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Original Article

Neuroprotective Effect of *Ginseng radix* on ICH - induced Rats

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Backgrounds : Intracerebral hemorrhage is one of the most devastating types of stroke. Ginseng radix, the root of Panax Ginseng, C. A. MEYER (Araliaceae), is one of the most famous medicinal herbs with various therapeutic applications.

Objectives : In the present study, the effect of aqueous extract of Ginseng radix on intracerebral hemorrhage-induced neuronal cell death in rats was investigated.

Materials and Methods : Step-down avoidance task, Nissl staining, immunohistochemistry for caspase-3 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay were used for this study.

Results : The present results show that hemorrhage-induced lesion volume and apoptotic neuronal cell death in the striatum were significantly suppressed by treatment with Ginseng radix, resulting in enhancement of short-term memory.

Conclusions : We have shown that Ginseng radix has a neuroprotective effect on stroke, and aids the recovery from central nervous system sequelae following stroke.

Key Words: Intrastriatal hemorrhage, *Ginseng radix*, short-term memory, apoptotic neuronal cell death

Introduction

Cerebrovascular accident is a primary cause of disability in industrialized countries and is the third leading cause of death in North America, Europe, and Asia[1]. Of these, intracerebral hemorrhage is a devastating clinical condition, accounting for 15% of all stroke hospitalizations [2].

Intracerebral hemorrhage is one of the most devastating types of stroke, which commonly occurs in the striatum, thalamus, cerebellum, and pons[3]. Intracerebral hemorrhage is associated with severe neurological deficits and a considerable mortality rate. In hemorrhagic stroke, brain damage occurs through multiple mechanisms. These mechanisms include direct tissue destruction, the space-occupying effect of the hematoma, ischemic damage to adjacent tissue, clot-derived toxic factors, and edema formation[4].

Recently, apoptosis, also known as programmed cell death, has become known as an important form of intracerebral hemorrhage-induced neuronal cell death[5,6]. It has also been reported that neuronal cell death in the parenchyma occurs by an apoptotic mechanism in ICH rats and is associated with the induction of caspase-3 in cells adjacent to the hematoma[6]. Caspases, which make up a family of cysteinyl proteases encompassing 14 members, are essential players in apoptotic cell death

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both as initiators (caspase-2, -8, -9, and -10) and executioners (caspase-3, -6, and -7)[7].

Ginseng radix, the root of Panax Ginseng, C. A. MEYER (Araliaceae), is one of the most famous medicinal herbs with various therapeutic applications. *Ginseng radix* has also been used to restore and to enhance well-being in traditional medicine, and these effects are described as increasing resistance against noxious or stressful influences without impairing physiological functions[8]. In addition, it has been reported that *Ginseng radix* improves the learning capacity in animals[9,10] and possesses a protective effect against apoptotic cell death.[11,12] However, the effect of *Ginseng radix* on intracerebral hemorrhage-induced neuronal cell death has not been reported yet.

In the present study, to investigate whether aqueous extract of *Ginseng radix* on intracerebral hemorrhage-induced neuronal cell death in rats, the step-down avoidance task, Nissl staining, immunohistochemistry for caspase-3, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining were used.

Materials and Methods

1. Animals

Male Sprague-Dawley rats weighing 280 ± 10 g (8 weeks of age) were used for the experiment. The experimental procedures were performed in accordance with the animal care guidelines of the National Institute of Health (NIH) and the Korean Academy of Medical Sciences. The animals were housed under laboratory conditions at a controlled temperature (20 ± 2℃) and maintained under light-dark cycles, each consisting of 12 h of light and 12 h of darkness (lighting from 07:00 to 19:00 h) with food and water made available ad libitum. Animals were divided into five groups: the sham-operation group, the hemorrhage-induction group, the hemorrhage-induction and 10 mg/kg *Ginseng radix*-treated group, the hemorrhage-induction and 50 mg/kg *Ginseng radix*-treated group, and the hemorrhage-induction and 100 mg/kg *Ginseng radix*-treated group (n = 5 in each group).

