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Gypenoside attenuates white matter lesions induced by chronic cerebral hypoperfusion in rats

Guanglin Zhang [a,b,1], Zhenwei Zhao [a,1], Li Gao [a,1], Jianping Deng [a], Benhan Wang [b], Dunquan Xu [a], Bolin Liu [c], Youzhi Qu [a], Jia Yu [a], Jian Li [a], Guodong Gao [a,*]

[a] Institute for Functional Neurosurgery P.L.A and Institute for Functional Brain Disorders, Tang Du Hospital, The Fourth Military Medical University, Xi'an 710038, PR China
[b] Department of Neurosurgery, 153rd Hospital of P.L.A, Zhengzhou 450042, PR China
[c] Department of Neurosurgery, Xijing Institute of Clinical Neuroscience, Xijing Hospital, The Fourth Military Medical University, Xi'an 710038, PR China

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ABSTRACT

Cerebral white matter lesions (WMLs) are frequently observed in vascular dementia and Alzheimer's disease and are believed to be responsible for cognitive dysfunction. The cerebral WMLs are most likely caused by chronic cerebral hypoperfusion and can be experimentally induced by permanent bilateral common carotid artery occlusion (BCCAO) in rats. Previous studies found the involvement of oxidative stress and astrocytic activation in the cerebral WMLs of BCCAO rats. Gypenoside (GP), a pure component extracted from the *Gyrostemma pentaphyllum* Makino, a widely reputed medicinal plants in China, has been reported to have some neuroprotective effects via anti-oxidative stress and anti-inflammatory mechanisms. In the present study, we investigated the protective effect of GP against cerebral WMLs and the underlying mechanisms for its inhibition of cognitive decline in BCCAO rats. Adult male Sprague–Dawley rats were orally administered daily doses of 200 and 400 mg/kg GP for 33 days after BCCAO, and spatial learning and memory were assessed using the Morris water maze. Following behavioral testing, oxygen free radical levels and antioxidative capability were measured biochemically. The levels of lipid peroxidation and oxidative DNA damage were also assessed by immunohistochemical staining for 4-hydroxynonenal and 8-hydroxy-2′-deoxyguanosine, respectively. Activated astrocytes were also assessed by immunohistochemical staining and Western blotting with GFAP antibodies. The morphological changes were stained with Klüver–Barrera. Rats receiving 400 mg/kg GP per day performed significantly better in tests for spatial learning and memory than saline-treated rats. GP 400 mg/kg per day were found to markedly scavenge oxygen free radicals, enhance antioxidant abilities, decrease lipid peroxide production and oxidative DNA damage, and inhibit the astrocytic activation in corpus callosum and optic tract in BCCAO rats. However, GP 200 mg/kg per day had no significant effects. GP may have therapeutic potential for treating dementia induced by chronic cerebral hypoperfusion and further evaluation is warranted.

1. Introduction

Alzheimer's disease (AD) and vascular dementia (VD), which are also vascular dysfunction diseases (Liu et al., 2007), are the two primary types of dementia prevalent in the elderly population (Royall, 2002; Gunstad et al., 2005). Cerebral white matter lesions (WMLs), a neurodegenerative condition characterized by hyper-intense signals on magnetic resonance images, are frequently linked with AD and VD and are responsible for the cognitive dysfunction of the elderly (Burton et al., 2004). Chronic cerebral hypoperfusion is most likely the cause of such cerebral WMLs, given that cerebral blood flow was found to decrease in these patients (de la Torre, 2004). Permanent bilateral common carotid arteries occlusion (BCCAO) in rats is a suitable experimental model of chronic cerebral hypoperfusion, and it can successfully imitate pathophysiologic features of human AD and VD (Shibata et al., 2004; Farkas et al., 2007).

The cerebral white matter consists of lipid-rich contents of the myelin sheath, which is a massive source of reactive oxygen and nitrogen species (Sommani et al., 2007). Previous studies have demonstrated that chronic cerebral hypoperfusion can induce the generation of reactive oxygen and nitrogen species, as well as the activation of inflammatory glial cells (Wang et al., 2007). These reactive species and inflammatory glial cells are toxic to brain tissues, which cause particularly severe damage to the white matter (Watanabe et al., 2006). Thus, attenuating the oxidative stress and

* Corresponding author at: Institute for Functional Neurosurgery P.L.A and Institute for Functional Brain Disorders, Tang Du Hospital, The Fourth Military Medical University, Xinsi Road, Baqiao District, Xi'an 710038, PR China. Tel./fax: +86 29 84777435.
E-mail address: zglin127s@163.com (G. Gao).
[1] These authors contributed equally to this work.

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suppressing the activation of inflammatory glial cells are potential approaches for treating cerebral WMLs and alleviating the cognitive impairment.

The Gypenoside (GP), C54H92O23 (Fig.1), is extracted as a pure component from the Chinese folk medicine of *Gyrostemma pentaphyllum* Makino. Previous pharmacological studies have demonstrated that GP possesses a series of protective effects, such as anti-inflammatory (Attawish et al., 2004), anti-oxidative stress (Wang et al., 2006), and anti-neuronal apoptosis effects (Wei et al., 2005). Additionally, GP is being evaluated in clinical practice to treat some neurological diseases such as stroke and traumatic brain injury in China. The results of these studies strongly suggest that GP may have therapeutic potential as a treatment for cognitive impairment caused by chronic cerebral hypoperfusion. Moreover, although GP can exert some neuroprotective activities under pathophysiological conditions, the precise underlying mechanisms are not fully understood. The present study was aimed at determining the potential protective effects of GP on cerebral WMLs and the related mechanisms for its alteration of cognitive function.

2. Materials and methods

2.1. Animals and surgical preparation

All animal experiments were approved by the Animal Care and Use Committee of the Fourth Military Medical University and complied with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). Adult male Sprague–Dawley rats weighing 250–300 g were used in this study (Experimental Animal Center, the Fourth Military Medical University). The animals used in this study were group-housed in our animal room maintained at 23 ± 1 °C with a 12 h light/dark cycle, and they were allowed free access to water and food. The rats were acclimatized to housing conditions for at least 4 d before use. The chronic cerebral hypoperfusion model was induced as previously described (Farkas et al., 2007). Briefly, rats were anesthetized using sodium pentobarbital (50 mg/kg, ip). A ventral midline incision was made, and the bilateral common carotid arteries were exposed and gently separated from the carotid sheath and vagus nerve. In the rats assigned to the ischemic groups, each artery was double ligated with a 4–0 silk suture and cut between the ligations. During the surgical procedure, the body temperature of each rat was kept stable at 37.5 ± 0.5 °C using a heating pad. The sham-operated control group received the same operation without ligation.

