

Protective Effect of *Shenqi-wan* on Traumatic Brain Injury-induced Delayed Apoptosis in Rat Hippocampal Dentate Gyrus

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ABSTRACT

Shenqi-wan, Oriental herbal medicine formulation, has been traditionally used for delayed mental and physical development in children, complications of diabetes, and glomerulonephritis. In the present study, the protective effect of the aqueous extract of *Shenqi-wan* against traumatic brain injury (TBI) in the rat hippocampal dentate gyrus was 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 conducted. In the present results, it was shown that apoptotic cell death and cell proliferation in the dentate gyrus were significantly increased following TBI in rats and that the aqueous extract of *Shenqi-wan* suppressed the TBI-induced increase in apoptosis and cell proliferation in the dentate gyrus. Based on the present results, it is possible that the aqueous extract of *Shenqi-wan* has a neuroprotective effect on TBI-induced neuronal cell death.

Key words: *Shenqi-wan*, hippocampal dentate gyrus, traumatic brain injury, apoptosis, cell proliferation

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 (Engberg and Teasdale, 1998; Firsching and Woischneck, 2001; Masson et al., 2001; Adekoya et

al., 2002). Similar data can be found from a variety of demographic and cultures (Song et al., 1997; Gururaj, 2002). Also TBI patients take much of economic and social burden, especially to care of neuronal disabilities (Sosin et al., 1995; McGarry et al., 2002; Hawley et al., 2003).

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

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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 (Clifton et al., 2001; Ikonomidou and Turski, 2002; Narayan et al., 2002)

There are two waves of neuronal cell death in secondary brain injury after TBI. First wave 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 (Lenzlinger et al., 2001). 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 is targeted for therapies (Liou et al., 2003).

Apoptosis has long been identified as an evolutionarily conserved process of active cell elimination during development. Its 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 several gene expression and protein synthesis (Lockshin, 1969; Webster and Gross, 1970; Marovitz et al., 1976). 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 (Graham et al., 2000).

Shenqi-wan, an Oriental herbal medicine formulation, has been traditionally used for delayed mental and physical development in children, complications of diabetes, and treatment of glomerulonephritis patients (Wang and Zhang, 1987; Chen et al., 1997). However, the effect of the aqueous extract of *Shenqi-wan* on TBI has not been reported yet. In the present study, the neuroprotective effects of the aqueous extract of *Shenqi-wan* 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

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 ($20 \pm 2^\circ\text{C}$) and lighting (07.00~19.00 hours) conditions and were supplied with food and water *ad libitum*. The rats were divided five groups (n=10 in each group): the sham-operation group, the TBI-induction group, the TBI-induction with 50 mg/kg *Shenqi-wan*-treated group, the TBI-induction with 100 mg/kg *Shenqi-wan*-treated group, and the TBI-induction with 200 mg/kg *Shenqi-wan*-treated group.

Preparation of the aqueous extract of Shenqi-wan

The ingredients of *Shenqi-wan* are as follows: *Rehmanniae Radix* 16 g, *Dioscorae Radix* 8 g, *Corni Fructus* 8 g, *Alimatis Rhizoma* 6 g, *Moutan Cortex Radicis* 6 g, *Hoelen* 6 g, *Maximowicziae Fructus* 8 g, and *Cervi Cornu* 4 g. All ingredients were obtained from the Kyung Dong marketplace (Seoul, Korea). After washing, to obtain the aqueous extract of *Shenqi-wan*, 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 *Shenqi-wan* treated groups orally received the aqueous extract of *Shenqi-wan* 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).

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).

Step-down avoidance task

In order to evaluate the short-term memory ability, we determined the latency of the step-down avoidance task. On the 9th day from the beginning of *Shenqi-wan* 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 1 sec 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.

Tissue preparation

The animals were sacrificed immediately after step-down avoidance task. 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 μm thickness were made with a freezing microtome (Leica, Nussloch, Germany).

Determination of DNA fragmentation by TUNEL staining

For visualization of DNA fragmentation, a marker of apoptotic cell death, TUNEL staining was per-

formed 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 μg/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[®].

Bax immunohistochemistry

For visualization of Bax expression, Bax immunohistochemistry was performed. 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). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.02% 3,3'-diaminobenzidine (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).