2. Drugs

Ginseng radix used in this experiment was obtained from the KT&G. After washing, *Ginseng radix* was immersed in cold water for 12 h. To obtain the aqueous extract, 300 g of *Ginseng radix* was added to distilled water, heat-extracted at 80℃, concentrated using a rotary evaporator and lyophilized. The resulting powder, weighing 22.5 g, was diluted with saline. After filtering through a 0.45 m syringe filter, animals of the *Ginseng radix*-treated groups received aqueous extract of *Ginseng radix* at the respective doses intraperitioneally once a day for 10 days. Those of the sham-operation group received intraperitoneally equivalent amount of saline once a day for the same duration of time(from 2nd day to 11th day after surgery).

3. Induction of intrastriatal hemorrhage

For induction of intrastriatal hemorrhage, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p. Sigma Chemical Co., St. Louis, MO, USA) and placed in a stereotaxic frame as a previously described method13). Through a hole drilled in the skull, a 26-gauge needle was implanted into the striatum at the following coordinates: 2.6 mm lateral to midline, 0.7 mm anterior to coronal suture, depth 4.5 mm from the surface of the brain, and 1 μL of saline containing 0.2 U collagenase (Type 4; Sigma Chemical Co.) was infused over 1 min. The needle remained in place for an additional 3 min following the infusion, and then was slowly withdrawn. Animals of the sham-operation group received an equivalent dose of physiological

saline with the same method.

4. Step-down avoidance task

In order to evaluate the short-term memory ability, the latency in the step-down avoidance task was assessed. At the 9th day after surgery, rats were trained in a step-down avoidance task. Rats were placed on a 7 25 cm platform with 2.5 cm height. The platform faced a 42 25 cm grid of parallel 0.1 cm-caliber stainless steel bars spaced 1 cm apart. In training session, the animals received 0.5 mA, scramble footshock for 20 sec immediately upon stepping down. The rat's task was to learn and remember that stepping on the grid delivered a shock to them. The retention time was determined at the 11th day after surgery by re-testing the rats on their step-down avoidance task[14,15]. The latency for rats to step down was measured as the time in took the rats to place all four paws on the grid.

5. Tissue preparation

The rats were sacrificed on the 11th day after surgery. Animals were first fully anesthetized with Zoletil 50® (10 mg/kg, i.p.; Vibac Laboratories, Carros, France), transcardially perfused with 50 mM phosphate-buffered saline (PBS), and then fixed with a freshly prepared solution consisting of 4% paraformaldehyde (PFA) in 100 mM phosphate buffer (PB, pH 7.4). The brains were then removed, postfixed in the same fixative overnight, and transferred into a 30% sucrose solution for cryoprotection. Coronal sections of 40 m thickness were made using a freezing microtome (Leica, Nussloch, Germany).

6. Nissl staining

For the determination of hemorrhage volume, Nissl staining was performed as a previously described method[13,16]. Sections were mounted on a gelatin-coated slide and allowed to air-dry overnight. The slides were then rehydrated in double-distilled water, submerged in 0.5% cresyl violet solution for 5 min, and then gradually dehydrated for 5 min in successive baths of ethanol (i.e. 50%, 75%, 90%, 95%, and 100%). Each slide was then given 15 min baths in 100 % xylene two times and coverslipped.

7. Caspase-3 immunohistochemistry

For visualization of caspase-3 expression, caspase-3 immunohistochemistry was performed as a previously described method[17]. Sections were incubated with mouse anti-caspase-3 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight, and then for another 1 h with biotinylated mouse secondary antibody. 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. TUNEL staining

For visualization of apoptotic cell death, TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) as a previously described method[17]. Briefly, the sections were finally mounted onto gelatin-coated slides and slides were air-dried overnight at room temperature. The sections were post-fixed in ethanol-acetic acid (2:1) and rinsed. Then, the sections were incubated with proteinase K (100 μg/ml), 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 nickel-DAB. Mayer's hematoxylin

(Dako, Glostrup, Denmark) was used for counter-staining. The slides were air-dried overnight at room temperature, and coverslips were mounted using Permount® (Fisher Scientific).

9. Data analysis

In the step-down avoidance task, the latency-time was determined and latency over 180sec was counted as 180sec.

Images were captured with video camera attached to light microscope (Olympus, Tokyo, Japan) and the data were analyzed using Image-Pro Plus® software (Media Cybernetics Inc., Silver Spring, MD, USA).

