2004. Vol. 25. No. 4. 95-107
Korean Journal of Oriental Medicine

Original Articles

Paeonia Radix decreases Intracerebral Hemorrhage-induced Neuronal Cell Death via Suppression on Caspase-3 Expressionin Rats

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Objective : The inappropriate or excessive apoptosis has been known to be associated with neurodegenerative disorders including intracranial hemorrhage(ICH). *Paeoniae radix*, in traditional Korean medicine, has played its role as blood-nourisher and yin-astringent. In the present study, the effect of *Paeoniae radix* on the inhibition of neurodegeneration in the brain of rats after artificial ICH and on the resulting apoptosis was investigated.

Methods : 30 rats were divided into 6 equal groups ; the sham-operation group, the hemorrhage-induction group, the hemorrhage-induction with 10, 50, 100, and 200 mg/kg *Paeoniae radix*-treated group, respectively. Stereotactic surgery was performed and collagenase was infused to induce ICH in the region of CA1 of hippocampus of rats. The sham group took only saline infusion. For 7 days after the surgery, 4 testing groups had intraperitoneal injections of *Paeoniae radix* extract. The step-down inhibitory avoidance task, measurement of neurodegeneration degree in the CA1 region of the hippocampus, and detection of caspase-3 and newly generated cells in the dentate gyrus were done after animal sacrifice.

Results : Rats receiving *Paeoniae radix* extract showed increased latency time in the inhibitory avoidance task. The extension of neuron-deprived areas in the CA1 region was significantly suppressed in the *Paeonia* treated groups. Also expressions of caspase-3 in the CA1 region and cortex were significantly inhibited in the *Paeonia* treated groups. The cell proliferation was evaluated by means of BrdU methods and proved to be decreased in the *Paeonia treated* groups.

Conclusion : These results suggest that *Paeoniae radix* has potential to suppress short-term memory loss after devastating neurologic accidents. Also it was proved that *Paeoniae radix* has a neuroprotective effect and alleviates central nervous complications following intracerebral hemorrhage. Furthermore, it may imply that this medicinal plant can be widely used for vascular dementia and other neurodegenerative disorders.

Key Words: *Paeoniae radix*, intracranial hemorrhage, stereotactic surgery, apoptosis, memory loss, neurodegeneration.

Introduction

Cerebrovascular diseases are a primary cause of disability in industrialized countries and stroke is the third leading cause of death and one of the leading causes of adult disability in North America, Europe, and

Received 28 December 2004; received in revised from 7 October 2004; accepted 12 November 2004
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Asia[1]. Of these, intracerebral hemorrhage (ICH) is a devastating clinical condition, accounting for 15% of all stroke hospitalizations[2]. ICH is associated with severe neurological deficits and a considerable mortality rate. ICH shows a consistent neurological deficit, hematoma volume, brain swelling, cortical hypoperfusion[2], and neuronal cell death[3,4].

Apoptosis, also known as programmed cell death, is from the Greek meaning 'to fall away from,' which was inspired from the cells that break into small membrane-surrounded fragments (apoptotic bodies) after characteristic morphological alteration[5]. This programmed cell death plays critical roles in a wide variety of physiological processes during fetal development and in adult tissues. In most cases, physiological cell death occurs by apoptosis as opposed to necrosis[5,6].

It has been reported that inappropriate or excessive apoptosis is closely implicated in neurodegenerative disorders including stroke[3]. Apoptosis is involved in the pathophysiology of cell death after ICH[7] and is associated with the activation of caspases-3[3].

Paeoniae radix is the root of Paeonia japonica MIYABE, which is a perennial plant classified in the family Paeoniaceae. That medicinal plant is commonly used for nourishing the blood, activating circulation, alleviating pain, regulating menstruation, and for treating liver disease and cancer[8]. Recently it has also been proved that *Paeonia radix* can improve blood flow through its endothelium-dependent vasodilatory action on the aorta[9] and reduce fatigue under exercise and resting conditions[10] as well as that fact that it has potentially anti-aging and anti-carcinogenic effects by inhibiting oxidative DNA cleavage induced by various oxidative chemicals[11,12] and by an inhibitory effect on thrombosis and platelet aggregation[13]. However, the effect of *Paeoniae radix* on intracerebral hemorrhage-induced neuronal cell death has not been reported yet.

In the present study, the effects of *Paeoniae radix* on intracerebral hemorrhage-induced neuronal cell death in the hippocampal CA1 region and cerebral cortex and on cell proliferation in the dentate gyrus of rats were investigated using step-down inhibitory avoidance task, Nissl staining, and immunohistochemistry for caspase-3 and 5-bromo-2' -deoxyuridine (BrdU).

