Ginseng radix Suppresses Ischemia-induced Increase in c-Fos Expression and Apoptosis in the Hippocampal CA1 Region in Gerbils

Keung Ryol Park, Mi-Hyeon Jang[1], Chang-Ju Kim[1], Choong-Yeol Lee*

Departments of Physiology, College of Oriental Medicine, Kyungwon University,
1 : Departments of Physiology, College of Medicine, Kyung Hee University

Ginseng radix, the root of Panax ginseng C.A.Meyer (Araliaceae), has traditionally been used for the treatment of various disorders including cerebrovascular accident (CVA). In the present study, the effect of Ginseng radix on c-Fos expression and apoptosis in the hippocampal CA1 region of gerbils following transient global ischemia was investigated via immunohistochemistry for c-Fos and caspase-3 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Enhanced c-Fos-, TUNEL-, and caspase-3-positivities were detected in the hippocampal CA1 region in ischemic gerbils. Administration of the aqueous extract of Ginseng radix suppressed this ischemia-induced increment in the numbers of c-Fos-, TUNEL-, and caspase-3-positive cells. These results suggest that Ginseng radix has an inhibitive effect on the induction of c-Fos expression and apoptosis seen following transient global ischemia.

Key words : Ginseng radix, ischemia, c-Fos, apoptosis, caspase-3

Introduction

Ginseng radix, the root of Panax ginseng C.A.Meyer (Araliaceae), is one of the best known Oriental medicinal herbs, with numerous therapeutic applications. Ginseng radix is known to possess a number of pharmacological effects including hypotensive, cardiotonic, sedative, aphrodisiac, antiaging, and antioxidant actions[1-3].

Cerebral ischemia results from a variety of causes that impair cerebral blood flow and leads to the deprivation of both oxygen and glucose. When persistent and critical, it creates a pathological situation which precipitates in neuronal death in the brain through apoptotic changes[4-6]. It has been shown using an animal model of transient global ischemia that pyramidal neurons in the hippocampal CA1 region are particularly vulnerable to ischemic insult, while those of other regions of the hippocampus are spared[7]. Recent studies have indicated that cerebral ischemia induces a variety of changes in intracellular signaling which may subsequently alter gene expression. Expression of immediate early genes (IEG) is known to be enhanced following transient global ischemia[8,9]. Particular attention has been focused on c-fos, which functions as a transcription factor and is promptly induced after ischemic insults, especially in the CA1 region. Numerous studies have shown that prolonged c-fos induction gives way to neuronal death after ischemia[10-13].

It has been well established that cerebral ischemia results in a selective loss of hippocampal CA1 neurons and that neuronal death following ischemic brain injury is associated with internucleosomal DNA fragmentation. It has also been reported that ischemia induces apoptotic cell death in hippocampal CA1 region[4,6,14]. Apoptosis, also known as programmed cell death, plays an indispensable role in the development and maintenance of cellular homeostasis in all organisms[15]. Thompson reported that apoptosis is a form of cell death that occurs in several pathological situations and that it constitutes part of a common mechanism in cell replacement, tissue remodeling, and removal of damaged cells[16].

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) is widely used to detect DNA fragmentation, a characteristic of apoptotic cell death[17]. Another important characteristic of apoptosis is the activation of caspase-3, the most widely studied member of the caspase family and one of the key executors of apoptosis[18]. Recent studies have shown that the pattern of occurrence of apoptosis as visualized by TUNEL staining is similar to that seen through caspase-3 immunostaining[6].

* To whom correspondence should be addressed at : Choon-Yeol Lee, Department of Physiology, College of Oriental Medicine, Kyungwon University, 65 San, Bokjeong-dong, Seongnam-shi, Kyunggi-do, Korea.
· E-mail : cylee@@kyungwon.ac.kr, · Tel : 031-750-5419.
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It was reported that Ginseng radix protects learning disability and neuronal loss in the hippocampus following forebrain ischemia in gerbils[19]. In the present study, the effects of Ginseng radix on c-Fos expression and apoptosis in the CA1 region of the hippocampus after transient global ischemia in gerbils were investigated via immunohistochemistry for c-Fos and caspase-3 and TUNEL assay.

Materials and Methods

1. Animals and treatments

Adult male Mongolian gerbils (11 - 13 weeks of age) were used in the experiment. The experimental procedures were performed in accordance with the animal care guidelines of NIH and the Korean Academy of Medical Sciences. Animals were housed under controlled temperature (20 ± 2℃) and lighting (lights on from 07:00 to 19:00 h) conditions, with food and water made available ad libitum. Ginseng radix was purchased from Hanaro Mart (Seoul, Korea). To obtain the aqueous extract of Ginseng radix, 200 g of Ginseng radix was added to distilled water, and extraction was performed by heating at 80 ℃, concentrating with a rotary evaporator, and lyophilizing. The resulting powder, weighing 30 g (a collection rate of 15%), was diluted with saline to the appropriate concentration.