BrdU Immunohistochemistry

For detection of newly generated cells in the dentate gyrus, 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 × standard saline citrate (SSC) at 65°C for 2 h, denaturated in 2 N HCl at 37°C for 30 min, and rinsed twice in 100 mM sodium borate (pH 8.5). Afterwards, the sections were incubated overnight at 4°C with a BrdU-specific mouse monoclonal antibody (1 : 600; Boehringer Mannheim, 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). 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®.

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-Pro® Plus 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

Effect of the aqueous extract of *Shenqi-wan* on step-down avoidance task

The latency time of the step-down avoidance task was 229.85±37.45 sec, 69.88±33.68 sec, 201.63±44.81 sec, 201.38±39.64 sec, and 196.88±32.69 sec in the sham-operation group, the TBI-induction group, the TBI-induction with 50 mg/kg *Shenqi-wan*-treated group, the TBI-induction with 100 mg/kg *Shenqi-wan*-treated group, and the TBI-induction with 200 mg/kg *Shenqi-wan*-treated group, respectively.

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

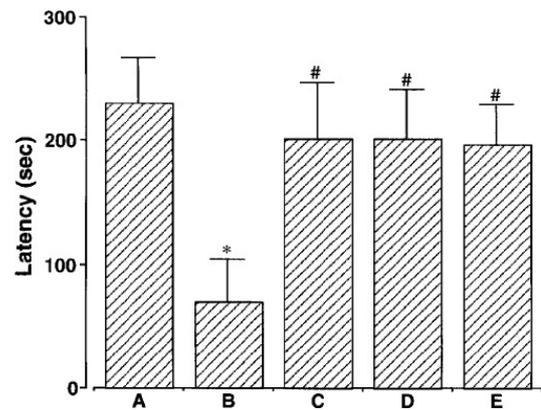


Fig. 1. Effect of *Shenqi-wan* on the latency of the step-down avoidance task. The data are presented as the mean ± standard error of the mean (S.E.M.). *represents $p < 0.05$ compared to the sham-operation group. #represents $p < 0.05$ compared to the TBI-induction group. (A) Sham-operation group, (B) TBI-induction group, (C) TBI-induction with 50 mg/kg *Shenqi-wan*-treated group, (D) TBI-induction with 100 mg/kg *Shenqi-wan*-treated group, (E) TBI-induction with 200 mg/kg *Shenqi-wan*-treated group.

Effect of the aqueous extract of *Shenqi-wan* on the number of TUNEL-positive cells in the hippocampal dentate gyrus

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, 288.92±46.15/mm², 289.09±26.88/mm², 155.09±20.97/mm², and 139.10±27.04/mm² in the sham-operation group, the TBI-induction group, the TBI-induction with 50 mg/kg *Shenqi-wan*-treated group, the TBI-induction with 100 mg/kg *Shenqi-wan*-treated group, and the TBI-induction with 200 mg/kg *Shenqi-wan*-treated group, respectively.

The present results showed that TBI-induced apoptotic neuronal cell death in the hippocampal dentate gyrus and treatment with the aqueous extract of *Shenqi-wan* significantly suppressed the ischemia-induced apoptosis of neuronal cells as dose-dependent manner (Fig. 2).

Effect of the aqueous extract of *Shenqi-wan* treatment on the number of Bax-positive cells in the hippocampal dentate gyrus

Photomicrographs of Bax-positive cells in the hippocampal dentate gyrus are presented in Fig. 3. The number of Bax-positive cells was 36.84±15.69/mm², 459.59±73.25/mm², 346.71±63.97/mm², 266.50±56.51/mm², and 184.02±19.06/mm² in the sham-