Materials and Methods

1. Animals and treatments

Male Sprague-Dawley rats weighing 230±10g (7 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 six groups: the sham-operation group, the hemorrhage-induction group, the hemorrhage-induction and 10 mg/kg *Paeoniae radix*-treated group, the hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group, the hemorrhage-induction and 100 mg/kg *Paeoniae radix*-treated group, and the hemorrhage-induction and 200 mg/kg *Paeoniae radix*-treated group (n = 5 in each group).

Paeoniae radix used in this experiment was obtained from the department of oriental pharmacology in Kyung Hee Medical Center (Seoul, Korea). After washing, *Paeoniae radix* was immersed in cold water for 12 h. To obtain the aqueous extract, 200 g of *Paeoniae radix* was added to distilled water, heat-extracted at 80℃, concentrated using a rotary evaporator and lyophilized. The resulting powder, weighing 25 g, was diluted with saline. After filtering through a 0.45 (m syringe filter,

animals of the *Paeoniae radix*-treated groups received the extract of *Paeoniae radix* at respective doses intra-peritoneally, and those of the sham-operation group received equivalent amounts of saline once a day for 7 days. Following its respective treatment, each animal was injected intra-peritoneally with BrdU (50mg/kg; Sigma Chemical Co., St. Louis, MO, USA) once a day for 7 consecutive days.

2. Stereotaxic surgeries and drug injection protocol

For induction of hemorrhage, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.; Sigma Chemical Co.) and placed in a stereotaxic frame[14]. Through a hole drilled in the skull, a 26-gauge cannula aimed 1.0 mm above area CA1 of the dorsal hippocampus. The coordinates used were as follows: A 4.3, L 4.0, V 2.0, according to the method of Izquierdo et al.[14], 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 were received an equivalent dose of physiological saline with the same method.

3. Step-down inhibitory avoidance task

In order to evaluate the short-term memory ability, the latency-time of the step-down inhibitory avoidance task was determined. Two days after surgery, rats were trained in a step-down inhibitory 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 sessions, the animals received a 0.5 mA, 20 sec scramble footshock immediately upon stepping down, and no footshock was given in test sessions. Retention time was tested on six days after surgery[14,15]. The latency of the rats to step down placing all four paws on the grid was measured.

4. Tissue preparation

The rats were sacrificed on the 7th day of the experiment. 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).

5. Nissl staining

For the determination of degree of neuronal degeneration in the CA1 region of the hippocampus, Nissl staining was performed as previously described [16,17]. For Nissl staining, sections were mounted on gelatin-coated slides and stained with cresyl violet.

6. Caspase-3 immunohistochemistry

For visualization of caspase-3 expression, caspase-3 immunohistochemistry was performed[17]. Sections were drawn from each brain and incubated overnight with mouse anti-caspase-3 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) 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.

7. BrdU immunohistochemistry

For detection of newly generated cells in the dentate gyrus, BrdU incorporation, generally used as an indicator of DNA synthesis, was visualized via a previously described immunohistochemical method[17]. Average 10 sections within the hippocampal region spanning from Bregma -3.30 mm to Bregma -4.16 mm were selected from each brain. The sections were first permeabilized by incubating in 0.5% Triton X-100 in PBS for 20 min. They were then incubated in 50% formamide-2 x standard saline citrate 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 4℃ 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, Burlingame, CA, USA). The sections were then incubated for another 1 h with VECTASTAIN® Elite ABC Kit (1:100; Vector Laboratories, Burlingame, CA, USA). For staining, the sections were incubated in a reaction mixture consisting of 0.02% 3,3' -diaminobenzidine containing nickel chloride (40 mg/ml) (nickel-DAB) and 0.03% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6) for 5 min.

After BrdU-specific staining, counter-staining was performed on the same sections using a mouse anti-neuronal nuclei (NeuN) antibody (1:300; Chemicon International, Temecula, CA, USA). The sections were washed three times with PBS, incubated for 1 h with a biotinylated anti-mouse secondary antibody, and processed with VECTASTAIN® Elite ABC Kit. For staining, the sections were incubated in a reaction mixture consisting of 0.02% DAB and 0.03% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6) for 5 min. The sections were then washed three times with PBS and 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. Data analysis

In the step-down inhibitory avoidance task, the latency-time was determined and latency over 180 sec was counted as 180 sec. The differences in the latency time among the groups were evaluated by a Kruskal-Wallis ANOVA and individual differences between groups were evaluated by Mann-Whitney U tests. Results are presented as median value (interquartile range).