Gerbils were divided into 8 groups (n = 5 in each group): the sham-operation group, the 10 mg/kg Ginseng radix-treated sham-operation group, the 50 mg/kg Ginseng radix-treated sham-operation group, the 100 mg/kg Ginseng radix-treated sham-operation group, the ischemia-induction group, the 10 mg/kg Ginseng radix-treated ischemia-induction group, the 50 mg/kg Ginseng radix-treated ischemia-induction group, and the 100 mg/kg Ginseng radix-treated ischemia-induction group.

2. Induction of transient global ischemia

To induce ischemia in gerbils, a surgical procedure based on previously described experimental procedures was performed20). In brief, animals were anesthetized with 3% isoflurane in 20% O2 77% N2, and the common carotid arteries (CCAs) were occluded using aneurysm clips for 5 min. The rectal temperature was maintained at 37℃ with a heat lamp and monitored for an additional 2 h to prevent hypothermia. The sham-operation group was treated in the same way without occlusion of the CCAs.

3. Histological procedure

For the sacrificial process, 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 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 μm thickness were made with a freezing microtome (Leica, Nussloch, Germany).

4. Immunohistochemistry for c-Fos and caspase-3 expression

Fos and caspase-3 immunostaining was performed according to the protocol described by Cho et al.[7] and Zhu et al.[6] Eight sections on average were selected from each brain region spanning from Bregma -3.30 mm to -4.16 mm. Free-floating tissue sections were incubated overnight with rabbit anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000 for visualization of c-Fos or with mouse anti-caspase-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000 for visualization of caspase-3 expression. The sections were then incubated for 1 h with biotinylated anti-rabbit secondary antibody or with anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA). The sections were subsequently incubated with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Immunoreactivity was visualized by incubating the sections in a solution consisting of 0.05% 3,3-diaminobenzidine and 0.01% H_2O_2 in 50 mM Tris-buffer (pH 7.6) for approximately 3 min. As the negative control, brain sections from the experiment were likewise processed using normal goat serum or normal horse serum in place of the primary antibody; no c-Fos- and caspase-3-like immunoreactivity was observed.[21]

5. TUNEL staining

For visualization of apoptotic cells, TUNEL assay was performed using In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) as per the manufacturer's protocol[14,22]. To begin the procedure, 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% H_2O_2, 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 DAB. Mayer's hematoxylin (DAKO, Glostrup, Denmark) was used for counter-staining. The slides were air-dried overnight at room temperature, and cover slips were mounted using Permount.

6. Data analyses

To count the numbers of Fos-positive and TUNEL-positive

cells in the CA1 region of the hippocampus, cell counting was performed through a light microscope (Olympus, Tokyo, Japan). The area of the granular layer was traced using Image-ProPlus image analyzer (Media Cybernetics Inc., Silver Spring, MD, USA) under 40 x magnification. The numbers of Fos-positive and TUNEL-positive cells inside the pyramidal cell layer were counted hemilaterally in the CA1 region of the selected hippocampal slices.

7. Statistical analyses

The results are expressed as mean ± standard error mean (S.E.M.). Data were analyzed by one-way ANOVA followed by Scheffé's post-hoc test using SPSS. Differences were considered statistically significant for $p < 0.05$.

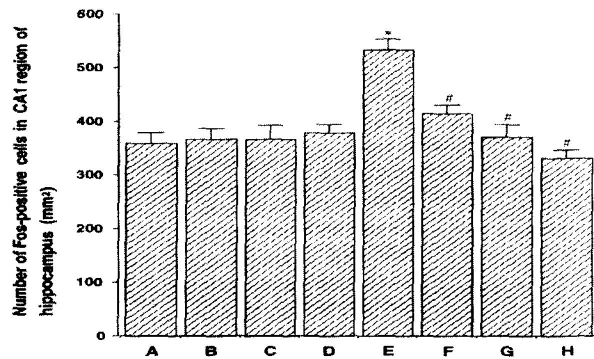


Fig. 1. Effect of Ginseng radix on c-Fos expression in the hippocampal CA1 region after transient global ischemia. Above: Photomicrographs of Fos-positive cells in each group. The scale bar represents 25 μm. Below: The number of c-Fos-positive cells in each group. Values are presented as the mean ± S.E.M. * represents p (0.05 compared to the sham-operation group. # represents p (0.05 compared to the ischemia-induction group. A, Sham-operation group; B, 10 mg/kg Ginseng radix-treated sham-operation group; C, 50 mg/kg Ginseng radix-treated sham-operation group; D, 100 mg/kg Ginseng radix-treated sham-operation group; E, Ischemia-induction group; F, 10 mg/kg Ginseng radix-treated ischemia-induction group; G, 50 mg/kg Ginseng radix-treated ischemia-induction group; H, 100 mg/kg Ginseng radix-treated ischemia-induction group.

