Panax ginseng extract attenuates neuronal injury and cognitive deficits in rats with vascular dementia induced by chronic cerebral hypoperfusion

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Graphical Abstract

Panax ginseng extract might be developed as an effective drug for the prevention of vascular dementia. Treatment with Panax ginseng extract for 8 weeks significantly improved behavioral function and increased neuronal density and VEGF and bFGF protein expression in the hippocampal CA3 area. Furthermore, Panax ginseng extract reduced the number of glial fibrillary acidic protein-immunoreactive cells, and it decreased apoptosis by upregulating Bcl-2 and downregulating Bax protein expression. The effect of Panax ginseng extract was dose-dependent and similar to that of nimodipine, a commonly used drug for the treatment of vascular dementia. These findings suggest that Panax ginseng extract is neuroprotective against vascular dementia induced by chronic cerebral hypoperfusion, and therefore might have therapeutic potential for preventing and treating the disease.

Key Words: nerve regeneration; Panax ginseng extract; chronic cerebral hypoperfusion; vascular dementia; learning and memory; vascular endothelial growth factor; basic fibroblast growth factor; apoptosis; neural regeneration

Abstract

Panax ginseng is a slow-growing perennial plant. Panax ginseng extract has numerous biological activities, including antitumor, anti-inflammatory and antistress activities. Panax ginseng extract also has a cognition-enhancing effect in rats with alcohol-induced memory impairment. In this study, we partially occluded the bilateral carotid arteries in the rat to induce chronic cerebral hypoperfusion, a well-known model of vascular dementia. The rats were then intragastrically administered 50 or 100 mg/kg Panax ginseng extract. Morris water maze and balance beam tests were used to evaluate memory deficits and motor function, respectively. Protein quantity was used to evaluate cholinergic neurons. Immunofluorescence staining was used to assess the number of glial fibrillary acidic protein-positive cells. Western blot assay was used to evaluate protein levels of vascular endothelial growth factor, basic fibroblast growth factor, Bcl-2 and Bax. Treatment with Panax ginseng extract for 8 weeks significantly improved behavioral function and increased neuronal density and VEGF and bFGF protein expression in the hippocampal CA3 area. Furthermore, Panax ginseng extract reduced the number of glial fibrillary acidic protein-immunoreactive cells, and it decreased apoptosis by upregulating Bcl-2 and downregulating Bax protein expression. The effect of Panax ginseng extract was dose-dependent and similar to that of nimodipine, a commonly used drug for the treatment of vascular dementia. These findings suggest that Panax ginseng extract is neuroprotective against vascular dementia induced by chronic cerebral hypoperfusion, and therefore might have therapeutic potential for preventing and treating the disease.

Key Words: nerve regeneration; Panax ginseng extract; chronic cerebral hypoperfusion; vascular dementia; learning and memory; vascular endothelial growth factor; basic fibroblast growth factor; apoptosis; neural regeneration

Introduction

Chronic cerebral hypoperfusion is a pathological condition associated with diseases such as Alzheimer’s disease and vascular dementia (VD) (Li and Zhang, 2015). Animal models of chronic cerebral hypoperfusion can be prepared by performing two-vessel occlusion, resulting in a pathology similar to VD (Sun et al., 2015; Huang et al., 2016; Lv et al., 2016). In the rat with two-vessel occlusion, cerebral blood flow is disrupted, resulting in chronic hypoperfusion in the cortex, hippocampus and white matter. The ensuing neuronal injury produces suboptimal metabolism and cognitive dysfunction. Accumulating evidence shows that cerebral ischemia is a major cause of the development of cognitive decline and dementia in the elderly, which involves multiple pathophysiological processes (Ma et al., 2016). The brain regions supplied by the middle cerebral artery, such as the parietal cortex, hippocampus and striatum, are strongly affected by cerebral ischemia. In particular, hippocampal neurons, known to play an important role in learning and memory processes, are vulnerable to ischemic neuronal injury, thereby resulting in severe learning and
memory deficits. Functional and morphological alterations in the hippocampus, such as changes in neurons, astrocytes and synapses, are the most important factors contributing to cognitive dysfunction. Neuronal death in the hippocampus is a major contributor to memory decline in the elderly (Burke et al., 2014). In addition, the vulnerability of hippocampal CA3 pyramidal neurons plays a key role in the onset of cognitive impairment (Counts et al., 2014). Astrocytes also perform critical functions in the brain, such as promoting neo-vascularization, regulating neuronal activity, and supporting synaptogenesis and neurogenesis, which play a role in recovery following ischemic injury. Changes in astrocytes following ischemia may result from direct cellular injury or might occur in response to injury in other central nervous system structures (Lana et al., 2017). However, the pathogenic mechanisms that underlie VD remain to be identified.