The maximum lateromedial longitudinal extent of neuronal degeneration in the selected region of the lateral band of CA1 region was measured as previously described[16]. This method measured the maximum lateromedial longitudinal extent (in (m) of the layers deprived of neurons or layers containing only small dense nuclei with loss of Nissl substance in cell bodies. Images were captured with video camera attached to light microscope (Olympus, Tokyo, Japan) and data were analyzed using Image-Pro Plus® software (Media Cybernetics Inc., Silver Spring, MD, USA).

The numbers of caspase-3-positive cells were assessed under the microscope using a magnification of (100 and counted in 2-3 representative quadrants of 300 (300 (m of the cerebral cortex on a blind basis and the values were expressed as number of cells per mm^2 of the cerebral cortex[18].

The area of the dentate granular layer in the selected region of the hippocampus was measured using the Image-Pro Plus® software (Media Cybernetics). The number of caspase-3-positive cells in the hippocampal CA1 region and BruU-positive cells in the hippocampal dentate gyrus were counted and expressed as number of cells per mm^2.

Statistical analysis of Nissl staining, caspase-3 and BrdU immunohistochemistry were performed using one-way ANOVA followed by Duncan post-hoc test. Results are presented as mean±standard error mean (S.E.M). Differences were considered significant for $p < 0.05$.

Results

1. Effect of *Paeoniae radix* on step-down inhibitory avoidance task

The latency time was about 180 (180/180) sec in the sham-operation group, 18 (18/19) sec in the hemorrhage-induction group, 41 (11/180) sec in the hemorrhage-induction and 10 mg/kg *Paeoniae radix*-treated group, 180 (180/180) sec in the hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group, 180 (142/180) sec in the hemorrhage-induction and 100 mg/kg *Paeoniae radix*-treated group, and 180 (180/180) sec in the hemorrhage-induction and 200 mg/kg *Paeoniae radix*-treated group.

In the present results, latency time was significantly decreased in the rats of the hemorrhage-induction group and *Paeoniae radix* treatment increased latency time significantly.

2. Effect of *Paeoniae radix* on neuronal dege neration in the CA1 region

The maximal longitudinal lateromedial extension of areas deprived of Nissl stained neurons in the CA1 region was about 91.80±8.39 (m in the sham-operation group, 1492.55±161.81 (m in the hemorrhage-induction group, 820.13±133.12 (m in the hemorrhage-induction and 10 mg/kg *Paeoniae radix*-treated group, 491.15±111.98 (m in the hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group, 545.48± 109.86 (m in the hemorrhage-induction and 100 mg/kg *Paeoniae radix*-treated group, and 696.93± 144.70 (m in the hemorrhage-induction and 200 mg/kg *Paeoniae radix*-treated group.

In the present results, the maximal longitudinal

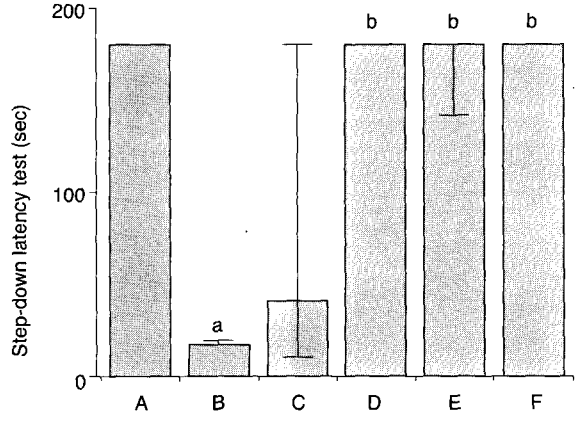

Fig. 1. Effect of *Paeoniae radix* on the latency time of the step-down inhibitory avoidance task.

A, sham-operation group; B, hemorrhage-induction group; C, hemorrhage-induction and 10 mg/kg *Paeoniae radix*-treated group; D, hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group; E, hemorrhage-induction and 100 mg/kg *Paeoniae radix*-treated group; F, hemorrhage-induction and 200 mg/kg *Paeoniae radix*-treated group. a represents $p< 0.05$ compared to the sham-operation group, b represents $p < 0.05$ compared to the hemorrhage-induction group.