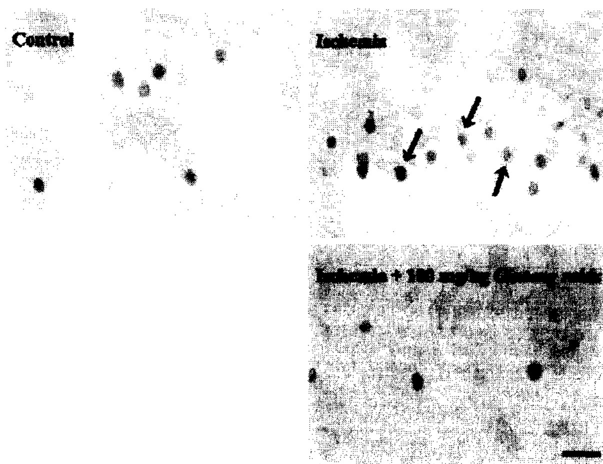
Results

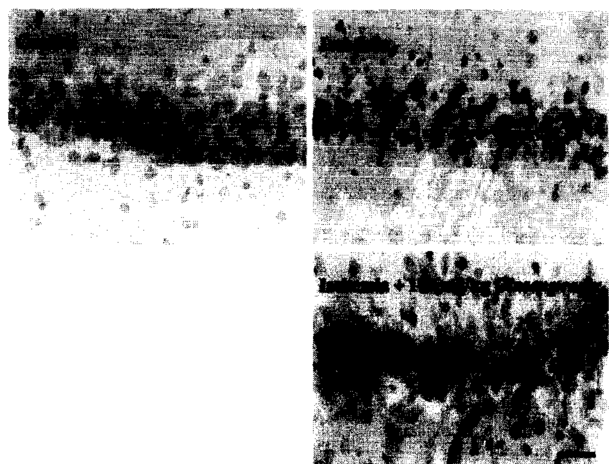
1. Effect of Ginseng radix on c-Fos expression after global ischemic injury

Images of Fos-positive cells in the CA1 pyramidal cell layer of each group are shown in Fig. 1. The number of Fos-positive cells was 359.53 ± 19.73/mm^2 in the sham-operation group, 366.40 ± 20.45/mm^2 in the 10 mg/kg Ginseng radix-treated-sham-operation group, 367.10 ± 24.68/mm^2 in the 50 mg/kg Ginseng radix-treated-sham-operation group, and 378.10 ± 15.38/mm^2 in the 100 mg/kg Ginseng radix-treated-sham-operation group. This figure was increased to 532.59 ± 20.73/mm^2 in the ischemia group, but the increase was curbed to 415.47 ± 14.11/mm^2 in the group treated with 10 mg/kg Ginseng radix after ischemia induction, to 370.67 ± 23.82/mm^2 in the group treated with 50 mg/kg Ginseng radix after ischemia induction, and to 332.05 ± 14.86/mm^2 in the group treated with 100 mg/kg Ginseng radix after ischemia induction (Fig. 1).

2. Effect of Ginseng radix on apoptosis after global ischemic injury

Images of TUNEL-positive cells in the CA1 pyramidal cell layer of each group are shown in Fig. 2. The number of TUNEL-positive cells was 62.95 ± 16.90/mm^2 in the sham-operation group, 53.43 ± 13.56/mm2 in the 10 mg/kg Ginseng radix-treated sham-operation group, 50.38 ± 16.35/mm^2 in the 50 mg/kg Ginseng radix-treated sham-operation group, and 62.59 ± 18.82/mm^2 in the 100 mg/kg Ginseng radix-treated sham-operation group. This figure was increased to 1480.87 ± 135.80/mm^2 in the ischemia group, but the increase was curbed to 1271.71 ± 142.99/mm^2 in the group treated with 10 mg/kg Ginseng radix after ischemia induction, to 1039.66 ± 116.38/mm^2 in the group treated with 50 mg/kg Ginseng radix after ischemia induction, and to 819.94 ± 93.94/mm^2 in the group treated with 100 mg/kg Ginseng radix after ischemia induction (Fig. 2).