There is a great demand for the development of disease-modifying drugs for VD that could attenuate or even reverse the neurodegenerative process by targeting a major hallmark of the disease. Chinese medicine has a long history of preventing and treating cardiovascular and cerebrovascular diseases as well as other related brain diseases (Gao et al., 2017; Guo et al., 2017). These medicines are therapeutically effective and are deemed safe because of their low toxicity and few side effects (Lin et al., 2017; Mei et al., 2017).

Ginseng is a slow-growing perennial plant that belongs to the Araliaceae family and Panax genus. Panax ginseng extract (PGE), is taken orally as a traditional medicine in East Asian countries (Lin et al., 2017; Ma et al., 2017). It is one of the most renowned herbs, with more than 5,500 years of use in East Asia (Lin et al., 2017). It has been reported to possess a variety of biological activities, including antitumor (Zhang et al., 2008), anti-inflammatory (Wang et al., 2015) and antistress activities (Kaneko and Nakanishi, 2004). PGE also has a cognition-enhancing effect in rats with alcohol-induced memory impairment (Shin et al., 2016). In addition, its safety and tolerability for long-term human consumption is well-established (Okita et al., 2011; Furumura et al., 2012).

In this study, to help evaluate the effect of PGE on VD, we used nimodipine, an L-type voltage-dependent Ca\(^{2+}\) channel antagonist, as a positive control. Nimodipine is commonly used for treating VD (Baskys and Cheng, 2012). A previous study reported that nimodipine improves the symptoms of cognitive impairment, increases regional cerebral blood flow, reduces hippocampal inflammatory factor levels, and alleviates neuronal injury (Zhang et al., 2012).

In this study, we examine the effects and mechanism of action of PGE in a rat model of VD produced by bilateral common carotid artery occlusion. Our aim is to advance our understanding of the mechanisms by which PGE protects against the development and progression of VD.

**Materials and Methods**

**Animals**

A total of 100 healthy clean male Wistar rats, 4–5 months old and weighing 320–350 g, were purchased from the Experimental Animal Center of Guizhou Medical University, China [License No. SCXK (qian) 2018-0001]. Rats were housed at 50–70% humidity and a temperature of 22–24°C under a 12/12-hour light/dark cycle. All experiments were conducted in accordance with the guidelines of the Ministry of Health of China and the Animal Care Committee of Guizhou Medical University in China. The study protocol was approved by the Experimental Animal Research Committee of Guizhou Medical University in China. Food and water were freely available during all stages of the experiment. The rats were randomly divided into the following five groups (n = 20 each): sham, VD model, 50 mg/kg PGE, 100 mg/kg PGE and nimodipine.

**Preparation of the VD model**

Rats were acclimated to the facility for a week prior to surgery. The rat model of VD was established as previously described by Gupta et al. (2016). In brief, the rats were intraperitoneally anesthetized with 3 mL/kg of 10% (w/v) chloral hydrate. Through a midline cervical incision, the bilateral common carotid arteries were exposed and gently separated from the carotid sheath and vagus nerve. Each artery of the rats assigned to the ischemic group was ligated with a 5–0 silk suture. As a control, sham-operated rats underwent the identical surgery, but without suture insertion. After recovery from anesthesia, the rats were returned to the animal facility and given free access to food and water. An observer blinded to the identity of the groups assessed neurological deficits at 24 and 48 hours with the forelimb akinesia test, while the spontaneous rotational test was used to evaluate ischemic insult (Nishino et al., 1994). Rats not showing behavioral deficits at these time points were excluded from the study.