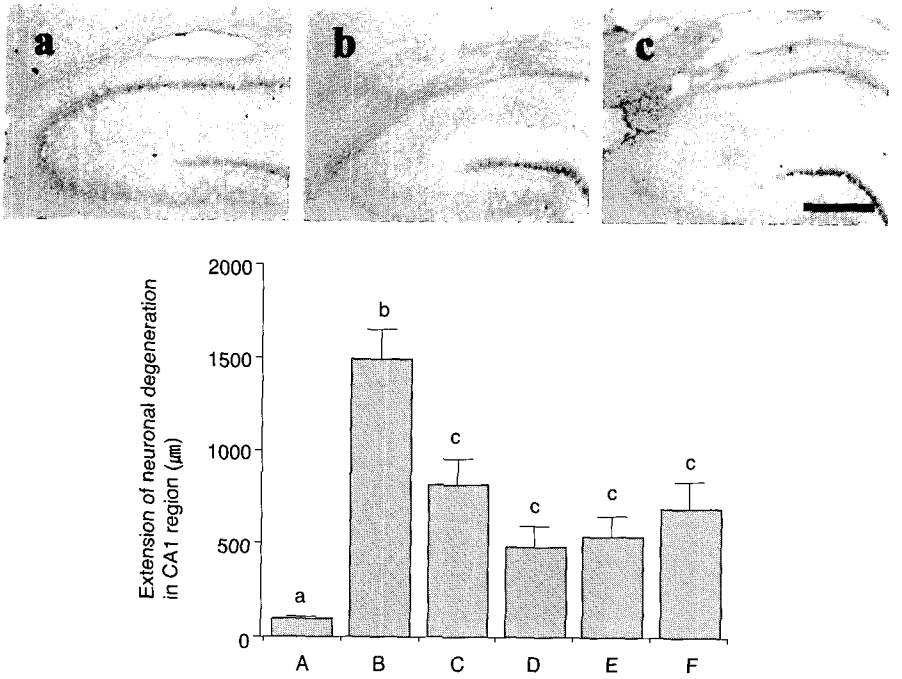

Fig. 2. Effect of *Paeoniae radix* on neuronal degeneration in the CA1 region.

Above: Photomicrographs of the Nissl stained neurons in the CA1 region in each group. a, sham-operation group; b, hemorrhage-induction group; c, hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group. Scale bar represents 250 (m. Below: Maximal longitudinal lateromedial extension of areas deprived of Nissl stained neurons in the CA1 region in each group. A, sham-operation group; B, hemorrhage-induction group; C, hemorrhage-induction and 10 mg/kg *Paeoniae radix*-treated group; D, hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group; E, hemorrhage-induction and 100 mg/kg *Paeoniae radix*-treated group; F, hemorrhage-induction and 200 mg/kg *Paeoniae radix*-treated group.

lateromedial extension of areas deprived of Nissl stained neurons in the CA1 region was significantly increased in the rats of the hemorrhage-induction group and *Paeoniae radix* treatment suppressed hemorrhage-induced neuronal degeneration in the CA1 region.

3. Effect of *Paeoniae radix* on the caspase-3 expression in the CA1 region

The number of caspase-3-positive cells in the CA1 region was about $47.13 \pm 8.55/mm^2$ in the sham-operation group, $297.34 \pm 57.93/mm^2$ in the he morrhage-induction group, $185.54 \pm 29.28/mm^2$ in the hemorrhage-induction and 10 mg/kg *Paeoniae radix*-treated group, $106.30 \pm 10.90/mm^2$ in the hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group, $187.83 \pm 26.69/mm^2$ in the hemorrhage-induction and 100 mg/kg *Paeoniae radix*-treated group, and $191.08 \pm 29.60/mm^2$ in the hemorrhage-induction and 200 mg/kg *Paeoniae radix*-treated group.

In the present results, expression of caspase-3 in the CA1 region was significantly increased in the rats of the hemorrhage-induction group and *Paeoniae radix* treatment significantly suppressed hemorrhage-induced caspase-3 expression in the CA1 region.