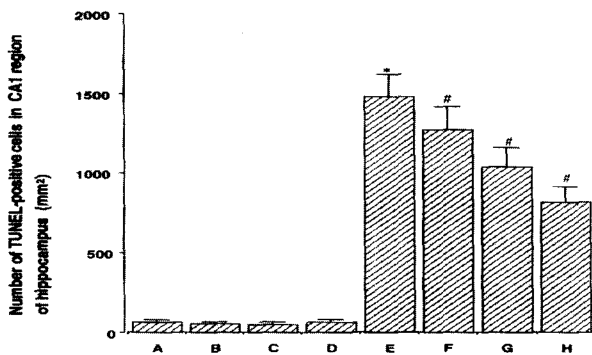
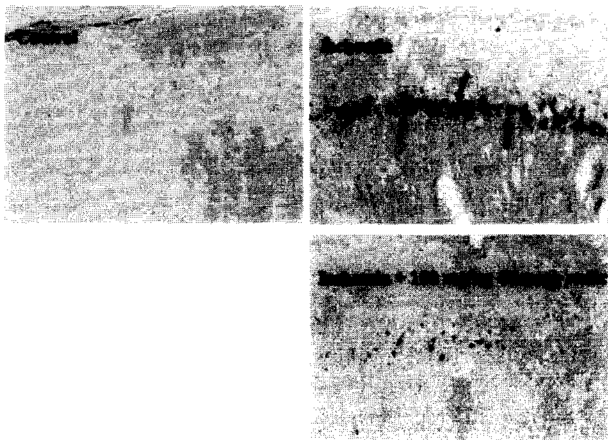


Fig. 2. Effect of Ginseng radix on apoptosis in the hippocampal CA1 region after transient global ischemia. Above: Photomicrographs of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells. The scale bar represents 25 μm. Below: The number of TUNEL-positive cells in each group. Values are presented as the mean ± S.E.M. * represents $p < 0.05$ compared to the sham-operation group. # represents $p < 0.05$ compared to the ischemia-induction group. A, Sham-operation group; B, 10 mg/kg Ginseng radix-treated sham-operation group; C, 50 mg/kg Ginseng radix-treated sham-operation group; D, 100 mg/kg Ginseng radix-treated sham-operation group; E, Ischemia-induction group; F, 10 mg/kg Ginseng radix-treated ischemia-induction group; G, 50 mg/kg Ginseng radix-treated ischemia-induction group; H, 100 mg/kg Ginseng radix-treated ischemia-induction group.

3. Effect of Ginseng radix on caspase-3 expression after global ischemic injury

Images of caspase-3-positive cells in the CA1 pyramidal cell layer of each group are shown in Fig. 3. The number of caspase-3-positive cells was 69.46 ± 13.74/mm^2 in the sham-operation group, 67.17 ± 13.28/mm^2 in the 10 mg/kg Ginseng radix-treated sham-operation group, 66.41 ± 18.78/mm^2 in the 50 mg/kg Ginseng radix-treated sham-operation group, and 64.12 ± 17.40/mm^2 in the 100 mg/kg Ginseng radix-treated sham-operation group. This figure was increased to 1081.53 ± 98.01/mm^2 in the ischemia group, but the increase was curbed to 974.23 ± 94.34/mm^2 in the group treated with 10 mg/kg Ginseng radix after ischemia induction, to 709.25 ± 90.68/mm^2 in the group treated with 50 mg/kg Ginseng radix after ischemia induction, and to 637.19 ± 93.43/mm^2 in the group treated with 100 mg/kg Ginseng radix after ischemia induction (Fig. 3).