2.2. Drug preparation and experimental design

GP (colorless powder, purity>99%) was obtained from the Department of Phytochemistry, Shanghai Institute of Materia Medica.A total of 57 rats were randomly assigned to four groups: sham-operated animals administered with saline (sham-operated group, $n = 12$), BCCAO animals administered with saline (BCCAO + saline group, $n = 15$), BCCAO animals administered with 200 mg/kg GP per day (BCCAO + GP200 group, $n = 15$), and BCCAO animals administered with 400 mg/kg GP per day (BCCAO + GP400 group, $n = 15$). The dosage volume of all groups was 10 ml/kg. At 3 h post-surgery, all rats orally received the initial administration of 400 mg/kg or 200 mg/kg GP dissolved in saline solution or a matched volume of normal saline according to the above-mentioned experimental plan every 24 h up to 33 days afterwards. The animals were tested in the Morris water maze task from day 29 to day 33 after surgery, and the brain tissues were harvested after the behavioral testing.

2.3. Morris water maze

Spatial learning and memory were evaluated using the Morris water maze task (Morris, 1984). The apparatus is consisted of a circular water

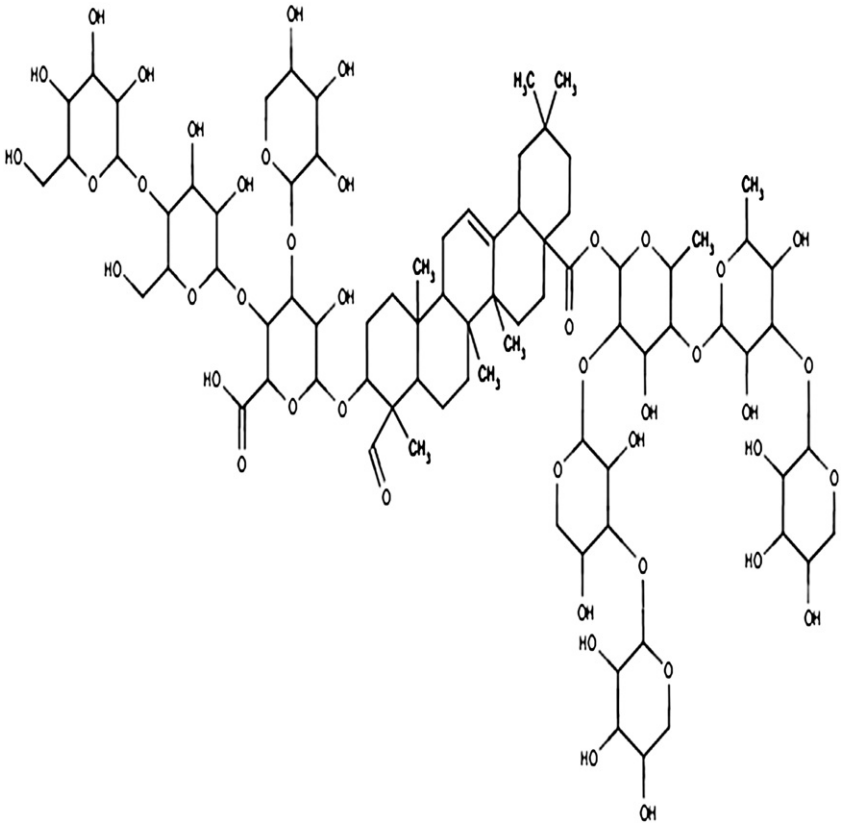


Fig. 1. Chemical structure of Gypenoside.

tank 120 cm in diameter and 50 cm in height, and a platform, 9 cm in diameter and 30 cm in height. The top of the platform was approximately 1.5 cm below the surface of the water. The temperature of the swimming pool water was maintained at 23 ± 2 °C. To make the water opaque, 1 kg of powdered milk was added. Each rat performed two trials per day for five consecutive days. Rats were randomly placed into the pool from each quadrant, facing the wall of the pool. Swimming paths of the rats were monitored by a video camera linked to a computer through an image analyzer. For each training trial, the latency to escape onto the hidden platform and the path length were recorded. The rats were given a maximum of 60 s to find the hidden platform. If the rat failed to find the platform within 60 s, the training was terminated and a maximum score of 60 s was assigned. The rat was then guided to the hidden platform by hand, and it was allowed to stay on the platform for 20 s before being removed from the water. A probe test, in which the hidden platform was removed, was conducted immediately after the last trial on training day 5. The rats were released into the water from the opposite quadrant with respect to the training quadrant. The rats were allowed to swim for 60 s in the pool before they were removed from the water. The time spent in the target quadrant where the platform had been set during training was recorded. The training in the visible platform was performed after spatial training was completed. Rats were given two trials per day similar to those described above for the hidden platform trial, except that the escape platform was elevated 2 cm above the water surface. The experimenter conducting the Morris water maze task was blinded to the treatment groups.

2.4. Biochemical examinations

Following the behavioral testing, six or eight rats from each group were randomly chosen to be decapitated under anesthesia. After the rats were sacrificed, the brain corpus callosum and optic tract were removed on ice. The tissues were rapidly frozen and stored at 80 °C until assays were performed. Brain tissues were homogenized, and the supernatant was used to spectrophotometrically determine the level of malondialdehyde (MDA) and the activity of superoxide dismutase (SOD) according to the procedure provided with the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, P.R. China). MDA, the degradation product of preoxidation lipids, reacts with thiobarbituric acid to form a pink chromogen that can be assayed by the method of Buege and Aust (1978). The MDA level reflects the degree of cell destruction by free radicals. The SOD activity was assessed using the procedure of Kakkar et al. (1984). The SOD activity represents the capability of cells to clear free radicals.

2.5. Western blotting analysis

Western blotting assays were performed as previously described (Guo et al., 2008). The corpus callosum and optic tract tissues were dissected and homogenized in T-PER buffer containing protease inhibitors. After homogenization, aliquots containing 50 μg of protein were subjected to 12% SDS-PAGE. The protein bands were transferred onto 0.22 μM nitrocellulose filter membranes (Millipore, Bedford, USA), which were incubated with GFAP antibodies diluted (1:500; Boster, Wuhan, P.R. China) in blocking solution overnight at 4 °C, followed by incubation with secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-mouse, 1:5000 for GFAP. Blots were visualized using the ECL system (BestBio Inc, Shanghai, China). Equal protein loading was confirmed by measuring α-tubulin.

2.6. Immunohistochemical assay

Following the behavioral experiments, six or seven rats from each group were randomly chosen, anesthetized with sodium pentobarbital (50 mg/kg, ip), and perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were rapidly removed, fixed in 4% paraformaldehyde for 48 h, and embedded in paraffin. Coronal brain sections (5 μm) were obtained. 0.09pt?>Sections were immunohistochemically stained for 4-hydroxynonenal (4-HNE) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) to identify lipid peroxidation and DNA damage, respectively. Briefly, the slides were placed in 0.3% H_2O_2 in methanol for 11 min at room temperature to quench endogenous peroxidase activity. After blocking nonspecific reactions with bovine serum albumin, the slides were incubated with goat monoclonal anti-4-HNE (1:500; Millipore, U.S.A) or goat monoclonal anti-8-OHdG (1:200; Abcom, UK.) overnight at 4 °C. The slides were washed with PBS three times for 20 min and incubated with diluted biotinylated secondary antibody for 1 h at room temperature. Then, the sections were incubated overnight in a solution containing the rabbit anti-GFAP primary antibody (1:500; Boster, Wuhan, P.R. China) at 4 °C. Slides were then washed again with PBS three times for 20 min and incubated with diluted biotinylated secondary antibodies for 1 h at room temperature. The color reaction was developed conventionally with DAB and H_2O_2. In each study, a set of sections was stained simultaneously in a similar way without the primary antibody as a negative control. The 4-HNE, 8-OHdG and GFAP positive cells of the corpus callosum (0.26 mm posterior to bregma and 0.16 mm lateral to midline) and optic tract (0.5 mm posterior optic chiasma) area were counted at 400× magnification in five continuous visual fields from each animal. The number of positive cells per mm^2 in each rat was recorded as the mean of the three coronal sections. An Olympus microscope with a video camera system linked to a computer was used to obtain digitized images. The researcher evaluating the histology results was blinded to the treatment group of the rats.