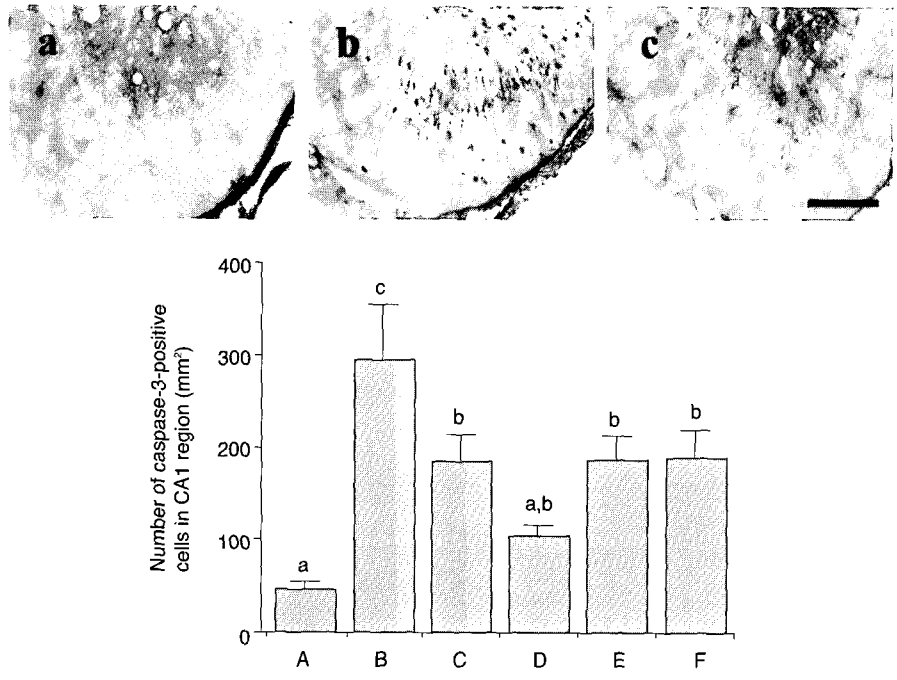

Fig. 3. Effect of *Paeoniae radix* on apoptosis in the CA1 region. Above: Photomicrographs of caspase-3-positive cells in the CA1 region in each group. a, sham-operation group; b, hemorrhage-induction group; c, hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group. Scale bar represents 100 (m. Below: Number of caspase-3-positive cells per mm2 of cross-sectional area in the CA1 region in each group. A, sham-operation group; B, hemorrhage-induction group; C, hemorrhage-induction and 10 mg/kg *Paeoniae radix*-treated group; D, hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group; E, hemorrhage-induction and 100 mg/kg *Paeoniae radix*-treated group; F, hemorrhage-induction and 200 mg/kg *Paeoniae radix*-treated group.

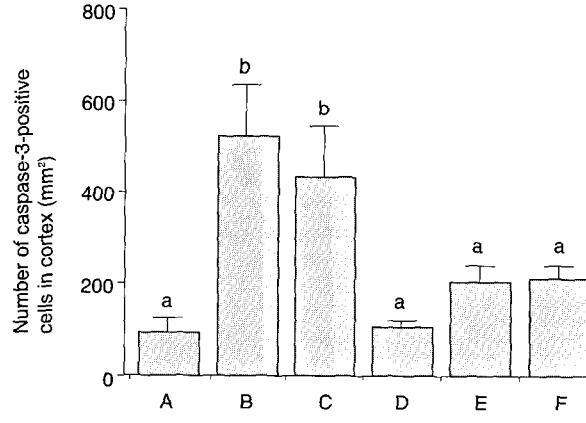

Fig. 4. Effect of *Paeoniae radix* on apoptosis in the cerebral cortex. A, sham-operation group; B, hemorrhage-induction group; C, hemorrhage-induction and 10 mg/kg *Paeoniae radix*-treated group; D, hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group; E, hemorrhage-induction and 100 mg/kg *Paeoniae radix*-treated group; F, hemorrhage-induction and 200 mg/kg *Paeoniae radix*-treated group.

4. Effect of *Paeoniae radix* on the caspase-3 expression in the cerebral cortex

The number of caspase-3-positive cells in the cerebral cortex was about 97.88±28.59/mm² in the sham-operation group, 527.27± 109.95/mm² in the hemorrhage-induction group, 437.04±109.97/mm² in the hemorrhage-induction and 10 mg/kg *Paeoniae radix*-treated group, 111.11±10.85/mm² in the hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group, 208.08±36.52/mm² in the hemorrhage-induction and 100 mg/kg *Paeoniae radix*-treated group, and 218.84±25.16/mm² in the hemorrhage-induction and 200 mg/kg *Paeoniae radix*-treated group.

In the present results, expression of caspase-3 in the cerebral cortex was significantly increased in the rats of the hemorrhage-induction group and *Paeoniae radix* treatment significantly suppressed hemorrhage-induced caspase-3 expression in the cerebral cortex.