The hemorrhage volume in the striatum was calculated as a previously described method[18,19]. The third dimension of the volume [V(ref)] was obtained by multiplying the sum of the measured areas [ΣA_i] by the inverse sampling frequency of the sections [k] and the section thickness [t]: $V(ref) = \Sigma A_i \; k \; t$.

The numbers of caspase-3- and TUNEL-positive cells were assessed under the microscope using a magnification of 20, and counted in 4 fields (each area 250 250 m) immediately adjacent to the needle injection in damage site, which was defined by the presence of erythrocytes or necrosis[20]. The values were expressed as the number of cells per mm^2 of counting area.

10. Statistical analysis

Statistical analysis of the differences in the latency among the groups were evaluated by a Kruskal-Wallis ANOVA and individual differences between groups were evaluated by Mann-Whitney U tests. The results are presented as the median value (interquartile range). Statistical analysis of Nissl staining, caspase-3 immunohistochemistry and TUNEL staining were performed using one-way ANOVA followed by Duncan *post-hoc* test. The results are presented as the mean±standard error mean (S.E.M). Differences were considered significant at $p < 0.05$.

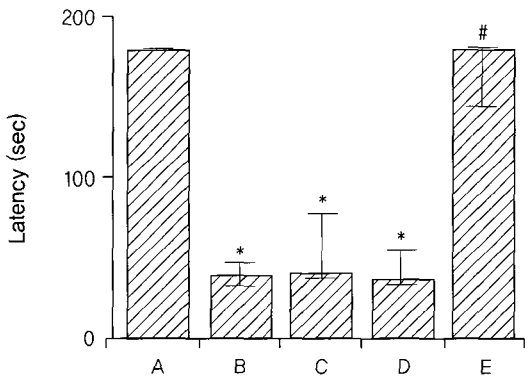

Fig. 1 Effect of Ginseng radixon the latency in step-down avoidance task
(A) Sham-operation group, (B) hemorrhage-induction group, (C) hemorrhage-induction and 10 mg/kg Ginseng radix-treated group, (D) hemorrhage-induction and 50 mg/kg Ginseng radix-treated group, (E) hemorrhage-induction and 100 mg/kg Ginseng radix-treated group. * represents $p < 0.05$ compared to the sham-operation group, # represents $p < 0.05$ compared to the hemorrhage-induction group.

Results

1. Effect of Ginseng radix on step-down avoidance task

The latency time was 180 (180/180) sec in the sham-operation group, the hemorrhage-induction group was 39 (32/46) sec, the hemorrhage-induction and 10 mg/kg *Ginseng radix*-treated group was 40 (36/77) sec, the hemorrhage-induction and 50 mg/kg *Ginseng radix*-treated group was 36 (32/54) sec, and the hemorrhage-induction and 100 mg/kg *Ginseng radix*-treated group was 180 (144/180) sec.

In the present results, latency was significantly decreased in the rats of the hemorrhage-induction group, in contrast, treatment with 100 mg/kg *Ginseng radix* increased latency significantly.(Fig. 1)

2. Effect of Ginseng radix on hemorrhage volume in the striatum

The hemorrhage volume of areas deprived of Nissl stained neurons in the striatum was 0.06 ± 0.01 mm^3 in the sham-operation group, the hemorrhage-induction group was 27.64 ± 3.77 mm^3, the hemorrhage-induction and 10 mg/kg *Ginseng radix*-treated group was 12.69 ± 2.55 mm^3, the hemorrhage-induction and 50 mg/kg *Ginseng radix*-treated group was 11.58 ± 1.18 mm^3, and the hemorrhage-induction and 100 mg/kg *Ginseng radix*-treated group was 9.44 ± 1.21