2.7. Klüver–Barrera staining

Sections of the corpus callosum and optic tract were treated with Klüver–Barrera staining. The myelin areas of both sides in three sections per animal were stained with Luxol Fast Blue. The severity of white matter changes was graded as 0 (normal), grade 1 (disarrangement of the nerve fibers), grade 2 (formation of marked vacuoles), or grade 3 (disappearance of myelinated fibers) by two independent investigators blinded to the treatment groups (Wakita et al., 2002; Ueno et al., 2009).

2.8. Statistical analysis

The results are expressed as mean ± S.E.M. Differences in the escape latency in the Morris water maze were analyzed statistically using analysis of General Linear Model with repeated measures followed by the Bonferroni test using SPSS16.0. The other data were analyzed by one-way ANOVA followed by the Tukey test using SPSS16.0. Statistically significant difference were defined at $P<0.05$.

3. Results

3.1. GP reduces axonal damage

Klüver–Barrera staining showed increased number of vacuoles and demyelinated fibers in the corpus callosum and optic tract in the rats of the hypoperfused saline group and GP 200 mg/kg group. However, fewer vacuoles and demyelinated fibers were found in the GP 400 mg/kg group (Fig. 2A). As shown in Fig. 2B, there was a significant reduction in the grading score of WMLs in the GP 400 mg/kg group compared to the hypoperfused saline group ($P<0.01$). However, the grading score of WMLs of the GP 200 mg/kg group did not differ significantly from the hypoperfused saline group ($P>0.05$).

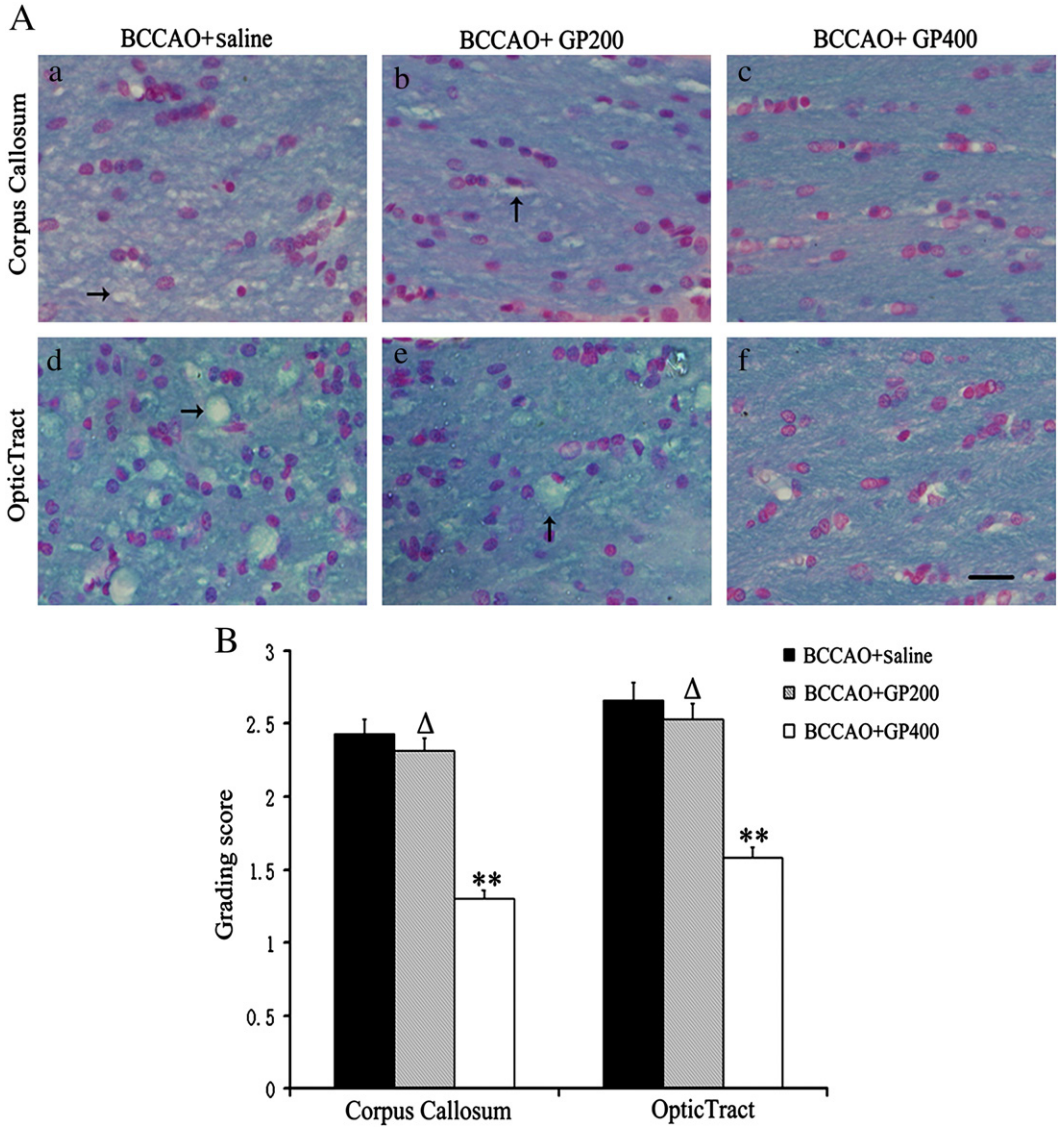


Fig. 2. Effect of GP on morphological changes in the corpus callosum and optic tract induced by chronic cerebral hypoperfusion. (A) Representative photomicrographs of Klüver–Barrera staining in the corpus callosum and optic tract after chronic cerebral hypoperfusion and treatment. Scale bar = 40 μm. Magnification 400×. (B) The histogram of the grading scores for the corpus callosum and optic tract of each group of rats. Values are expressed as mean ± S.E.M. sham-operated group, $n = 6$; BCCAO + saline group, $n = 7$; BCCAO + GP200 group, $n = 7$; BCCAO + GP400 group, $n = 7$. $^{\Delta}P > 0.05$ vs. BCCAO + saline group; $^{**}P < 0.01$ vs. BCCAO + saline group. The red staining is the nuclei of glial cells. The arrows point to the vacuolations in the corpus callosum and optic tract.