5. Effect of *Paeoniae radix* on cell proliferation in the hippocampal dentate gyrus

The number of BrdU-positive cells in the dentate gyrus was about 245.85±25.20/mm² in the sham-operation group, 625.99± 83.48/mm² in the hemorrhage-induction group, 512.19±79.68/mm² in the hemorrhage-induction and 10 mg/kg *Paeoniae radix*-treated group, 335.80±41.79/mm² in the hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group,

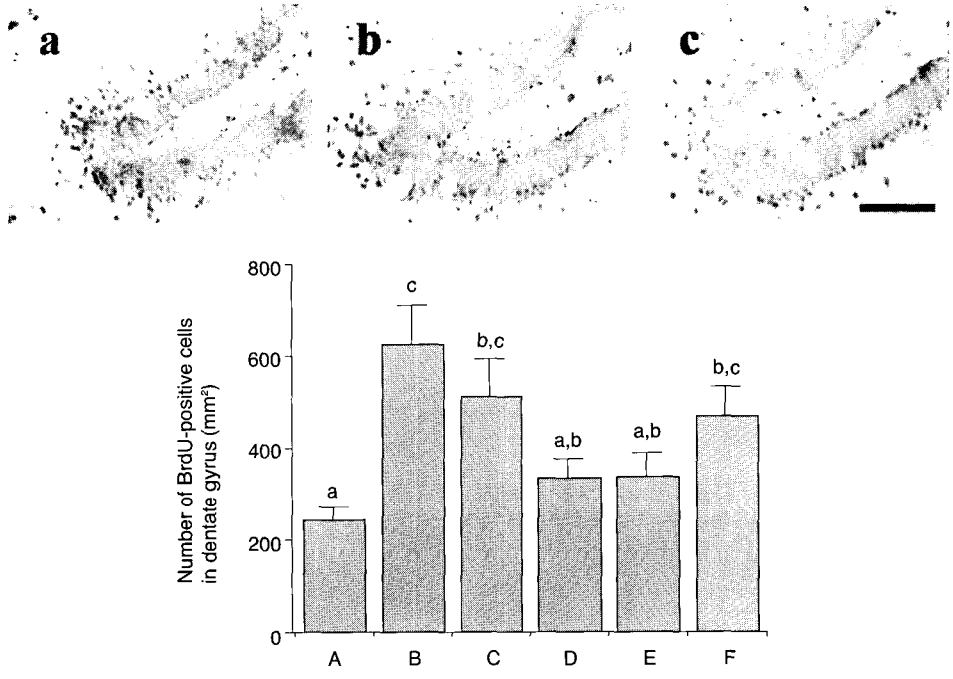

Fig. 5. Effect of *Paeoniae radix* on cell proliferation in the hippocampal dentate gyrus. Above: Photomicrographs of 5-bromo-2'-deoxyuridine (BrdU)-positive cells in the dentate gyrus in each group. a, sham-operation group; b, hemorrhage-induction group; c, hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group. Scale bar represents 100 (m. Below: Number of BrdU-positive cells per mm2 of cross-sectional area in the dentate gyrus in each group. A, sham-operation group; B, hemorrhage-induction group; C, hemorrhage-induction and 10 mg/kg *Paeoniae radix*-treated group; D, hemorrhage-induction and 50 mg/kg *Paeoniae radix*-treated group; E, hemorrhage-induction and 100 mg/kg *Paeoniae radix*-treated group; F, hemorrhage-induction and 200 mg/kg *Paeoniae radix*-treated group.

340.51±48.60/mm^2 in the hemorrhage-induction and 100 mg/kg *Paeoniae radix*-treated group, and 471.12± 62.31/mm^2 in the hemorrhage-induction and 200 mg/kg *Paeoniae radix*-treated group.

In the present results, cell proliferation in the dentate gyrus was significantly increased in the rats of the hemorrhage-induction group and *Paeoniae radix* treatment significantly decreased hemorrhage-induced enhancing of cell proliferation in the dentate gyrus.

Discussion

Intracranial hemorrhage(ICH) remains a rigorous cerebrovascular event with a mortality rate approaching 50%. Therapies have been largely ineffective in reducing morbidity and mortality, and clinical trials have lagged far behind those for patients with ischemic stroke[2]. The pathophysiology of ICH is complex and brain damage occurs through multiple mechanisms. Hemorrhage into the brain initially results in a mass effect with compression of the adjacent microva sculature by the hematoma as well as direct tissue destruction. This is followed by development of brain edema, clot-derived toxic factors and possibly damage caused by raised intracranial pressure with local reductions in cerebral blood flow[2,19]. Currently, there is no medical therapy available for these patients except neurosurgical evacuation of the hematoma. Patients who survive ICH are usually severely disabled; only 10% are capable of living independently after 30 days and only 20% after 6 months[2].

