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Original Article

Effects of polydatin from Huhuang Shaoshang Liniment on oxidative damage and inflammatory response in rats with cerebral ischemia-reperfusion

Qiankun Sha[1*], Yan Huang[2*], Faying Zhou[3], Yong Wang[4], Wei Fang[5], Jing Li[6]

[1]Department of Pharmacy, Chongqing Yangdu Biology Institute, Chongqing 408000, China; [2]Department of Pharmacy, The People's Hospital of Nanchuan, Chongqing 408499, China; [3]Department of General Surgery, Qianjiang Central Hospital of Chongqing, Chongqing 409000, China; [4]Department of Dermatology, Qijiang District Traditional Chinese Medicine Hospital of Chongqing, Chongqing 401420, China; [5]Department of Pharmacy, Chongqing Sanxia Central Hospital, Chongqing 404100, China; [6]Department of Pharmacy, People's Hospital of Chongqing, Banan District, Chongqing 401320, China. [*]Equal contributors and co-first authors.

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Abstract: Objective: To analyze the effect of polydatin (PD) from Huhuang Shaoshang Liniment on oxidative damage and inflammatory response in rats with cerebral ischemia-reperfusion (CI/R), and the effect of PD on NF-E2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1)/NAD(P)H quinone oxidoreductase 1 (NQO1) pathway. Methods: The rat model of focal CI/R was established using suture-occlusion method and treated with intraperitoneal injection of PD (25 mg/kg and 50 mg/kg). High performance liquid chromatography (HPLC) was used to determine the PD content in three batches of Huhuang Shaoshang Liniment. The neurological function scores of each group were observed at 24 h, 48 h, and 72 h postoperatively. The hematoxylin and eosin (HE) staining was conducted to observe the morphological structure of brain tissue, the triphenyltetrazolium chloride (TTC) staining was used to determine the size of cerebral infarction, and the neuronal apoptotic index was calculated using image analysis system under the optical microscope. The expressions of the Nrf2/HO-1/NQO1 pathway and neuronal apoptosis-related proteins in brain tissue were measured using Western blot. Results: The PD content in three batches of Huhuang Shaoshang Liniment was detected by HPLC, and the average result showed that the product contained 0.73 mg PD per 1 mL. The PD 50 mg/kg group and 25 mg/kg group showed lower neurological function scores at 24 h, 48 h, and 72 h postoperatively, lower percentage of cerebral infarction area on the ischemic side and apoptotic index, lower interleukin (IL)-1β, IL-6, malondialdehyde (MDA), and lactate dehydrogenase (LDH) levels, and lower Bax protein expression ($P < 0.05$), and showed higher IL-4, IL-10, superoxide dismutase (SOD), glutathione peroxidase (GPx), Nrf2, HO-1, NQO1, and c-Myc protein expression than the CI/R group ($P < 0.05$). Conclusion: PD from Huhuang Shaoshang Liniment can alleviate neurological damage, improve neuronal morphology and structure, reduce cerebral infarction area, alleviate oxidative damage and inflammatory response, and inhibit neuronal cell apoptosis in CI/R model rats.

Keywords: Cerebral ischemia-reperfusion, polydatin, Nrf2/HO-1/NQO1 pathway, oxidative damage, inflammatory response

Introduction

Ischemic stroke is a reduction in blood flow to the brain caused by vascular obstruction and spasm, resulting in hypoxic-ischemic necrosis of brain tissue, which can be accompanied by varying degrees of hemiparesis, neurological impairment, aphasia, and functional loss [1, 2]. Cerebral ischemia-reperfusion injury (CIRI) is the primary pathological mechanism of injury in ischemic stroke, which refers to the failure of brain function, tissue and organ function to recover in time after the recovery of blood supply following ischemic episodes, but aggravates structural damage and dysfunction of organs and tissues [3, 4]. The mechanism of CIRI is complex and involves multiple factors such as oxidative stress damage, inflammatory response, and neuronal apoptosis. It has been found that CIRI can activate free radical chain reactions, cause excessive inflammatory responses, and aggravate the degree of cerebral

ischemic injury [5]. Therefore, the key to prevent and treat ischemic stroke is to clarify the molecular characteristics of CIRI and mitigate CIRI-mediated neural damage, inflammatory response, and oxidative stress injury.