**Drug administration**

PGE in concentrated form was purchased commercially from Xi’an Season Biotechnology, Co., Ltd. (Xi’an, China). PGE was produced from the roots of fully mature six-year-old Chinese ginseng plants. PGE contains the following sapogenin fractions: 30.1% ginsenoside (G)-Rb1, 13.9% G-Rb2, 14.4% G-RC, 6.1% G-Rd, 13.9% G-Re, 4.7% G-Rf, 11.5% G-Rg1, 2.6% G-Rg2 and 2.8% G-Rg3 (Ban et al., 2012). PGE was dissolved in saline solution (0.9% NaCl). After the operation, the rats in the VD model group were treated with normal saline (2.0 mL) for 8 weeks. In the 50 and 100 mg/kg PGE groups, the rats underwent middle cerebral artery occlusion (MCAO) and were treated with PGE at the indicated dose by gavage for 8 consecutive weeks. In the nimodipine group, rats underwent MCAO and were treated with nimodipine (20 mg/kg/day, gavage; Sigma-Aldrich, St. Louis, MO, USA). The extracts were filtered and concentrated under vacuum at 60°C. The residue was dissolved in 100% ethanol and filtered and concentrated again.

**Behavioral testing**

**Morris water maze (MWM) test**

Rats were tested for spatial learning and memory using the MWM as previously described (Weitzner et al., 2015). The apparatus consisted of a circular tank (opaque), and four points around the edge were arbitrarily designated north, south, east and west to divide the pool into four corresponding quadrants (northeast, southeast, northwest and southwest). An escape platform was submerged approximately
2 cm below the water (22 ± 1°C) surface and placed in the northeast quadrant of the maze. Extramaze cues consisted of laboratory furniture and lights (held constant throughout the experiment). A video camera was mounted above the center of the pool, and all behaviors were recorded for subsequent analyses. The rats were trained for 4 consecutive days with 4 trials a day from any of the two starting points separated by 90°. The maximum trial duration was 120 seconds, and the inter-trial interval was 60 seconds, during which the rat remained on the escape platform. If the rat did not find the platform within the allowed time, it was guided to the finish by the observer. In each trial, the latency to escape onto the hidden platform was recorded by the observer. A 90-second probe trial was conducted 24 hours following the last test day, and swimming distance in the target quadrant was recorded.

**Balance beam test**

The balance beam test is a test of motor coordination (Zhou et al., 2013). After completion of the MWM test, the balance beam test was administered. Rats were trained to traverse a cylindrical beam with a length of 200 cm and a diameter of 2.5 cm. A black platform (7.0 cm × 4.0 cm) was positioned at one end of the beam at the start point, and a black plastic box (15 cm × 15 cm × 8.0 cm) was placed at the other end as an incentive to traverse the beam. The apparatus was suspended 90 cm above a cushion, which protected rats from injury due to falls, and was positioned 50 cm from a wall. Rats were moved into the testing room one hour before the test to adapt to the environment. The time required to traverse the beam during two trials was recorded. The maximal transversal time allowed was 60 seconds.

**Determination of choline acetyltransferase (ChAT) and acetylcholinesterase (AcH) activities**

To evaluate the neuroprotective effect of PGE on the cholinergic system, following behavioral testing, ten rats from each group were decapitated, and the brains were quickly removed. The hippocampi were dissected according to a stereotaxic brain atlas (Vállez García et al., 2015). Some brain tissues were flash-frozen with liquid nitrogen and stored at −80°C for subsequent use. The others were rinsed in cold physiological saline to remove blood, and a 10% (wt/vol) homogenate was prepared using a tissue homogenizer (3 × 10 seconds, with 30-second intervals). The homogenate was centrifuged at 4000 × g for 30 minutes at 4°C. The supernatant was assayed for ChAT and AcH activities according to the manufacturer’s protocol. Protein concentrations were determined using the Quantity Protein assay kit (Jiancheng Institute of Biotechnology, Nanjing, China).