Apoptosis is a morphological phenomenon, which is characterized by cytoplasmic condensation, nuclear pyknosis, chromatin condensation, DNA fragmentation, cell rounding, membrane blebbing, cytoskeletal collapse, and the formation of membrane bound apoptotic bodies that are rapidly phagocytosed and digested by macrophages or neighboring cells[20]. The studies in the past decade have revealed a framework and key players in this pathway. Caspases, a family of cysteine proteases (abbreviated from Cysteine Aspartyl-specific Preotease), are responsible directly or indirectly for the morphological and biochemical changes that characterize the phenomenon of apoptosis[5,6]. Diverse regulators of the caspases have also been discovered; adaptor proteins such as Apaf-1 (required for the activation of caspases), and a family of mitochondria-associated proteins termed the Bcl-2 family[6]. Inputs from signal transduction pathways into the core of the cell death machinery have also been identified, demonstrating ways of linking environmental stimuli to cell death responses or cell survival maintenance. Defects in apoptotic cell death regulation contribute to many diseases, including disorders where cell accumulation occurs including cancer or where cell loss occurs such as stroke, heart failure, neurodegeneration and AIDS[5]. It is well established that apoptotic neuronal cell death is closely implicated in pathogenesis of intracerebral hemorrhage[3,7].

The hippocampal formation is an important brain structure, playing a central role in learning and memory formation[21]. The dentate gyrus within the hippocampal formation is one of the few brain structures where neurogenesis has been demonstrated in adult mammals including humans. These newly generated cells eventually differentiate into mature neurons, demonstrating morphological and biochemical features shown by the surrounding neurons. These neurons in the adult brain are essential for the formation of memory[17]. The CA1 neurons in hippocampus are very vulnerable against a variety of damages that lead to neuronal death including stroke probably through a common mechanism of calcium influx[18].

Neurogenesis, which is upregulated by neural injury in the adult mammalian brain, is associated in the repair of the injured brain and functional recovery[22].

Neurogenesis encompasses cell proliferation, survival, migration, and neuronal differentiation. Cell proliferation in the hippocampal dentate gyrus is known to be enhanced by learning, serotonin, estrogen, antidepressants and physical exercise. Generation of new cells in the adult is also enhanced by pathological events such as seizures and ischemic insults. Such upregulation of cell proliferation occurring during pathological situation is thought to be a compensatory response to lesion-induced cell death in the brain[22,23]. Lee et al proved that cell proliferation was significantly increased following transient ischemia in the hippocampus of gerbils[23].

The major limitation of ICH research has been the lack of reproducible animal models. A widely used method that produces ICH is injection of bacterial collagenase into the brain. This enzyme digests the collagen present in the basal lamina of blood vessels and causes bleeding into the surrounding brain tissue. Although the collagenase method is a simple means of producing hemorrhage and is reproducible, bacterial collagenase causes a significant inflammatory reaction and likely differs from the mechanism that produces ICH in humans[2].

In Korean traditional medicine, *Paeoniae radix* is classified as a blood-tonifier with Angelicae radix and Rehmanniae radix. Since the use was mentioned in Shennong Bencao Jing (神農本草經 ; Shennong Materia Medica), it has been one of the most popular and important herbs in Korean traditional medicine. Its taste and property are sour, bitter and slightly cold. Sour taste acts as astringent as well as bitter taste discharge heat in the body. It enters the liver and spleen meridians. The functions are to nourish blood and astringe yin, smooth the liver, relieve pain, subdue the hyperactive liver-yang, relieve muscular spasms, tranquilize the mind, lower blood pressure and cease sweating[24,25]. Therefore, *Paeonia radix* is applied to blood deficiency and Yin collapse syndrome that is manifested as abdominal and epigastric pain, caused by disharmony between the liver and stomach or stagnation of liver chi, irregular menstruation and menorrhagia, or spasms due to blood deficiency. In many Chinese medicinal formulas, *Paeonia radix* is widely used for these various and effective pharmaceutical actions. For stroke diseases, *Paeonia radix* has been applied in case that the yin is deficient and the liver is overactive[24,25,31].