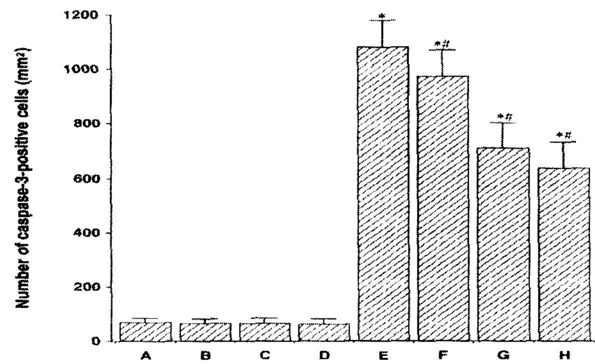


Fig. 3. Effect of Ginseng radix on caspase-3 expression in the hippocampal CA1 region after transient global ischemia. Above: Photomicrographs of caspase-3-positive cells in each group. The scale bar represents 25 μm. Below: The number of caspase-3-positive cells in each group. Values are presented as the mean ± S.E.M. * represents $p < 0.05$ compared to the sham-operation group. # represents $p < 0.05$ compared to the ischemia-induction group. A, Sham-operation group; B, 10 mg/kg Ginseng radix-treated sham-operation group; C, 50 mg/kg Ginseng radix-treated sham-operation group; D, 100 mg/kg Ginseng radix-treated sham-operation group; E, Ischemia-induction group; F, 10 mg/kg Ginseng radix-treated ischemia-induction group; G, 50 mg/kg Ginseng radix-treated ischemia-induction group; H, 100 mg/kg Ginseng radix-treated ischemia-induction group.

Discussion

Stroke is produced by the occlusion of one of the cerebral arteries, resulting in focal ischemia and infarction[4,5,23]. It is one of the major causes of disability in adults, especially old-aged individuals. For these reasons and others, there is a growing effort to understand the mechanisms of cell death in these situations and to prevent or ameliorate the deleterious effects of cerebral ischemia.

Increased induction of c-fos gene by transient global ischemia is a well documented phenomenon in various experimental models[8,9,24,25]. Increased expression of c-fos mRNA in CA1 neurons are observed 30 - 60 min after transient bilateral occlusion of the common carotid arteries in gerbils. In studying c-fos mRNA expression via Northern blot analysis, c-fos expression was observed to be increased 4 h after transient four-vessel occlusion in rats. In similar studies, increased expression of c-fos mRNA in CA1 neurons of the rat hippocampus was observed 3 days after transient four-vessel occlusion[24,25]. Likewise, in the present study, transient global ischemia significantly increased c-Fos expression in the CA1 region of gerbils.

In various studies, c-fos gene expression has been associated with delayed neuronal cell death, and it has been shown that prolonged c-fos induction precedes neuronal death after ischemia[10-12]. Preston et al. reported that the c-Fos protein plays a causative role in the initiation of apoptosis[13].

The 0loss of cells following ischemic insult to the brain results from a wide range of noxious stimuli including deprivation of oxygen and glucose needed for normal cell survival, increased concentrations of excitotoxic amino acids,

and release of cytotoxic mediators. Despite the variety of causative stimuli, cell death is considered to occur by two basic mechanisms: necrosis and apoptosis. Apoptosis can be distinguished from necrosis by morphological and biochemical features, most notably the laddering pattern observed upon electrophoresis of genomic DNA which results from internucleosomal cleavage[26-28]. TUNEL assay is intended to detect DNA fragmentation, a hallmark of apoptosis[17]. Kawase et al. have shown that significant DNA fragmentation is observed in the vulnerable hippocampal CA1 region after global ischemia by TUNEL staining and that the time course of increasing TUNEL-positive cells clearly reveals the occurrence of DNA fragmentation[28]. Pulera et al. also reported that TUNEL-positive cells are significantly increased in neonatal rats following cerebral ischemia induction[29]. In the present study, TUNEL-positive cells were significantly increased in the hippocampal CA1 region following transient global ischemia. In addition, immunohistochemistry for caspase-3, an enzyme which plays a significant role in the nuclear changes occurring in apoptosis[18] was performed in this study. Previous studies have reported that ischemia triggers apoptotic neurodegeneration which involves caspase-3 activation[30-32]. Ischemic insult was shown to enhance caspase-3 expression in the hippocampal CA1 region in this study.

Because of the involvement of apoptosis in ischemia-induced neuronal death, it appears logical that drugs which inhibit apoptosis may be of use in reducing ischemia-induced neuronal damage. Ginseng radix administration has been reported to prevent myocardial ischemia-reperfusion damage induced by hyperbaric oxygen administration[33], and it has also been shown that Ginseng radix has a protective effect against brain damage induced by transient global cerebral ischemia[34]. In addition, Ginseng radix is known to prevent learning disability and neuronal loss in gerbils following forebrain ischemia[19]. Ginsenosides (Rb1 and Rg1), a key components of Ginseng radix, are known to possess memory-enhancing effect in healthy animals[35] and protective effect on hippocampal neurons against ischemia-induced death[36].

In the present study, Ginseng radix treatment was shown to suppress increases in the numbers of c-Fos-positive, TUNEL-positive, and caspase-3-positive cells in the CA1 region of the hippocampus induced by transient global ischemia. These results reveal that the aqueous extract of Ginseng radix has a countering effect against ischemia-induced apoptosis. Based on the present results, it is appears that treatment with Ginseng radix may aid in the recovery from ischemic cerebral injury.

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