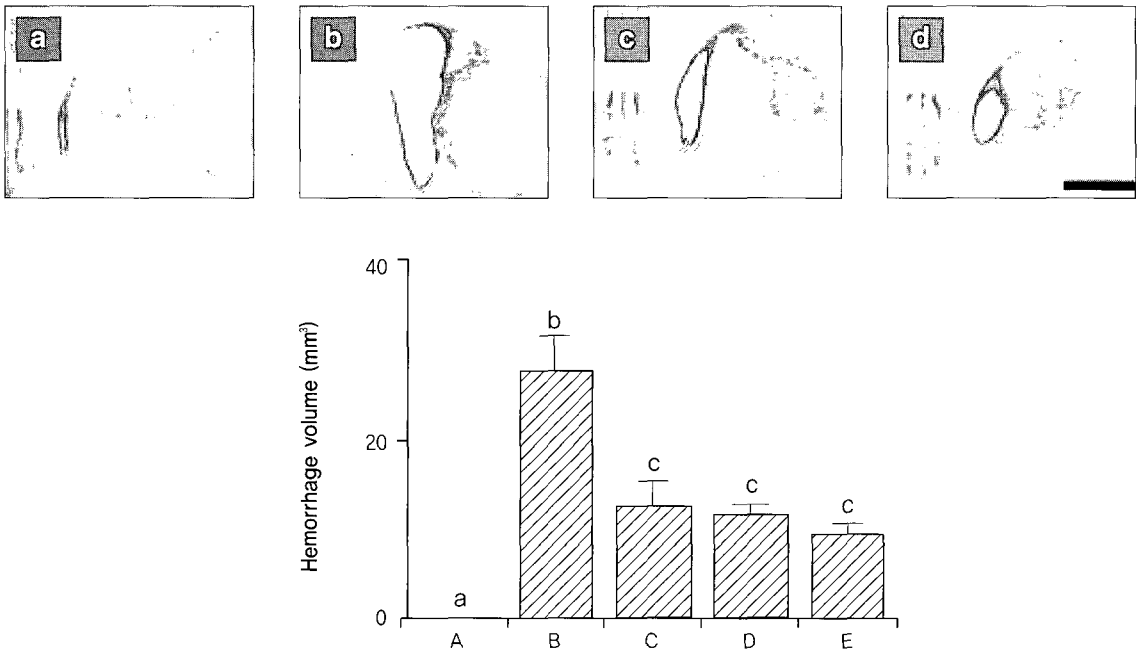

Fig. 2 Effect of Ginseng radix on hemorrhage volume in the striatum
Above: Photomicrographs of the Nissl stained neurons in the striatum. (a) Sham-operation group, (b) hemorrhage-induction group, (c) hemorrhage-induction and 10 mg/kg Ginseng radix-treated group, (d) hemorrhage-induction and 100 mg/kg Ginseng radix-treated group. The scale bar represents 800 m. Below: Hemorrhage volume in the striatum in each group. (A) Sham-operation group, (B) hemorrhage-induction group, (C) hemorrhage-induction and 10 mg/kg Ginseng radix-treated group, (D) hemorrhage-induction and 50 mg/kg Ginseng radix-treated group, (E) hemorrhage-induction and 100 mg/kg Ginseng radix-treated group.

mm^3.

In the present results, the hemorrhage volume in the striatum was significantly increased in the rats of the hemorrhage-induction group, in contrast, treatment with *Ginseng radix* suppressed hemorrhage-induced lesion volume in the striatum significantly as a dose-dependent manner.(Fig. 2)

3. Effect of Ginseng radixon the caspase-3 expression in the striatum

The number of caspase-3-positive cells in the striatum was 38.67 ± 9.85/mm^2 in the sham-operation group, the hemorrhage-induction group was 428.80 ± 64.80/mm^2, the hemorrhage-induction and 10 mg/kg *Ginseng radix*-treated group was 348.53 ± 52.96/mm^2, the hemorrhage-induction and 50 mg/kg *Ginseng radix*-treated group was 237.25 ± 29.55/mm^2, and the hemorrhage-induction and 100 mg/kg *Ginseng radix*-treated group was 156.53 ± 23.03/mm^2.