It is confirmed that the NF-E2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1)/NAD(P)H quinone oxidoreductase 1 (NQO1) signaling pathway exerts an important part in protecting the body from oxidative stress [6, 7]. The nuclear transcription factor Nrf2 is activated by signals such as antioxidants and oxygen radicals, enters the nucleus, binds to antioxidant response element (ARE) on the nucleic acid sequence, initiates downstream antioxidant gene expression such as NQO1 and HO-1, inhibits oxidative stress response, improves cellular resistance to oxidative stress, and thus reduces oxidative stress injury [8]. Therefore, how to upregulate the Nrf2/HO-1/NQO1 signaling pathway is particularly important in alleviating oxidative stress response induced by CIRI.

Huhuang Shaoshang Liniment is an external preparation for burns and scalds, which has the functions of purging fire and detoxifying, cooling blood and promoting blood circulation, reducing swelling and relieving pain, and eliminating dampness and astringing sores. The authors of this study have applied the preparation in clinical wound healing, archiving good effects [9]. Huhuang Shaoshang Liniment is mainly composed of 8 traditional Chinese medicines, including Polygonum cuspidatum, Coptis coptidis, Phellodendri Chinensis Cortex, buffalo horn, Carthami Flos, Angelica dahurica, Climbing Groundsel Herb, and Borneol, among which Polygonum cuspidatum is the sovereign drug in this prescription, which plays the main curative effect. Therefore, it is of great significance to study the therapeutic effect of Polygonum cuspidatum for understanding the mechanism of Huhuang Shaoshang Liniment. Polydatin (PD) is the main active ingredient of Polygonum cuspidatum, the sovereign drug in Huhuang Shaoshang Liniment, which may exert a crucial effect in promoting blood circulation and removing stasis and improving local circulation of wound [10]. We speculate that PD may also improve blood circulation to brain tissue. According to literature review, PD has multiple pharmacological effects, including anti-apoptosis, anti-oxidation, anti-inflammation, free radical scavenging, anti-platelet aggregation, and can protect against brain tissue damage and reduce the levels of lipid peroxidation in rats with cerebral ischemia [11]. However, it is still unknown regarding the biological mechanism of oxidative damage and inflammatory response of PD in CIRI. To further elucidate the therapeutic efficacies and possible mechanisms of PD on CIRI, this experiment was conducted to build a rat model of focal cerebral ischemia-reperfusion (CI/R), and to observe the effects of PD on neurological function, oxidative damage, inflammatory response, and neuronal apoptosis in CI/R rats.

Materials and methods

Experimental animals

This experiment complied with the relevant requirements of the Administrative Regulations of the People's Republic of China on Laboratory Animals. Forty-eight 8-week-old male Sprague-Dawley (SD) rats, weighing 250-280 g, were supplied by Shanghai Slack Laboratory Animal Co., Ltd. [Animal use license number: SYXK (Shanghai) 2018-0015]. The rats were kept for 1 week of adaptive feeding in clean and disinfected cages under artificial light at 20-25°C, 65-70% relative humidity, and noise less than 80 dB, with free water throughout the day and regular change of bedding. Every procedure of this study was implemented under the approval of the Animal Care and Use Committee of Chongqing Sanxia Central Hospital.

Methods

Determination of PD content in Huhuang Shaoshang Liniment: With resveratrol glycosides (China Institute for the Control of Pharmaceutical and Biological Products, 20 mg, purity > 98%) as the standard substance, high performance liquid chromatography (HPLC, Model 1260 HPLC instrument, Agilent technologies, USA) was used to determine the content of PD in Huhuang Shaoshang Liniment (Approval No. Z20010151, Chongqing Gankyil Bio-Technology Co., Ltd.). According to the methods reported in the previous literature [10], HPLC

was used with PD as the reference. The chromatography was performed on chromatographic column of ZORBAX Eclipse Plus C_{18} (250 mm × 4.6 mm, 5 μm), flowing phase of acetonitrile monohydrate (23:77), with flow rate of 1.0 $mL{\cdot}min^{-1}$, column temperature of 35°C, and detection wavelength of 306 nm.