**Western blot assay**

The hippocampal tissues were frozen and cut into 1.0-mm sections using a stainless-steel rat brain slicer, and homogenized in 0.5 mL of radioimmunoprecipitation assay buffer (150 mM NaCl, 1% N-40, 0.5% deoxycholate, 0.1% sodium dodecylsulfate, 50 mM Tris hydrochloride, 2 mM phenylmethylsulfonyl fluoride, pH 7.4). The homogenate was then transferred to small tubes and mixed by rotation at 4°C overnight. Solubilized protein was collected after centrifugation at 10,000 × g for 30 minutes. The supernatant from each tube was collected, and protein concentrations were quantified with the enhanced bicinchoninic acid protein assay kit (Boster Biotechnology, Wuhan, China). To detect vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), Bcl2 and Bax protein levels, tissue protein lysates from each group were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 10% gel and electrotransferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% skim milk powder in 0.1% phosphate buffered saline (PBS) at room temperature for 2 hours. Immunoblotting was performed using 2.0 μg/mL mouse anti-rat VEGF, bFGF, Bcl2 or Bax monoclonal antibody (1:200 dilution; Sigma-Aldrich) overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:200 dilution; Sigma) overnight at 4°C. Blots probed for β-actin or GAPDH served as a loading control. Immunoreactive proteins were visualized using enhanced chemiluminescence and a western blotting detection system (Beyotime Biotechnology, Haimen, China). Optical density values were quantified using BIO-1D software (Wilber Lourmat, Eberhardzell, Germany).

**Histopathological examination**

The remaining rats were intraperitoneally anesthetized with 3.5% chloral hydrate, 35 mg/100 g, and perfused through the aorta with pre-cooled physiological saline, followed by 4% paraformaldehyde in PBS (0.1 M, pH 7.4). The brains were immediately removed and postfixed in 4% paraformaldehyde for 2–4 hours, then dehydrated overnight in graded sucrose solutions (15%, 20% and 30%) until completely submerged. The dehydrated brains were embedded in Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek, Tokyo, Japan) under frozen conditions. Coronal slices were cut at 6 μm using a cryostat, and unbiased cell estimation was performed for the hippocampal CA3 region on every sixth section according to a systematic random sampling procedure. Approximately 105–137 consecutive sections were collected from the hippocampus in each rat and subjected to Nissl staining and glial fibrillary acidic protein (GFAP) immunofluorescence labeling.

**Nissl staining**

For Nissl staining, sections were immersed in 0.01% toluidine blue (Leagene Biotechnology, Beijing, China) for 15–20 minutes at room temperature, dehydrated twice using a graded series of ethanols (70%, 80%, 90% and 100%), permeabilized with xylene, wet-mounted onto glass slides, and immediately mounted using neutral resin. Sections were photographed using a light microscope (Olympus, Tokyo, Japan).

**Immunofluorescence staining**

To detect GFAP-immunoreactive cells in the hippocampal CA3 of the ischemic hemisphere, brain sections were incubated for 30 minutes in 2.0 N HCl to denature the DNA, and the reaction was neutralized in 0.1 M boric acid for 10 minutes. Thereafter, brain sections were rinsed in PBS containing 0.3% Triton for 30 minutes, preincubated in 10% normal goat serum for 2 hours at room temperature, incu-
Figure 1 Effect of PGE on performance in the Morris water maze.
(A) Escape latency in the training trials. (B) Percent distance in the target quadrant in the probe trials. (C) Representative pathways on the last day of training trials. Data are shown as the mean ± SD (n = 20; one-way analysis of variance followed by Student’s t-test). *P < 0.05, vs. sham group; †P < 0.05, vs. VD model group; ‡P < 0.05, vs. 50 mg/kg PGE group; §P < 0.05, vs. 100 mg/kg PGE group. PGE: Panax ginseng extract; VD: vascular dementia.

Figure 2 Effect of PGE on balance beam test scores. No statistically significant differences were found among the five groups. PGE treatment did not alter balance, as indicated by the time taken to traverse the elevated 2.5-cm (diameter) cylindrical beam (P > 0.05). Data are expressed as the mean ± SD (n = 20; one-way analysis of variance followed by Student’s t-test). Once: The first test; twice: the second test. PGE: Panax ginseng extract; VD: vascular dementia.

Figure 3 Effect of PGE on AChE and ChAT activities in the hippocampus.
Data are presented as the mean ± SD (n = 10; one-way analysis of variance followed by Student’s t-test). *P < 0.05, vs. sham group; †P < 0.05, vs. VD model group; ‡P < 0.05, vs. 50 mg/kg PGE group; §P < 0.05, vs. 100 mg/kg PGE group. PGE: Panax ginseng extract; VD: vascular dementia; AChE: acetylcholinesterase; ChAT: choline acetyltransferase.