In the present results, expression of caspase-3 in the striatum was significantly increased in the rats of the hemorrhage-induction group, in contrast, treatment with *Ginseng radix* significantly suppressed hemorrhage-induced caspase-3 expression in the striatum as a dose-

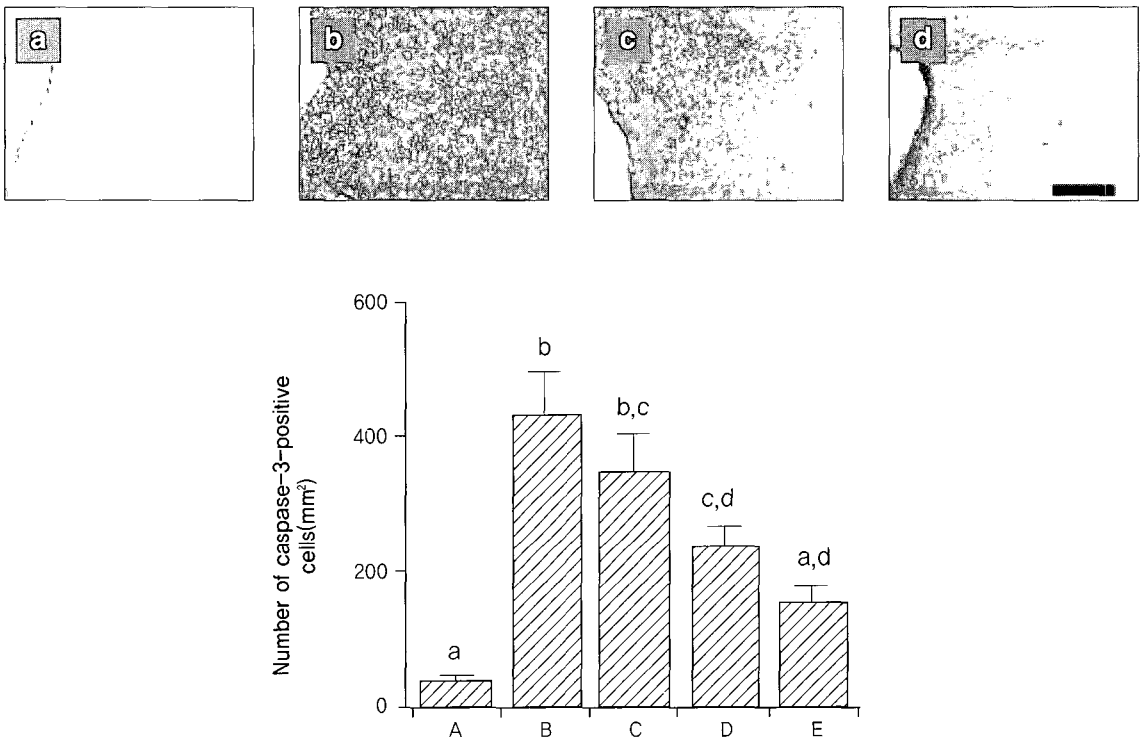

Fig. 3 Effect of Ginseng radix on caspase-3 expression in the striatum
Above: Photomicrographs of caspase-3-positive cells in the striatum. (a) Sham-operation group, (b) hemorrhage-induction group, (c) hemorrhage-induction and 10 mg/kg Ginseng radix-treated group, (d) hemorrhage-induction and 100 mg/kg Ginseng radix-treated group. The scale bar represents 100 m. Below: Number of caspase-3-positive cells per mm2in the striatum in each group. (A) Sham-operation group, (B) hemorrhage-induction group, (C) hemorrhage-induction and 10 mg/kg Ginseng radix-treated group, (D) hemorrhage-induction and 50 mg/kg Ginseng radix-treated group, (E) hemorrhage-induction and 100 mg/kg Ginseng radix-treated group.

dependent manner.(Fig. 3)

4. Effect of Ginseng radixon the number of TUNEL-positive cells in the striatum

The number of TUNEL-positive cells in the striatum was 34.67 ± 5.92/mm² in the sham-operation group, the hemorrhage-induction group was 191.67 ± 22.09/mm², the hemorrhage-induction and 10 mg/kg *Ginseng radix*-treated group was 141.67 ± 29.80/mm², the hemorrhage-induction and 50 mg/kg *Ginseng radix*-treated group was 82.33 ± 12.00/mm², and the hemorrhage-induction and 100 mg/kg *Ginseng radix*-treated group was 67.67 ± 6.54/mm².