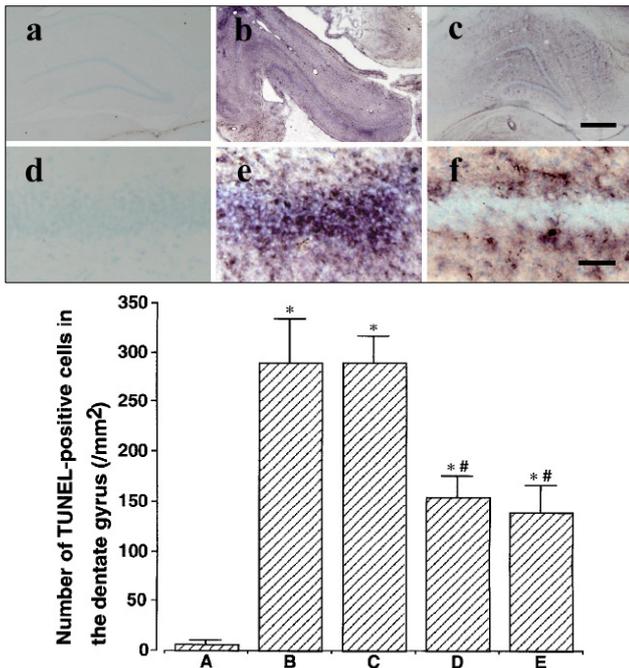


Fig. 2. Effect of *Shenqi-wan* on DNA fragmentation in the hippocampal dentate gyrus. Upper: Photomicrographs showing immunostaining for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells. (a)(d) Sham-operation group, (b)(e) TBI-induction group, (c)(f) TBI induction with 200 mg/kg *Shenqi-wan*-treated group. (a)(b)(c) magnified $\times 40$. The scale bar of (c) represents $500 \mu\text{m}$. (d)(e)(f) magnified $\times 400$. The scale bar of (f) represents $50 \mu\text{m}$. Lower: Number of TUNEL-positive cells in each group. The data are presented as the mean \pm S.E.M. *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 *Shenqi-wan*-treated group, (D) TBI-induction with 100 mg/kg *Shenqi-wan*-treated group, (E) TBI-induction with 200 mg/kg *Shenqi-wan*-treated group.

operation group, the TBI-induction group, the TBI-induction with 50 mg/kg *Shenqi-wan*-treated group, the TBI-induction with 100 mg/kg *Shenqi-wan*-treated group, and the TBI-induction with 200 mg/kg *Shenqi-wan*-treated group, respectively.

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

Effect of *Shenqi-wan*-treatment on the number of cell proliferation in the hippocampal dentate gyrus

Photomicrographs of BrdU-positive cells in the hippocampal dentate gyrus are presented in Fig. 4.

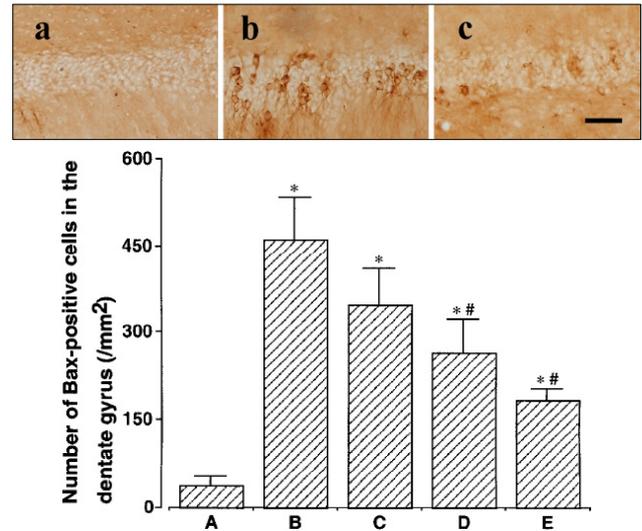


Fig. 3. Effect of *Shenqi-wan* on Bax expression in the hippocampal dentate gyrus. Upper: Photomicrographs showing immunostaining for Bax-positive cells. (a) Sham-operation group, (b) TBI-induction group, (c) TBI induction with 200 mg/kg *Shenqi-wan*-treated group. The scale bar represents $50 \mu\text{m}$. Lower: Mean number of Bax-positive cells in each group. The data are presented as the mean \pm S.E.M. *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 *Shenqi-wan*-treated group, (D) TBI-induction with 100 mg/kg *Shenqi-wan*-treated group, (E) TBI-induction with 200 mg/kg *Shenqi-wan*-treated group.

The number of BrdU-positive cells was $92.03 \pm 11.82/\text{mm}^2$, $661.11 \pm 106.67/\text{mm}^2$, $606.55 \pm 86.35/\text{mm}^2$, $310.61 \pm 56.26/\text{mm}^2$, and $316.78 \pm 50.31/\text{mm}^2$ in the sham-operation group, the TBI-induction group, the TBI-induction with 50 mg/kg *Shenqi-wan*-treated group, the TBI-induction with 100 mg/kg *Shenqi-wan*-treated group, and the TBI-induction with 200 mg/kg *Shenqi-wan*-treated group, respectively.

