroreport. 1995;7:61-64.
23. Zhang X, Satchell MA, Clark RSB, Nathaniel PD, Kochanek PM, Graham SH. Apoptosis. In Brain Injury. Edited by Clark RSB, Kochanek PM. Boston: Kluwer Academic Publishers; 2001;199-230.
24. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell. 1997;90:405-413.
25. Kaya SS, Mahmood A, Li Y, Yavuz E, Goksel M, Chopp M. Apoptosis and expression of p53 response proteins and cyclin D1 after cortical impact in rat brain. Brain Res. 1999;6;818(1) :23-33.
26. Wennersten A, Holmin S, Mathiesen T. Characterization of Bax and Bcl-2 in apoptosis after experimental traumatic brain injury in the rat. Acta Neuropathol (Berl). 2003;105(3): 281-288.
27. Liu J, Solway K, Messing RO, Sharp FR.

Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. J Neurosci. 1998;18:7768-7778.

28. Jiang W, Gu W, Brannstrom T, Rosqvist R, Wester P. Cortical neurogenesis in adult rats after transient middle cerebral artery occlusion. Stroke. 2001;32:1201-1207.
29. Takasawa K, Kitagawa K, Yagita Y, Sasaki T, Tanaka S, Matsushita K, Ohstuki T, Miyata T, Okano H, Hori M, Matsumoto M. Increased proliferation of neural progenitor cells but reduced survival of newborn cells in the contralateral hippocampus after focal cerebral ischemia in rats. J Cereb Blood Flow Metab. 2002;22:299-307.
30. Gould E, Tanapat P. Stress and hippocampal neurogenesis. Biol. Psychiatry. 1999;46:1472-1479.
31. Squire LR, Zola SM. Ischemic brain damage and memory impairment: a commentary. Hippocampus. 1996;6:546-552.
32. Zhangji. Jinguiyaolue. Jiwenshuju. Taibei. 1972; 383-385.
33. Judangye. Dangyeshimbeop. Daesung Publisher. Seoul. 1982;503-509.
34. Lee JH, Yang HU, Park SM. Effect of Schisandrae Fructus on Cultured Mouse Cerebral Neurons Damaged by Hydrogen Peroxide. Korean Journal of Oriental Physiology and Pathology. 2003;17(1):101-104.
35. Park SH, Han JH. A Study of Medicinal Plants for Applications in Functional Foods 1. Effects of Schizandrae fructus on the Regional Cerebral Blood Flow and Blood Pressure in Rats. Journal of the Korean Society of Food Science and Nutrition. 2004;33(1):34-40.
36. Kim SO, Yoon DH, Na CS. Effect of Cervus Elaphus-Herbal Acupuncture on Focal Ischemia Induced by Inserted Intraluminal Filament in MCA of Rats. The Korean Journal of Meridian and Acupoint. 2005;22(4):67-81.
37. Kim YT, Son YS, Jin SH, Han SW, Shim IS, Lim S, Lee HI. The effects of cervus elaphus on the growth and the interllectual development of animals. The Journal of Korean Acupuncture & Moxibustion Society. 2001;18 (5):122-134.
38. Shin HT, Chung SH, Lee JS, Kim SS, Shin HD, Jang MH, Shin MC, Bahn GH, Paik EK, Park JH, Kim CJ. Protective effect of Shenqi-wan against H2O2-induced apoptosis in hippocampal neuronal cells. Am J Chin Med. 2003;31(5):675-686.
39. Yang S, Zhou W, Zhang Y, Yan C, Zhao Y. Effects of LiuweiDihuang decoction on ion channels and synaptic transmission in cultured hippocampal neuron of rat. J Ethnopharmacol. 2006;30;106(2):166-172.