In the present results, the number of TUNEL-positive cells in the striatum was significantly increased in the rats of the hemorrhage-induction group, in contrast, treatment with *Ginseng radix* significantly suppressed hemorrhage-induced increase in the number of TUNEL-positive cells in the striatum as a dose-dependent manner.(Fig. 4)

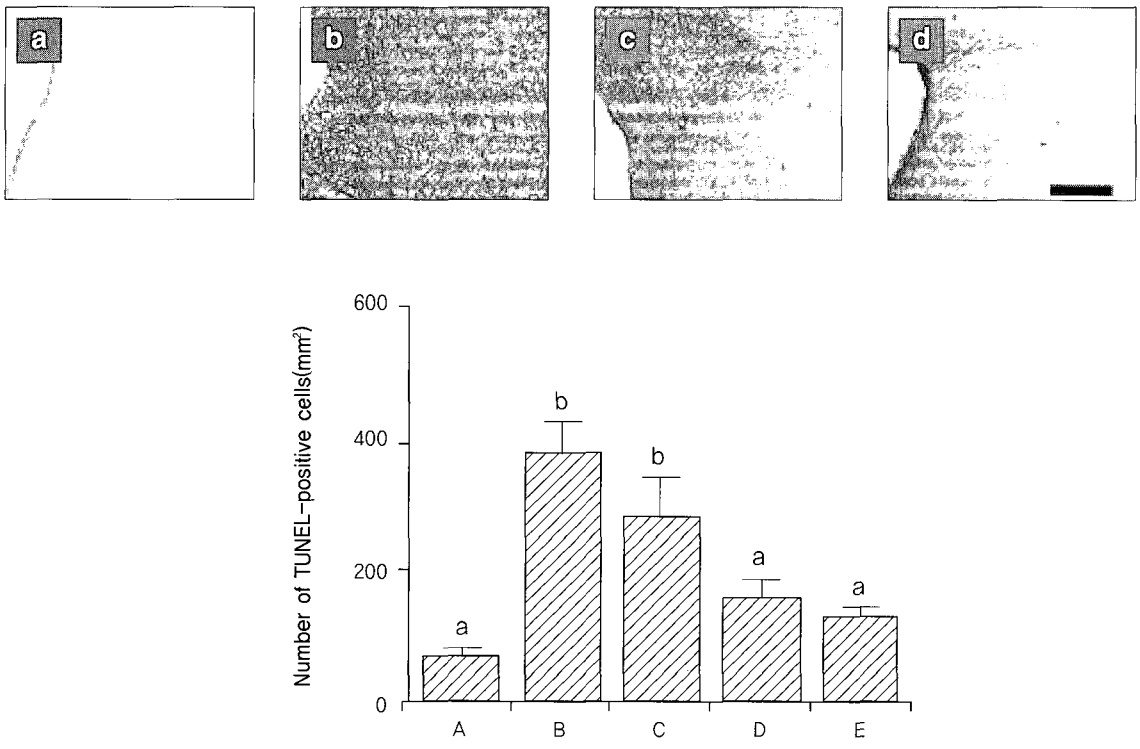

Fig. 4 Effect of Ginseng radix on the number of TUNEL-positive cells in the striatum
Above: Photomicrographs of TUNEL-positive cells in the striatum. (a) Sham-operation group, (b) hemorrhage-induction group, (c) hemorrhage-induction and 10 mg/kg Ginseng radix-treated group, (d) hemorrhage-induction and 100 mg/kg Ginseng radix-treated group. The scale bar represents 100 m. Below: Number of TUNEL-positive cells per mm2 in the striatum in each group. (A) Sham-operation group, (B) hemorrhage-induction group, (C) hemorrhage-induction and 10 mg/kg Ginseng radix-treated group, (D) hemorrhage-induction and 50 mg/kg Ginseng radix-treated group, (E) hemorrhage-induction and 100 mg/kg Ginseng radix-treated group.

Discussion

In hemorrhagic stroke, brain damage occurs through multiple mechanisms: direct tissue destruction, space-occupying effect of the hematoma, ischemic damage to adjacent tissue, clot-derived toxic factors, and edema formation[4].