Figure 4 Western blot assay for VEGF and bFGF protein expression in the brain.
Expression was calculated as the ratio of the optical density of the target protein to that of GAPDH. Values were presented as the mean ± SD (n = 10; one-way analysis of variance followed by Student’s t-test). *P < 0.05, vs. sham group; †P < 0.05, vs. VD model group; ‡P < 0.05, vs. sham group; ††P < 0.01, vs. 50 mg/kg PGE group; °P < 0.001, vs. VD model group. PGE: Panax ginseng extract; VD: vascular dementia; VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor.
bated with a mouse anti-GFAP antibody (1:200; Beyotime Biotechnology) at 4°C overnight, and then incubated with a Cy5-conjugated affinity-purified goat anti-mouse IgG (1:100; Sigma-Aldrich) in a humidified chamber for 1 hour at 37°C. GFAP-immunoreactive cells were counted with a laser scanning confocal microscope (Olympus).

Statistical analyses  
All statistical analyses were performed by an observer blinded to the experimental groupings. All data were analyzed using SPSS 19.0 software (IBM, Armonk, NY, USA). The data were expressed as the mean ± SD. One-way analysis of variance followed by Student’s t-test was used to assess statistical significance. A value of P < 0.05 was considered statistically significant.

Results  
PGE treatment prevented chronic cerebral hypoperfusion-induced learning and memory deficits in the rat model of VD  
To investigate the effects of PGE treatment on spatial learning in VD rats, the MWM test was performed, and learning ability was assessed by measuring escape latency (Figure 1). Rats in the sham group rapidly learned the location of the escape platform and quickly reached it. In contrast, rats in the VD model group spent a relatively greater amount of time exploring the margin of the pool during the testing period. In comparison, in the 50 and 100 mg/kg PGE groups as well as in the nimodipine group, the escape latency was significantly shortened compared with the VD model group (P < 0.05; Figure 1A). These results show that PGE alleviates the learning and memory impairment in rats with VD in a dose-dependent manner. In probe trials, the percent time in the target quadrant was shorter in the VD model group than in the sham group, but was longer after treatment with PGE. The PGE and nimodipine-treated rats swam a greater distance over the platform compared with rats in the VD model group (P < 0.05). There were no significant differences between the 100 mg/kg PGE and nimodipine groups (P > 0.05) (Figure 1B). The swimming paths recorded on the last day are shown in Figure 1C. Additionally, there was no significant difference in swimming speed among the groups (data not shown) (P > 0.05).

PGE treatment did not affect balance in the rat model of VD  
In the balance beam test, rats were placed on the beam to assess their balance and coordination by measuring the time required to traverse the beam. No statistically significant differences in traversal time were found among the five groups (P > 0.05; Figure 2).

PGE treatment restored cholinergic neuron levels in the hippocampus of rats with VD  
We examined AChE activity levels in the hippocampus to assess cholinergic function (Figure 3A). AChE activity was significantly higher in the VD model group compared with the sham group (P < 0.05). AChE activity was significantly lower in animals treated with PGE or nimodipine, compared with the VD model group. Furthermore, AChE activity was significantly lower in the 100 mg/kg PGE group than in the 50 mg/kg PGE group (P < 0.05). The hippocampal CA3 ChAT activity assay results are shown in Figure 3B. ChAT activity was significantly lower in the VD model group than in the sham group (P < 0.05). In rats administered PGE or nimodipine, ChAT activity was significantly higher compared with the VD model group (P < 0.05). PGE treatment dose-dependently reduced AChE activity and increased ChAT activity. In addition, AChE activity was lower and ChAT activity was significantly higher in the nimodipine group compared with the 100 mg/kg PGE group (P < 0.05).

PGE treatment increased the expression of VEGF and bFGF in the brain of rats with VD  
To clarify the mechanisms by which PGE alleviates neural tissue damage and promotes repair, we measured the expression of VEGF and bFGF proteins in the five groups. As shown in Figure 4, VEGF and bFGF protein levels were decreased in the VD model group compared with the sham group, and treatment with PGE (50 or 100 mg/kg) significantly blocked this reduction in expression (P < 0.05). Furthermore, protein levels of VEGF and bFGF were significantly higher in the 100 mg/kg PGE group compared with the 50 mg/kg PGE group (P < 0.01). However, there were no significant differences between the 100 mg/kg PGE and nimodipine groups (P > 0.05).