3.2. GP attenuates the oxidative damage induced by BCCAO

SOD is an important antioxidant enzyme that can protect brain tissues from oxidative stress damage. MDA, a product of lipid peroxidation, is used as an index of membrane oxidative injury. After testing cognitive function, the corpus callosum and optic tract of rats were separated to measure SOD and MDA levels (Figs. 3 and 4). As shown in Fig. 3, the SOD activity in the hypoperfused saline group rats was reduced after chronic cerebral hypoperfusion compared to sham-operated rats ($P < 0.01$). Meanwhile, the levels of MDA were shown in Fig. 4. The MDA content was significantly increased in the hypoperfused saline group compared to the sham-operated group ($P < 0.01$), suggesting that chronic cerebral hypoperfusion could induce oxidative injury. Compared with the hypoperfused saline group, repeated administration of 400 mg/kg GP per day significantly enhanced SOD activity and decreased MDA levels in the corpus callosum and optic tract ($P < 0.01$), whereas the administration of 200 mg/kg GP per day had modest effect, which did not differ significantly from the hypoperfused saline group ($P > 0.05$).

3.3. GP attenuates lipid peroxidation and DNA damage

Lipid peroxidation and oxidative DNA were assessed by immunostaining using anti-HNE and anti-8-OHdG antibodies. Immunohistochemical analysis of 8-OHdG and 4-HNE was used to determine the extent of oxidative DNA damage and lipid peroxidation, respectively (Imai et al., 2001; Deguchi et al., 2008). Positive staining for 4-HNE is seen in the neuronal perikarya and axons, whereas 8-OHdG positive staining predominantly occurs in the nuclei. As shown in Figs. 5 and 6, there were a number of 4-HNE and 8-OHdG positive cells in the hypoperfused saline group after chronic cerebral hypoperfusion. Chronic administration of 400 mg/kg GP per day significantly decreased the number of 4-HNE and 8-OHdG-stained cells in the corpus callosum and optic tract compared to the hypoperfused saline group ($P < 0.01$). Chronic administration of 200 mg/kg GP per day also decreased the numbers of 4-HNE and 8-OHdG-stained cells in the corpus callosum and optic tract, although the, differences were not statistically significant when compared to the hypoperfused saline group ($P > 0.05$).

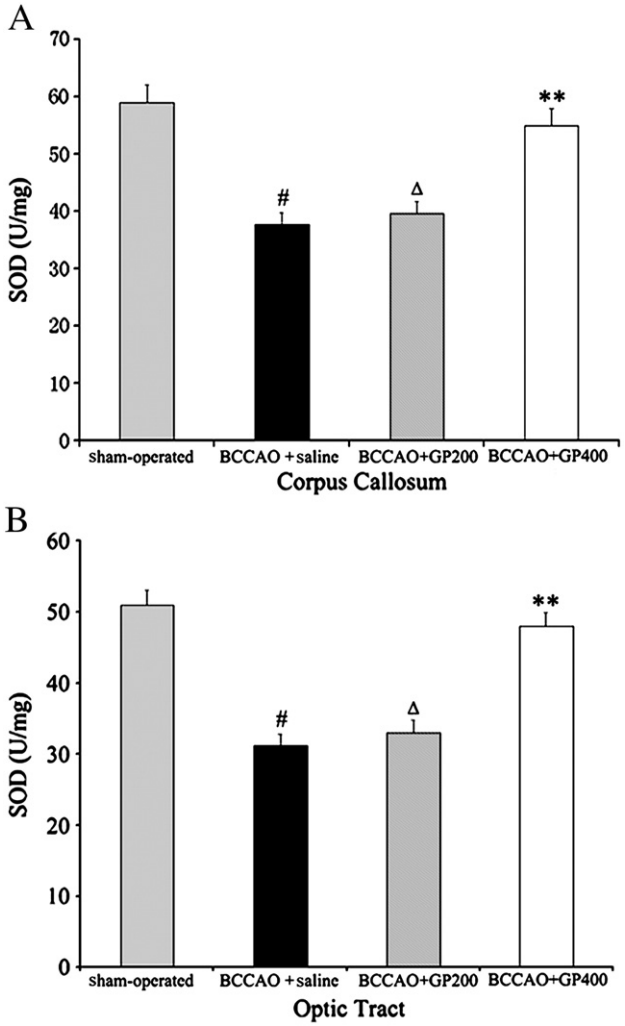


Fig. 3. Effect of GP on SOD levels in the corpus callosum (A) and optic tract (B) of rats with chronic cerebral hypoperfusion. Values are expressed as mean ± S.E.M. sham-operated group, $n = 6$; BCCAO + saline, $n = 8$; BCCAO + GP200 group, $n = 8$; BCCAO + GP400 group, $n = 8$. $P < 0.01$ vs. sham-operated group; $^{**}P < 0.01$ vs. BCCAO + saline group; $^{\Delta}P > 0.05$ vs. BCCAO + saline group.

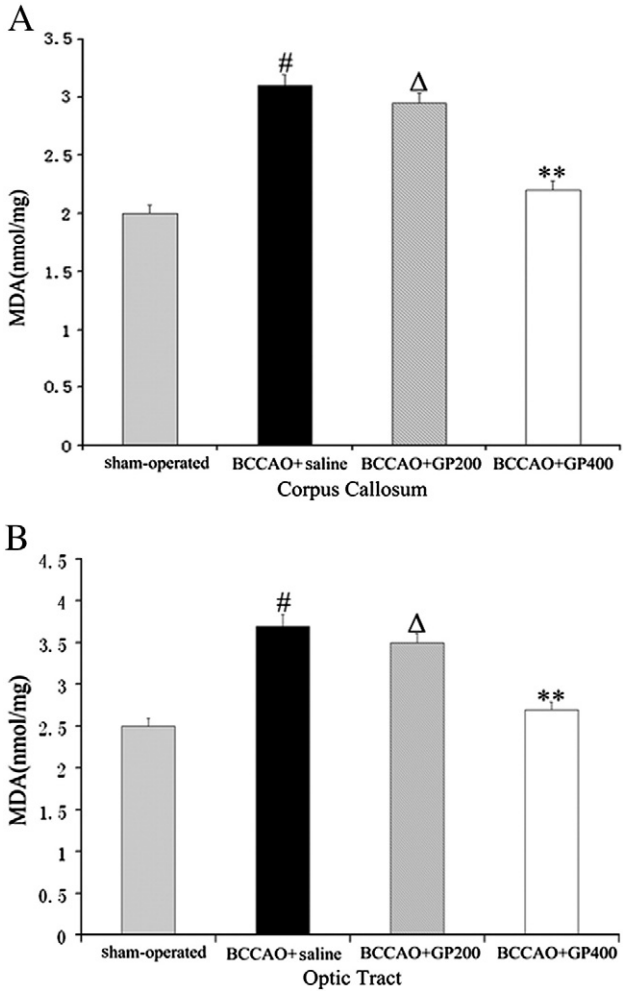


Fig. 4. Effect of GP on MDA levels in the corpus callosum (A) and optic tract (B) of rats with chronic cerebral hypoperfusion. Values are expressed as mean ± S.E.M. sham-operated group, $n = 6$; BCCAO + saline, $n = 8$; BCCAO + GP200 group, $n = 8$; BCCAO + GP400 group, $n = 8$. $P < 0.01$ vs. sham-operated group; $^{**}P < 0.01$ vs. BCCAO + saline group; $^{\Delta}P > 0.05$ vs. BCCAO + saline group.