Recently, the aqueous extract of *Paeoniae radix* has been proved to be effective for the treatment of stroke patients[26], and several studies suggested that *Paeoniae radix* has a neuroprotective effect on neuronal damage. The plant also has anti-platelet aggregation effect; therefore, it plays a preventive and pharmaceutical role in a variety of thromboembolic disorders including stroke. It was reported that Guizhifuling-wan (Geijibokryung-hwan ; 桂枝茯苓丸), consisting of *Paeoniae radix* (白芍药), *Cinnamomi ramulus* (桂枝), Poria cocos (茯苓), Mountan cortex radicis (牧丹皮), and *Persicae semen* (桃仁), inhibited platelet aggregation[27]. Siwu-tang (四物湯), a traditional Korean herbal formula, inhibited histamine release from rat mast cells, and *Paeoniae radix* is known to play a crucial effect in this formula[28]. Paeoniflorin, an active principle of *Paeoniae radix*, attenuated senile dementia and aging-induced cognitive dysfunction[29]. Activation of voltage-gated sodium channels allows sustained Na+ entry during stroke, and blocking of sodium channels exerts neuroprotective effect[30]. *Paeoniae radix* has protective effect in the hippocampal CA1 neurons by suppression on sodium channels during stroke[31].

We investigated in the present study whether the extract of *Paeoniae radix* has the effect of suppressing the neurodegeneration and following apoptosis as well as cell proliferation in the hippocampus of the rat brains.

In the memory-retention test, the hemorrhage-induction group showed reduction of latency times;

however, *Paeoniae radix* treatment significantly increased latency time, in this study, which implies that *Paeonia radix* has a suppressive effect to the neurodegeneration of the hippocampal area, which regulates short-term memory.

Nissl-staining is generally used for detecting the dying neurons, and it is generally accepted that hemorrhage increases neuronal cell loss[3]. In the present results, neuronal loss in the hippocampal CA1 region induced by intracerebral hemorrhage was alleviated by *Paeoniae radix* treatment.

Present results show that caspase-3, a crucial executor enzyme of apoptosis[16], significantly increased in the hippocampal CA1 region and cerebral cortex after intracerebral hemorrhage, and *Paeoniae radix* treatment reduced hemorrhage-induced increment of caspase-3 expression in the hippocampal CA1 region and cerebral cortex. It may be a possible mechanism that *Paeonia radix* can be used as medicine for rehabilitation after stroke.

In the present study, significant increase of cell proliferation was observed in the dentate gyrus following intracerebral hemorrhage and *Paeonia radix* treatment alleviated hemorrhage-induced increment of cell proliferation. Observed proliferation suggests a compensation for devastating cell death in a cerebrovascular episode. Decreased cell proliferation implies that *Paeonia radix* inhibited neuronal cell death in the hippocampus.

The present study demonstrates that the aqueous extract of *Paeoniae radix* reduces neuronal degene ration, caspase-3 expression, and cell proliferation in the intracerebral hemorrhagic rats resulting in enhancement of short-term memory. Based on the present results, it is possible that *Paeoniae radix* has a neuroprotective effect and alleviates central nervous complications following intracerebral hemorrhage.

In order to elucidate *Paeonia radix* has inhibition effect via suppressing the apoptosis and neurodegeneration mechanism after cerebral hemorrhage, an extensive clinical trial to human patients affected by ICH is required.

Conclusion

In order to investigate whether the extract of *Paeoniae radix* has the effect of suppressing the neurodegeneration and following apoptosis and cell proliferation in the hippocampus of the rat brains, stereotactic surgery and immunohistochemistry were performed. The results are as follows:

1. In the step-down inhibitory avoidance task, latency time was significantly decreased in the rats of the hemorrhage-induction group and *Paeoniae radix* treatment increased latency time significantly.

2. The maximal longitudinal lateromedial extension of areas deprived of Nissl stained neurons in the CA1 region was significantly increased in the rats of the hemorrhage-induction group and *Paeoniae radix* treatment suppressed hemorrhage-induced neuronal degeneration in the CA1 region.

3. The expression of caspase-3 in the CA1 region was significantly increased in the rats of the hemorrhage-induction group and *Paeoniae radix* treatment significantly suppressed hemorrhage-induced caspase-3 expression in the CA1 region.

4. The expression of caspase-3 in the cerebral cortex was significantly increased in the rats of the hemorrhage-induction group and *Paeoniae radix* treatment significantly suppressed hemorrhage-induced caspase-3 expression in the cerebral cortex.

5. Cell proliferation in the dentate gyrus was

significantly increased in the rats of the hemorrhage-induction group and *Paeoniae radix* treatment signi ficantly decreased hemorrhage-induced enhancing of cell proliferation in the dentate gyrus.

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