PGE treatment impacted the expression of neuronal apoptosis-related proteins in the hippocampus  
To further clarify the molecular mechanisms underlying the neuroprotective effect of PGE, we focused on two proteins involved in apoptotic death by western blot assay. Bcl-2 expression was significantly decreased in the VD model group compared with the sham group (P < 0.01), and treatment with PGE (50 or 100 mg/kg) significantly suppressed this reduction in expression level (P < 0.05) in a dose-dependent manner. In addition, Bax expression was significantly increased in the VD model group compared with the sham group (P < 0.01), and this was significantly prevented by treatment with PGE (50 or 100 mg/kg; P < 0.05) in a dose-dependent manner. No difference was found in the expression of Bcl-2 or Bax between the nimodipine and 100 mg/kg PGE groups (Figure 5).

PGE treatment attenuated histopathological changes in the hippocampal CA3 region of rats with VD  
Histological changes in the hippocampal CA3 region were examined by Nissl staining (Figure 6). In the sham group, the neurons exhibited a normal morphology, with distinct round or oval nuclei and nucleioli, and clear Nissl bodies in the cytoplasm, with no signs of interstitial edema (Figure 6A, B). In contrast, in the VD model group, degeneration and necrosis of a great number of neurons, with cell body shrinkage, pyknosis, breaking and dissolution of the nucleus, and interstitial edema were observed (Figure 6C). In comparison, in rats treated with PGE or nimodipine for 8 weeks, the numbers of degenerating and necrotic neurons were substantially reduced (Figure 6D–G).

PGE treatment reduced GFAP-immunoreactive cells in the hippocampal CA3 region in the rat model of VD  
To provide further insight into the molecular mechanisms
underlying the neuroprotective effect of PGE, we examined GFAP-immunoreactive cells by immunolabeling. The expression of GFAP in the rat hippocampal CA3 area is shown and quantified in Figure 7A–E. Compared with the weak constitutive expression in the sham group, GFAP-immunoreactive cells were significantly increased in the VD model group (P < 0.05). Treatment with 50 or 100 mg/kg PGE significantly reduced the number of GFAP-immunoreactive cells compared with the VD model group (P < 0.05). Moreover, compared with the 50 mg/kg PGE group, the number of GFAP-immunoreactive cells was significantly lower in the 100 mg/kg PGE group (P < 0.05). However, there were no significant differences between the 100 mg/kg PGE and nimodipine groups (P > 0.05; Figure 7F).

Discussion

In the present study, we used a rat model of VD which is widely used to simulate human ischemic injury-induced learning and memory impairment. The ischemic injury produced by MCAO had a strong impact on the brain. Administration of PGE significantly attenuated neurological injury by ameliorating central cholinergic function, improving blood circulation in the brain, delaying neuronal cell death, and by decreasing apoptosis in the hippocampal region.

Cognitive dysfunction as a result of Alzheimer’s disease or VD is a major contributor to morbidity in Western society (Brookmeyer et al., 2007). Although the underlying etiology of dementia is likely multifactorial, dysfunction of the cerebrovasculature is undoubtedly a contributing factor, especially in VD (Du et al., 2017). To date, pharmacological and immunologic interventions have met with limited success in reducing mild cognitive impairments and dementia in patient populations (Venkat et al., 2015). Patients with VD generally experience a decline in cognitive function due to ischemic, ischemic-hypoxic (Blair et al., 2017) or hemorrhagic brain lesions caused by cerebrovascular disease and cardiovascular pathologic changes (Gorelick et al., 2011). The hippocampus is one of the most important brain regions associated with learning and memory. In recent years, investigators have found that the hippocampus, especially the hippocampal CA3 area, is particularly susceptible to ischemic insult (Jung et al., 2012). Functional and morphological perturbations in the hippocampus, including changes in neurons, astrocytes and synapses, are among the most important factors contributing to cognitive dysfunction. Neuronal death in the hippocampus is a major contributor to memory decline in the elderly (Burke et al., 2014). Astrocytes also perform critical functions in the brain, such as promoting neovascularization, regulating neuronal activity, and supporting synaptogenesis and neurogenesis (Kim et al., 2017), which may influence recovery from ischemic injury. Changes in astrocytes following ischemia can result from direct cellular injury or in response to injury in other central nervous system structures (Becerra-Calixto and Cardona-Gómez, 2017).