3.4. GP attenuates the activation of inflammatory astrocytes

The anti-inflammatory effects of GP were reflected by quantitative analysis of the expression of GFAP (a marker of activated astrocytes) in the corpus callosum and optic tract, which were examined by immunohistochemistry and Western blotting. Fig. 7 showed the results of effects of GP on the activation of inflammatory astrocytes. As shown in Fig. 7A, GFAP-positive astrocytes in the corpus callosum and optic tract were rare in the sham-operated rats, but were markedly increased after chronic cerebral hypoperfusion ($P < 0.001$); and the GFAP content in the hypoperfused saline group was significantly higher than that in the sham-operated group ($P < 0.001$, Fig. 7B), suggesting that chronic cerebral hypoperfusion could induce the activation of astrocytes. However, the expression levels of activated astrocytes in the GP 200 and 400 mg/kg group were significantly lower than that in the hypoperfused saline group ($P < 0.05$ and $P < 0.01$, respectively). Chronic administration of 400 and 200 mg/kg GP per day significantly reduced the activation of astrocytes in the corpus callosum and optic tract.

3.5. GP improves spatial learning and memory deficits induced by BCCAO

The Morris water maze task was carried out for five consecutive days to investigate the spatial learning ability of the rats treated with GP. In the hidden platform trial, shown in Fig. 8A, the escape latencies for four groups were evaluated. On the first two days of the trial, the escape latencies did not differ significantly between the groups. On day 3, rats in the hypoperfused saline group took significantly longer time to find the platform than the sham-operated rats ($P < 0.01$), suggesting that chronic cerebral hypoperfusion significantly induced learning deficits in the BCCAO model rats. The escape latencies of rats receiving 200 mg/kg GP per day were shorter but not significantly different from that of the hypoperfused saline group rats ($P > 0.05$) on day 3 and 4. The rats treated with 400 mg/kg GP per day spent significantly less time in finding the platform than the hypoperfused saline group rats on day 3 ($P < 0.01$). In the probe trials, shown in Fig. 8B, memory was evaluated by analyzing the percentage of time spent in the target quadrant where the hidden platform had previously been available. The times that the hypoperfused saline group rats stayed in the platform region were notably shorter than that of the sham-operated rats ($P < 0.01$), suggesting that chronic cerebral hypoperfusion also significantly induced memory deficits in the BCCAO rats. Chronic administration of 400 mg/kg GP per day significantly increased the amount of time of the rats spent in the platform region compared with the hypoperfused saline group ($P < 0.01$). However, the group chronically administered 200 mg/kg GP per day failed to show a significant increase of time spent in the platform compared to the hypoperfused saline group ($P > 0.05$). Analysis of the animal performance in the visible

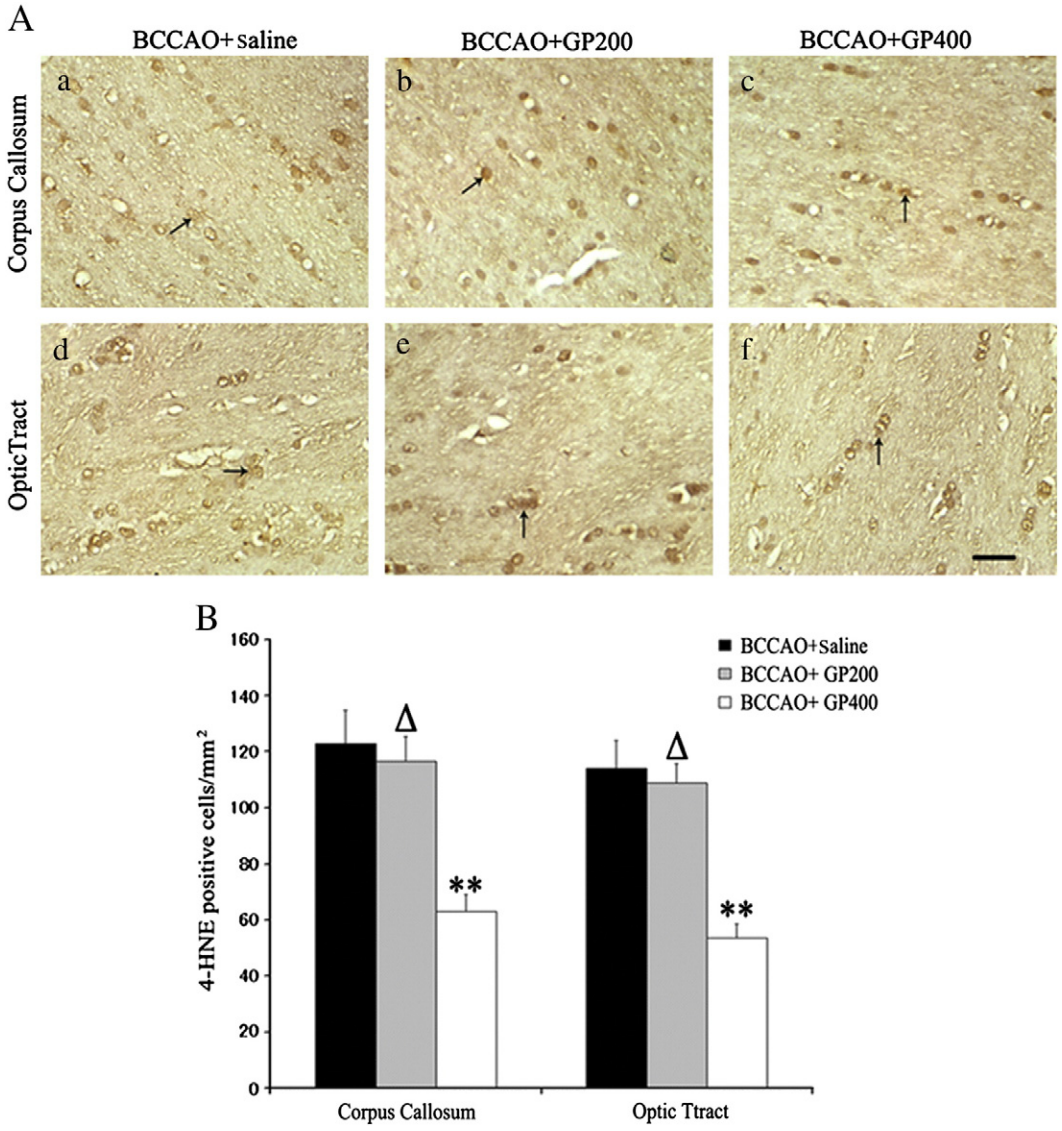


Fig. 5. Effect of GP on lipid peroxidation induced by chronic cerebral hypoperfusion. (A) Representative photomicrographs of 4-HNE immunohistochemical staining in the corpus callosum and optic tract after chronic cerebral hypoperfusion and treatment. Scale bar = 40 μm. Magnification 400×. (B) The quantitative number of 4-HNE-positive cells in the corpus callosum and optic tract. Values are expressed as mean ± S.E.M. sham-operated group, $n = 6$; BCCAO + saline group, $n = 7$; BCCAO + GP200 group, $n = 7$; BCCAO + GP400 group, $n = 7$. $^{**}P < 0.01$ vs. BCCAO + saline group; $^{\Delta}P > 0.05$ vs. BCCAO + saline group.

platform training showed that all rats reached the platform in a short period of less than 30 s, and there was no difference between any two groups, indicating that the difference in spatial performance between groups is unlikely to be associated with visual impairments.