The present results showed that TBI-induced the cell proliferation in the hippocampal dentate gyrus and treatment with the aqueous extract of *Shenqi-wan* significantly suppressed the ischemia-induced cell proliferation as dose-dependent manner (Fig. 4).

DISCUSSION

In the present results, the number of TUNEL-positive cells was increased in by induction of TBI and treatment with the aqueous extract of *Shenqi-wan* suppressed the number of TUNEL-positive cells in the hippocampal dentate gyrus. TUNEL staining has been used to identify DNA fragmen-

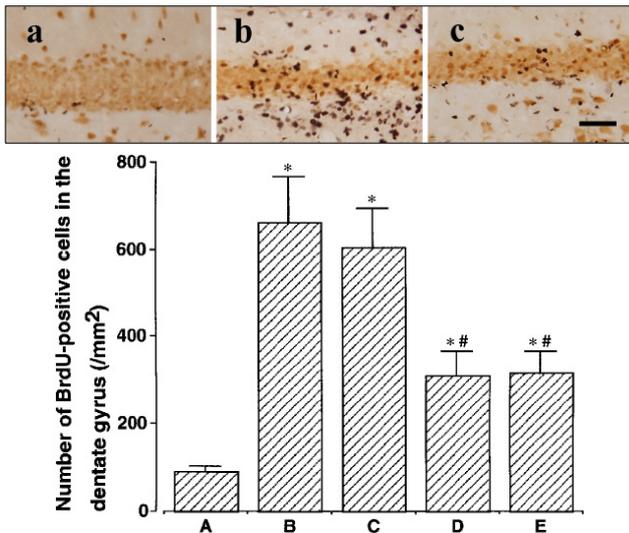


Fig. 4. Effect of *Shenqi-wan* on cell proliferation in the hippocampal dentate gyrus. Upper: Photomicrographs of 5-bromo-2'-deoxyuridine (BrdU)-positive cells. Sections were stained for BrdU (black) and neuronal nuclei (NeuN; brown). (a) Sham-operation group, (b) ischemia-induction group, (c) TBI induction with 200 mg/kg *Shenqi-wan*-treated group. The scale bar represents 50 μ m. Lower: Number of BrdU-positive cells in each group. The data are presented as the mean \pm S.E.M. *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 *Shenqi-wan*-treated group, (D) TBI-induction with 100 mg/kg *Shenqi-wan*-treated group, (E) TBI-induction with 200 mg/kg *Shenqi-wan*-treated group.

tation, however, TUNEL-staining labels both apoptotic and necrotic cells (Charriaut-Marlangue and Ben-Ari, 1995). As it is known that TBI dose 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 another involves the caspase-independent release of apoptotic factors from mitochondria (Zhang et al., 2001). 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 caspases-dependant pathway and Bcl-2 family. In caspases-dependant pathways, targeted caspase-3 that activates the process of apoptosis is irreversible (Zou et al., 1997). Kaya et al. (1999) reported that caspase-3 expression is induced mainly 2 or 3 days after induction of 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 (Wennersten et al., 2003). 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 (Liu et al., 1998; Jiang et al., 2001; Takasawa et al., 2002). Liu et al. (1998) 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 (Liu et al., 1998; Takasawa et al., 2002).

It is well documented that the hippocampus affects memory formation (Gould and Tanapat, 1999). Injury to the hippocampus is known to induce memory impairment (Squire and Zola, 1996). In step-down avoidance task presented in this study, TBI induced short-term memory impairment. Treatment with the aqueous extract of *Shenqi-wan* 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 *Shenqi-wan* is associated with the reduction of apoptotic neuronal cell death by *Shenqi-wan*.

Shin et al. (2003) reported that *Shenqi-wan* has neuroprotective effect on H₂O₂-induced damage in HiB5 cell line and they also showed that this neuroprotective effect of *Shenqi-wan* on excitotoxicity was induced by suppressing glutamate-activated and NMDA-activated ion currents in rat hippocampal CA1 neurons. Also Yang et al. (2006) reported that *Liuweidihuang* decoction, similar to *Shenqi-wan*, suppressed K⁺ and Ca²⁺ ion currents in cultured rat hippocampal neurons.

Here in this study, we have shown that *Shenqi-wan* 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 *Shenqi-wan* and their mechanisms may yield novel ideas with possible implications for further therapeutic approaches.

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