It is becoming increasingly clear that traditional Chinese herbs can play an important role in alleviating symptoms and dementia (Lin et al., 2017). In traditional oriental medicine, many herbal drugs and prescriptions have been used clinically for the treatment of stroke (Mei et al., 2017), Alzheimer’s disease and VD (Lin et al., 2017). Chinese medicine incorporates centuries of experience in dealing with dementia. Ginseng is a slow-growing perennial plant that belongs to the Araliaceae family and Panax genus. PGE is used frequently as a crude substance that is taken orally as a traditional medicine in Asian countries (Lin et al., 2017). PGE contains various ginseng saponin fractions, including ginsenoside (G)-Rb1, G-Rb2, G- Rc, G-Rd, G-Re, G-Rf, G-Rg1, G-Rg2 and G-Rg3 (Ban et al., 2012). These components might act synergistically with each other to enhance the activity of or counteract the toxic effect of other factors (Yun et al., 2001; Goudarzvand et al., 2016), resulting in cognitive and behavioral improvement.

The MWM test is a hippocampus-dependent memory task that is commonly used in the evaluation of cognitive status in rodents. The training trials are used to assess spatial or place learning, and the probe trials evaluate whether the animal remembers the position of the platform (Vorhees and Williams, 2006). Rodents with VD exhibit significant learning and memory deficits during the MWM test, with a longer escape latency (Huang et al., 2017). In this experiment, we found that the sham group rapidly learned the location of the platform and quickly reached the escape platform. In contrast, the VD model group exhibited a swimming behavior in which rats wasted time exploring the margin of the pool during the testing period. In comparison, the escape latency was significantly shorter in the PGE treatment groups and in the nimodipine group. There were no significant differences in body weight or swimming ability among the different experimental groups. Our findings indicate that PGE improves learning and memory abilities in rats with VD in a dose-dependent manner.

A study on the pathogenetic mechanisms of VD showed that, similar to AD, cholinergic abnormalities are associated with a disturbance in cognitive function in VD patients (Naddaf et al., 2013). Cholinergic neurons that project into the hippocampus play a critical role in learning and memory functions, and the cholinergic terminals in the presynaptic membrane are sensitive to ischemic insults (Jia et al., 2004). In this study, we examined the brain tissue levels of two critical central cholinergic markers, ChAT and AChE. PGE treatment increased ChAT activity while decreasing AChE levels. This suggests that drugs that target the cholinergic system might have therapeutic efficacy in VD patients. Inhibition of brain AChE increases synaptic concentrations of acetylcholine, which might alleviate cognitive dysfunction and neuropathology in patients with cerebral ischemic dementia.

Whether in the normal or ischemic brain, neurogenesis and angiogenesis usually accompany each other, and both are important for functional recovery (Beck and Plate, 2009). In recent years, it has become clear that strategies that enhance angiogenesis and improve cerebral blood circulation are critical for the treatment of VD (Tarkowski et al., 2002). VEGF is a key angiogenic factor in the ischemic brain (Tarkowski et al., 2002), and it also has neurotrophic and neuroprotective effects that promote recovery (Dzielko et al., 2013). bFGF induces cell proliferation and neovascularization in an autocrine and/or paracrine fashion (Sun et al., 2009). Previous studies have demonstrated that bFGF promotes axonal branch formation (Shi et al., 2002) and stimulates the prolif-
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**Figure 5 Western blot assay for Bax and Bcl-2 protein expression in the hippocampus.**
Expression was calculated as the ratio of the optical density of the target protein to that of β-actin. Values are presented as the mean ± SD (n = 10; one-way analysis of variance followed by Student’s t-test). **P < 0.01, vs. sham group; #P < 0.05, vs. VD model group; †P < 0.05, vs. 50 mg/kg PGE group. PGE: Panax ginseng extract; VD: vascular dementia.**

**Figure 6 Effect of PGE on hippocampal histopathology.**
(A) Neurons in the hippocampal CA3 area. The box indicates the analyzed area (Nissl staining, ×100). (A, B) Sham group: The neurons were arranged normally, and Nissl bodies in the cytoplasm were abundant (× 400). (C) VD model group: the number of neurons was substantially reduced, and they were sparsely arranged. Nissl bodies in the cytoplasm were decreased (× 400). (D) 50 mg/kg PGE group (× 400). (E) 100 mg/kg PGE group (× 400). (F) Nimodipine group (× 400). In rats given PGE or nimodipine treatment, the number of neurons was increased, with a greater number of Nissl bodies in the cytoplasm. (G) Quantitation of Nissl-positive cells. Data are shown as mean ± SD (n = 10; one-way analysis of variance followed by Student’s t-test). *P < 0.05, vs. sham group; #P < 0.05, vs. VD model group; †P < 0.05, vs. 50 mg/kg PGE group. PGE: Panax ginseng extract; VD: vascular dementia.