4. Discussion

In the present study, we explored the protective mechanisms of GP against the WMLs induced by chronic cerebral hypoperfusion and the beneficial effects of GP 200 and 400 mg/kg per day on cognitive function. Chronic administration of 400 mg/kg GP per day resulted in significantly increased scavenging of oxygen free radicals, enhanced antioxidant abilities, decreased the lipid peroxide products and oxidative DNA damage and reduced the activation of astrocytes in the corpus callosum and optic tract of rats with chronic cerebral hypoperfusion. All of these changes were associated with functional improvement in water maze performance.

Previous studies demonstrated that chronic cerebral hypoperfusion can induce cerebral WMLs (Nakaji et al., 2006). Other studies found a direct correlation between the cerebral WMLs induced by chronic cerebral hypoperfusion and the memory deficits (Dohmen et al., 2005). The cerebral WMLs occurs in Alzheimer's disease, vascular dementia, and Binswanger's disease and can be caused by combinations of vascular disease and hypoperfusion in the most distal vascular territories (Kim et al., 2007; Iadecola, 2004). On the other hand, it was shown that the white matter areas have varying degrees of vulnerabilities to chronic cerebral hypoperfusion, with the corpus callosum and optic tract being most easily injured (Wakita et al., 2002; Takamatsu et al., 1984). A number of axonal mechanisms of ischemic cerebral WMLs have been clearly shown by extensive studies (Goldberg and Ransom, 2003; Stys, 2004). The BCCAO rat can reproduce the condition of chronic cerebral hypoperfusion, which is regarded as a suitable model for studying the pathogenesis of cerebral WMLs (Kim et al., 2008). The BCCAO technique can decrease the blood flow in the white matter areas, which is closely associated with the lesions of the corpus callosum and optic tract (Nakaji et al., 2006). These cerebral blood flow reductions persist in white matter regions were much longer than in grey matter (Otori et al., 2003), and the impeded cerebral blood flow ultimately results in delayed WMLs and memory impairment (Sarti et al., 2002a). To better investigate the delayed cerebral WMLs and memory deficits, we

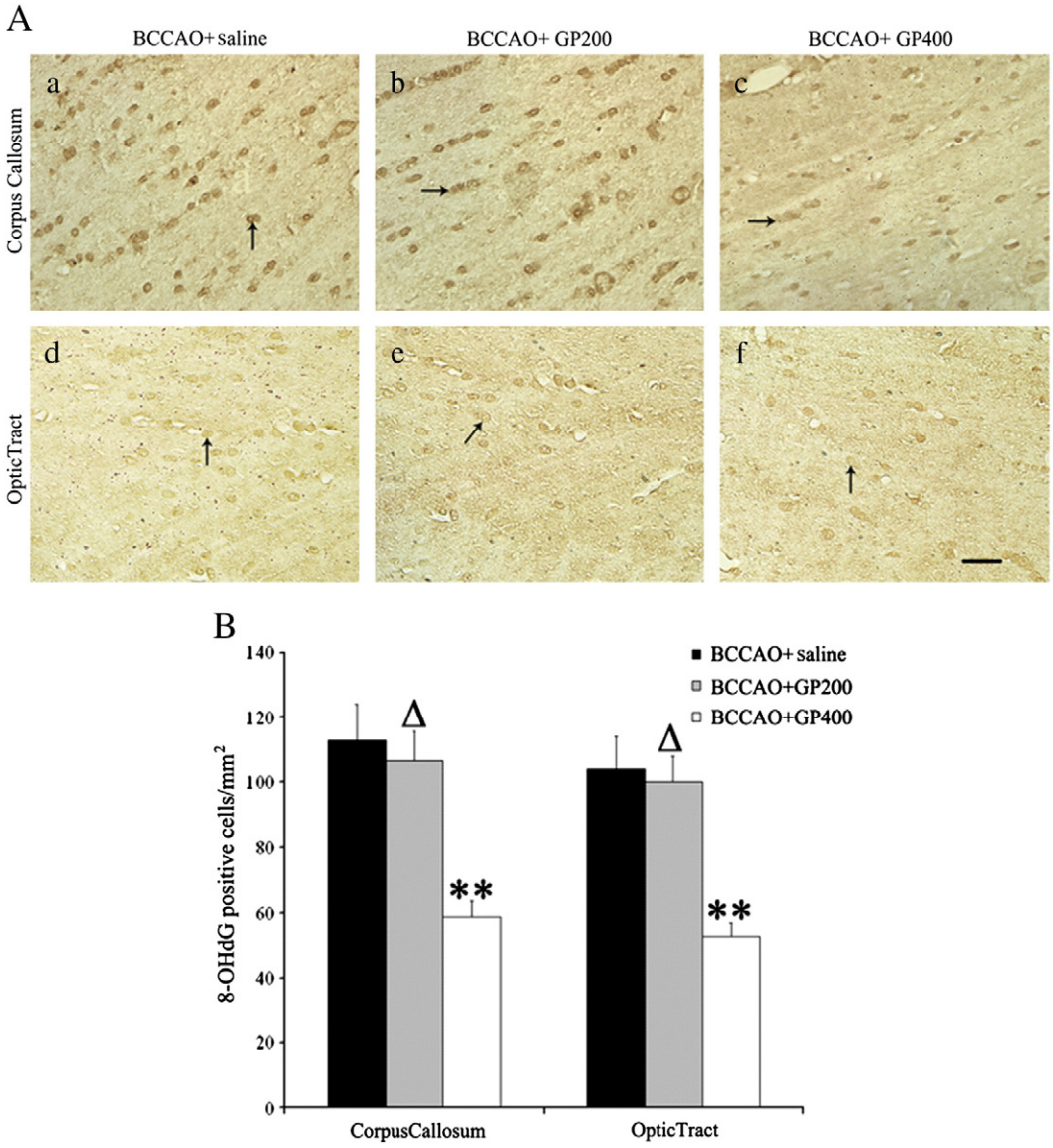
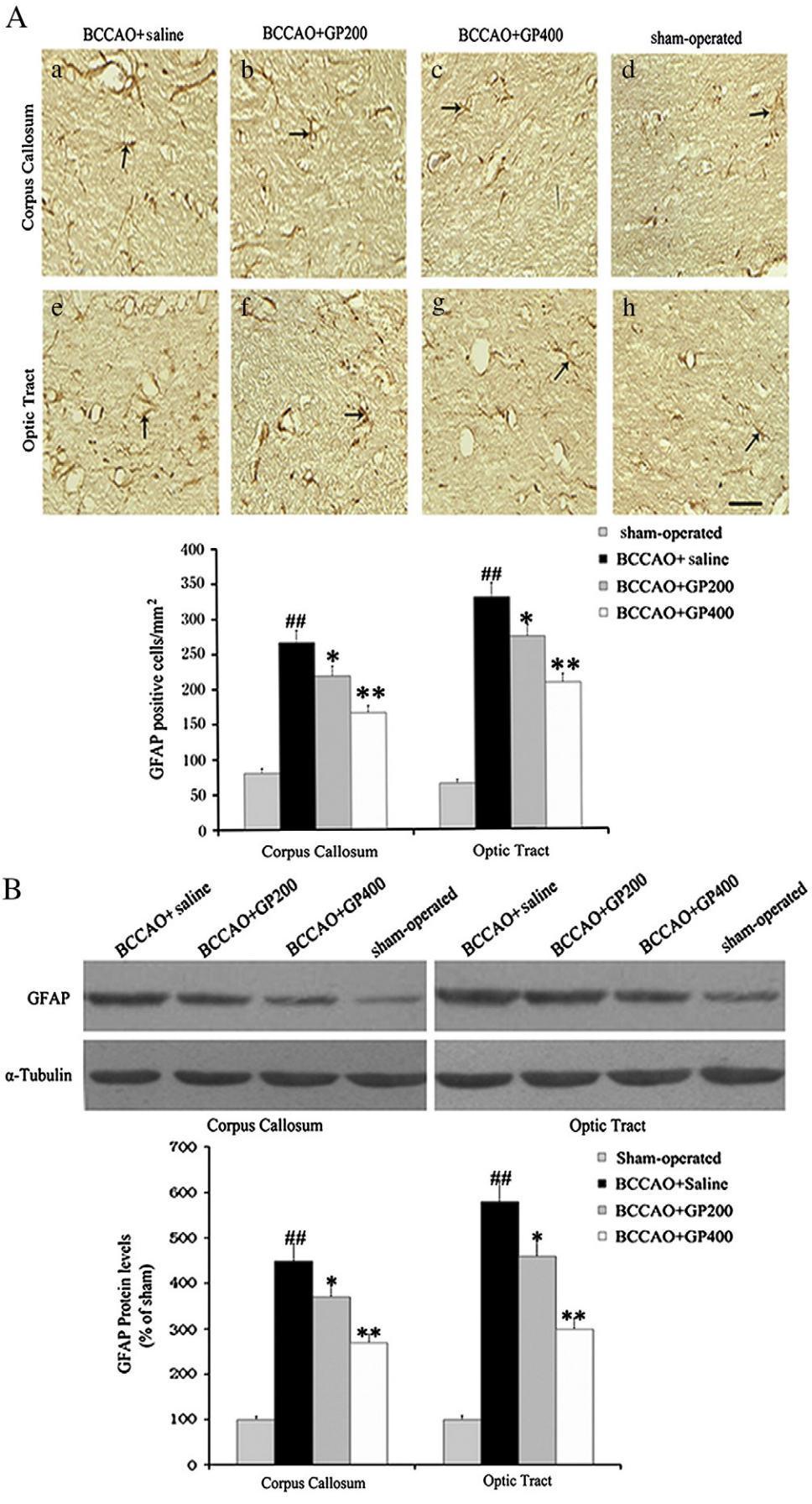