Brain cell death after intracerebral hemorrhage may be mediated, in part, by an apoptotic mechanism[5]. Apoptosis is a form of cell death that serves to eliminate dying cells in proliferating or differentiating cell populations, and thus plays a crucial role in normal development and tissue homeostasis[21]. On the other hand, inappropriate or excessive apoptosis has been implicated in several types of neurodegenerative disorders including stroke [5,22]. Apoptosis appears to play a key role in neuronal cell death following ischemic stroke[23]. Caspase-3 plays a pivotal role in apoptotic cell death[24] and is activated in the ischemia-reperfusion injury[25]. In addition, apoptotic cell death can be assessed by TUNEL staining, which detects DNA fragmentation, a characteristic of apoptotic cell death [22,26]. Activated caspase-3 expression and TUNEL-positive cells have been observed in intracerebral hemorrhage[21].

It has been reported that intrastriatal hemorrhage in rats induces neuronal cell death, and apoptosis is closely implicated in pathogenesis of intracerebral hemorrhage-induced neuronal cell death[5,21,27]. Apoptotic neuronal cell death induced by intracerebral hemorrhage begins at 24 hours after hemorrhage attack, reaches peak level at 3 days after hemorrhage, and continues at least 4 weeks[5].

Neurological deficit after intracerebral hemorrhage is induced by mechanical destruction by the hematoma, inflammatory response, brain edema, and impairment of cerebral blood flow[28-30].

Nissl-staining is generally used for detecting the dying neurons, and it is well known that hemorrhage increases neuronal cell loss[27]. In the present results, intrastriatal hemorrhage enhanced neuronal cell loss in the striatum, resulting in lesion volume shown by Nissl-staining. Treatment with *Ginseng radix* significantly reduced hemorrhage-induced lesion volume in the striatum.

In a step-down avoidance test, the rat's task was to learn and remember that stepping on the grid delivered a shock to them. A longer latency indicates that the rats remembered the electrical shock from their first training period, so they should avoid this in the future by taking a longer time (latency time) to step down on the grid. In the present results, hemorrhage-induction group showed reduction of latency, showing that intrastriatal hemorrhage induced short-term memory impairment. Treatment with high dose of *Ginseng radix* significantly increased latency near to control level, suggesting that *Ginseng radix* improved short-term memory.

In the present results, the numbers of TUNEL-positive and caspase-3-positive cells in the striatum were significantly increased following intracerebral hemorrhage, indicating that intracerebral hemorrhage inducedapoptotic neuronal cell death in the striatum. Treatment with *Ginseng radix* suppressed hemorrhage-induced apoptotic neuronal cell death in the striatum.

Traditionally, the aqueous extract of *Ginseng radix* has been used for the treatment of a wide variety of diseases including anemia, diabetes mellitus, insomnia with neurasthenia, gastritis, abnormality in blood pressure, dyspepsia, overstrain, and fatigue[31-33]. *Ginseng radix* is also known to possess various pharmacological effects including hypotensive, cardiotonic, sedative, aphrodisiac, anti-aging, and antioxidant activities[33-35]. In Oriental Medicine study, it is described that *Ginseng radix* has some effects of tonifying the five solid organs, resuscitating(opening the orifices), making patients stay calm and treating fidgetiness, and so on.[36,37] In this

study, there is less difference between pharmacological and Oriental Medicine effects of *Ginseng radix* so far. *Ginseng radix* exerts protective effect against myocardium damage due to ischemia-reperfusion injury[38]. It was reported that Bojung-ikgitang, comprising *Ginseng radix* and possessing the effect of reinforcing the vital energy[36], reduced the frequency of radiation-induced apoptosis[39]. It was also reported that *Ginseng radix* has a protective effect on 1-methyl-4-phenylpyridine (MPP^+)-induced apoptosis in PC12 cells[40]. Platelet aggregation plays a pivotal role in thromboembolic disorders including stroke, and *Ginseng radix* has inhibiting effect on platelet aggregation[41] and endotoxin-induced disseminated intravascular coagulation[42].

Here in this study, we have shown that the aqueous extract of *Ginseng radix* reduced hemorrhage-induced lesion volume, the number of TUNEL-positive cells, and caspase-3 expression in the rat striatum, resulting in enhancement of short-term memory. Based on the present results, it is possible that *Ginseng radix* has a neuroprotective effect on stroke, and aids the recovery from central nervous system sequelae following stroke.

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