**Figure 7 Effect of PGE on hippocampal GFAP-immunoreactive cells.**
(A–E) GFAP-immunoreactive cells were examined in the hippocampal CA3 region by immunofluorescence labeling. GFAP-positive cells are labeled red, and the labeling was mainly localized to the cytoplasm. Scale bars: 20 μm. (A) Sham group. (B) VD model group. (C) 50 mg/kg PGE group. (D) 100 mg/kg PGE group. (E) Nimodipine group. (F) Quantitation of GFAP-immunoreactive cells. Data are expressed as the mean ± SD (n = 10; one-way analysis of variance followed by Student’s t-test). *P < 0.05, vs. sham group; #P < 0.05, vs. VD model group; †P < 0.05, vs. 50 mg/kg PGE group. PGE: Panax ginseng extract; GFAP: glial fibrillary acidic protein.
eration and differentiation of neural precursors (Zhang et al., 2014). Therefore, bFGF has great potential for the treatment of central nervous system disorders. Our results show that VEGF and bFGF levels decreased dramatically after ischemic brain injury, and that PGE treatment significantly diminished this reduction, helping to maintain their levels.

The Nissl body is a structure unique to neurons, and the density of Nissl staining in the neuronal cytoplasm is used to evaluate neuronal damage (Kadar et al., 2009). Cognitive deficits are associated with damage to the hippocampal CA1 (Sugawara et al., 2002). Permanent occlusion of the bilateral common carotid arteries in rats results in significant pyramidal neuron loss in the hippocampal CA1 (Li et al., 2014). In addition, chronic cerebral hypoperfusion triggers reactive astrogliosis with detectable morphological changes and accumulation of GFAP in these glia (Lana et al., 2017). GFAP, one of the most highly synthesized proteins in the brain, is widely used to study the activation state of astrocytes and might play a role in the ischemic process (Panickar and Norenberg, 2005). In the current study, we found that PGE treatment significantly increased pyramidal neuron numbers and reduced astrocyte activation and proliferation in the hippocampal CA3. These changes were associated with improved cognitive performance in our rat model of VD. However, the mechanisms underlying the various neuroprotective effects of PGE require further investigation.

It is now well known that apoptosis during VD plays a major role in brain injury associated with dementia (Liu et al., 2017). Bcl-2 and Bax are the two primary proteins regulating apoptotic cell death, with opposing functions (Min et al., 2014). Bcl-2 is functionally an apoptosis suppressing factor, whereas Bax is an apoptosis promoting factor (Hwang et al., 2013). Bcl-2 inhibits cytochrome c release from mitochondria elicited by the pro-apoptotic molecule Bax, resulting in the inhibition of caspase activation and apoptotic death (Zhao et al., 2017). VD is a multifactorial disease, and the mechanisms of neuronal apoptosis in the hippocampus are important for an understanding of its pathogenesis. Therefore, we also examined the effects of PGE on the expression of Bcl-2 and Bax in the hippocampal CA3 region. VD significantly decreased Bcl-2 expression and increased Bax protein expression. PGE up-regulated Bcl-2 and down-regulated Bax, suggesting that the herbal medicine inhibits apoptosis, and therefore might have therapeutic potential for ischemic brain injury.

In summary, PGE possesses a potent neuroprotective activity against brain damage in VD. The ability of PGE to alleviate learning and memory deficits suggests a multifactorial mechanism that likely involves modulating neurotransmitter levels, protecting cerebral vessels from damage, promoting angiogenesis, and inhibiting apoptosis. These processes ultimately stimulate repair mechanisms that alleviate brain damage. Therefore, PGE may be a novel promising alternative medicine for the treatment of VD. However, further studies are needed to clarify the mechanisms underlying its neuroprotective effects.

Author contributions: ZSK and JDZ conceived and designed this study. JDZ, JJW, XHZ, YY, and ZSK performed the experiments. ZSK analyzed the data. JJW and XHZ provided reagents/materials/analysis tools. JDZ wrote the paper. All authors approved the final version of the paper.

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