Fig. 6. Effect of GP on oxidative DNA damage induced by chronic cerebral hypoperfusion (A) Representative photomicrographs of 8-OHdG immunohistochemical staining in the corpus callosum and optic tract after chronic cerebral hypoperfusion and treatment. Scale bar = 40 μm. Magnification 400×. (B) The quantitative number of 8-OHdG-positive cells in the corpus callosum and optic tract. Values are expressed as mean ± S.E.M. sham-operated group, $n=6$; BCCAO + saline group, $n=7$; BCCAO + GP200 group, $n=7$; BCCAO + GP400 group, $n=7$. $^{**}P<0.01$ vs. BCCAO + saline group; $^{\Delta}P>0.05$ vs. BCCAO + saline group.

administered 200 and 400 mg/kg GP per day to BCCAO rats for 33 days to investigate its therapeutic effects on cognitive impairment.

During the period of chronic cerebral hypoperfusion, the pathological changes of the brain are closely associated with oxidative stress (Chong et al., 2005). It is well known that excess free radicals damage the weakened antioxidant defense system of the brain, resulting in oxidative damage in human AD and VD (Almkvist and Tallberg, 2009; Hamel et al., 2008). In previous studies, the activity of SOD was found to be decreased after chronic cerebral hypoperfusion (Peng et al., 2007), but the diminished SOD only accounted partly for the accumulation of lipid hydrogen-peroxides (Huang et al., 2008). MDA is produced by free radical-catalyzed peroxidation of unsaturated fatty acids in the cell membrane, and its level reflects the degree of cell destruction by free radicals. The lipid peroxidation and cytotoxic byproduct, 4-HNE, can be used as a marker for oxidative neuronal damage (McCracken et al., 2000; Watanabe et al., 2006), and 8-OHdG is a hallmark of oxidative DNA damage caused by direct attacks by hydroxyl radicals (Cheng et al., 1992). By using these classic markers in the present study, we found that the levels of MDA, 4-HNE and 8-OHdG were markedly increased in the corpus callosum and optic tract of BCCAO rats. Chronic administration of 400 mg/kg GP per day significantly lessened free radical damage to cells, enhanced

Fig. 7. The expression of GFAP in the corpus callosum and optic tract. The corpus callosum and optic tract were analyzed by immunohistochemistry and Western blotting. (A) Representative photomicrographs of GFAP immunohistochemical staining and the quantitation of GFAP-positive astrocytes in the corpus callosum and optic tract. Scale bar = 40 μm. Magnification 400×. (B) Western blotting analysis of GFAP expression. The relative amount of GFAP was expressed as percentage of sham-operated group value, which is set to 100%. Values are expressed as mean ± S.E.M. sham-operated group, $n=6$; BCCAO + saline group, $n=7$; BCCAO + GP200 group, $n=7$; BCCAO + GP400 group, $n=7$. $^{\#\#}P<0.001$ vs. sham-operated group; $^{*}P<0.05$ vs. BCCAO + saline group; $^{**}P<0.01$ vs. BCCAO + saline group.


A
BCCAO+saline
BCCAO+GP200
BCCAO+GP400
sham-operated
Corpus Callosum
Optic Tract
a
b
c
d
e
f
g
h
sham-operated
BCCAO+saline
BCCAO+GP200
BCCAO+GP400
GFAP positive cells/mm²
400
350
300
250
200
150
100
50
0
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*
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Corpus Callosum
Optic Tract
B
BCCAO+saline
BCCAO+GP200
BCCAO+GP400
sham-operated
GFAP
α-Tubulin
Corpus Callosum
Optic Tract
Sham-operated
BCCAO+Saline
BCCAO+GP200
BCCAO+GP400
GFAP Protein levels (% of sham)
700
600
500
400
300
200
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0
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Corpus Callosum
Optic Tract

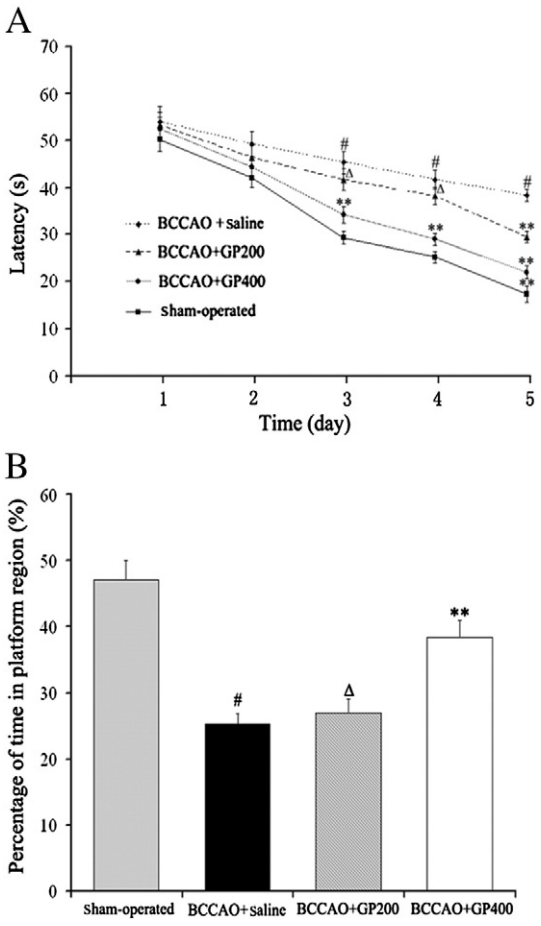

Fig. 8. Effect of GP on chronic cerebral hypoperfusion-induced deficits in spatial learning and memory as measured by the Morris water maze. (A) Change in the daily escape latency time. (B) The percentage of time in the platform region in the probe trial without the platform (day 5). Values are expressed as mean ± S.E.M. $P<0.01$ vs. sham-operated group; $^{**}P<0.01$ vs. BCCAO + saline group; $^{\Delta}P>0.05$ vs. BCCAO + saline group.

antioxidant abilities and reduced lipid peroxidation products and oxidative DNA damage in the corpus callosum and optic tract after chronic cerebral hypoperfusion. Chronic administration of 200 mg/kg GP per day had no significant effects.

Previous studies have showed that the activation of glial cells is closely associated with the subsequent development of cerebral WMLs and that the pharmacological suppression of inflammatory glial cells results in the attenuation of cerebral WMLs (Goldberg and Ransom, 2003; Wada-Isoe et al., 2004). The cellular basis for neuroinflammation would be activated microglia cells and astrocytes, which are also the major producers of inflammatory mediators (Koistinaho and Koistinaho, 2005; von Bernhardi, 2007). However, it was determined that relatively less activation of microglial is detected and largely limited to the initial observation period in BCCAO rats, whereas activated astrocytes persisted in white matter regions for a much longer time (Plaschke et al., 2001). Astrocytes constitute the main population of glial cells in the brain, representing over 20%–30% of the total cell volume and play key roles in performing many central nervous system functions (Cai et al., 2010). The activated astrocytes can release proinflammatory factors, reactive oxygen species and reactive nitrogen species, leading to abnormal breakdown of molecules (Buskila et al., 2005; Liu et al., 2007). The activation of astrocytes has been closely correlated with reduced functional recovery of BCCAO rats (Badan et al., 2003; Vicente et al., 2009). Some studies suggested that the activated astrocytes play a dual role in the pathological evolution of AD and VD, including promoting damage and mediating neuroprotection (O'Callaghan and Sriram, 2005; von Bernhardi, 2007). The activated astrocytes can secrete neurotrophic factors at the lesion sites and may provide a substrate for axonal regrowth at the early stage after chronic cerebral hypoperfusion. However, at the later stages, a scar-specific astrocytic phenotype emerges and hinders axonal regrowth (Maragakis and Rothstein, 2006). Recently, it has been widely recognized that the control of the activation of astrocytes is likely to be an important therapeutic target (Tateishi et al., 2002; Ueno et al., 2009).

In the present study, we found that the numbers and expression levels of activated astrocytes in the corpus callosum and optic tract after chronic cerebral hypoperfusion were apparently increased. Administration of GP 200 and 400 mg/kg per day significantly decreased the numbers and content of activated astrocytes in the corpus callosum and optic tract in BBCAO rats. The results indicated that GP exerted a potent anti-inflammatory effect via inhibiting the activation of astrocytes. Meanwhile, morphological changes in the corpus callosum and optic tract were found after chronic cerebral hypoperfusion, which is important for evaluating brain tissues damage and drug action. The marked axonal damage was visualized in the hypoperfused saline group as vacuolation and disarrangement of the myelin fibers. GP 400 mg/kg per day markedly attenuated the axonal damage; however, GP 200 mg/kg per day did not show notable effects. Our results were consistent with the notion that histological abnormalities are indicative of the decreases in cognitive function such as learning, memory and spatial discrimination reported in previous studies (Watanabe et al., 2006; Farkas et al., 2007).

Our results suggest that GP has therapeutic potential for cognitive deficits caused by decreased cerebral blood flow and may serves as a potential anti-dementia agent. However, there are certain limitations in the evaluation of the therapeutic effects of GP on cognitive impairment in the current study. Besides the effects of anti-oxidative stress and anti-astrocytic activation discussed here, multiple mechanisms may be involved in the protective properties of GP, such as increased cerebral blood flow, facilitated neuron energy metabolism, and improved cholinergic neuron functions. Meanwhile, it has been demonstrated that GP can inhibit platelet aggregation and thrombus formation (Dong, 2006) and ameliorate mitochondrial failure (Yao et al., 2005). These actions may also help to explain how GP improves cognitive function. Further investigation is needed to explore the neuroprotective mechanisms underlying the effects of GP. Furthermore, the white matter alteration is often associated with damage of the hippocampal CA1 region, making it difficult to understand if cognitive alteration is a consequence of white matter damage, hippocampal CA1 damage or both (Sarti et al., 2002b). Therefore, the role of hippocampal CA1 damage in the beneficial effects of GP, especially in relation to WMLs needs verification.

In conclusion, our findings indicate that chronic administration of GP ameliorated oxidative damage, reduced the astrocytic activation in the corpus callosum and optic tract after chronic cerebral hypoperfusion, which may underlie the improvement of cognitive function. Therefore, GP may be an effective treatment of dementia induced by chronic cerebral hypoperfusion and should undergo further evaluation.

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