Model building and grouping: Except for the Sham group, CI/R model was built using suture method in other groups [12]. SD rats were injected intraperitoneally with 2% pentobarbital sodium (0.3 mL/100 g body weight), and were successfully anesthetized in 10-15 min. Rats were then fixed in the supine position, and the neck was disinfected. An incision was made in the middle of the neck to expose the common cervical artery, external carotid artery and internal carotid artery. Next, the ligation of external carotid artery was performed. Heparin-soaked sutures were inserted from the internal carotid artery to the anterior cerebral artery. The sutures were tightened, and the wound was sutured layer by layer. After ischemia for 2 h, the sutures were then opened, and were pulled out slowly to resume blood perfusion, and the wound was sutured again. The right common carotid artery, internal carotid artery and external carotid artery were isolated in Sham group, and the wound was closed without ligation, stoma, or insertion of sutures. The PD 25 mg/kg and 50 mg/kg groups were intraperitoneally injected with 25 and 50 mg/kg of PD, respectively immediately after successful modeling, and the equal volume of 0.9% normal saline (AmyJet Scientific Inc., Wuhan, China) was injected intraperitoneally in Sham and CI/R groups.

Neurological impairment score: The neurological function of rats was assessed with a five-point scale [13] at 24, 48 and 72 h postoperatively, with no symptoms of neurological deficit scoring 0, failure to fully stretch left forepaw scoring 1, circling to the left scoring 2, falling to the left scoring 3, and inability to walk spontaneously and losing consciousness scoring 4.

Determination of cerebral infarction area using Image-ProPlus 6.0 image analysis system: Following neurological function examination, 6 rats randomly selected from each group were decapitated after anesthesia, and cerebellum, olfactory bulb, and lower brainstem were removed, routinely sectioned along the coronal plane for 2 mm, and incubated in 2% TTC solution (Wuxi Puhe Biological Medicine Technology Co., Ltd., China) at 37°C for 15 min. The sections were taken and placed in 10% formalin solution overnight in the dark. Photographs were taken, and the Image-ProPlus 6.0 image analysis system (Media Cybernetics, Inc., USA) was applied for the calculation of the cerebral infarction area. The cerebral infarction area on the ischemic side (%) = area of infarction area/brain area on the ischemic side × 100%.

Hematoxylin-eosin (HE) staining: After fixing with 4% paraformaldehyde, the brain tissues of rats were dehydrated with ethanol gradient after washing, transparented with xylene, and embedded in paraffin. Afterwards, the tissues were stained with hematoxylin-eosin (HE) staining kit (Wuhan Boster Biological Technology, Ltd., China) and mounted. The morphology and structure of the tissues were observed with a CX21 optical microscope (Olympus, Japan). The pathological score of HE stained brain tissue was quantified according to the following criteria [14]: no lesions were observed under light microscope - 0; the pathological manifestations such as focal gray and white matter mild edema, cytolysis, focal proliferation of a few cells (mainly glial cells), little inflammatory cell infiltration, and mild gray matter atrophy were observed under light microscope - 1 point; pathological manifestations such as multi-focal gray and white matter edema, cytolysis, degeneration and necrosis, focal proliferation of more cells (mainly glial cells), and more inflammatory cell infiltrations were observed under light microscope - 2 points; diffuse gray and white matter edema, cytolysis, degeneration and necrosis, focal proliferation of a large numbers of cells (mainly glial cells), and large numbers of inflammatory cell infiltrations were observed under light microscope - 3 points.

TUNEL staining: The paraffin sections were dewaxed, rehydrated, digested with proteinase K, treated with the TUNEL reaction solution and the complex solution, developed with diaminobenzidine, and observed under optical microscope, and the apoptotic index of brain tissue

was calculated using the Standard 1.6 image analysis system (Olympus, Japan).

Enzyme-linked immunosorbent assay (ELISA) and UV-Vis spectrophotometry: The remaining 6 rats in each group were given intraperitoneal injection of 0.3% pentobarbital sodium (1 mL/100 g) for anesthesia and decapitated. The brain tissues were stripped and ground into homogenate and centrifuged at low temperature for 15 min to harvest the supernatant. The contents of interleukin (IL)-1β, IL-6, IL-4, and IL-10 were determined using ELISA. The levels of glutathione peroxidase (GPx), superoxide dismutase (SOD), lactate dehydrogenase (LDH), and malondialdehyde (MDA) were measured by UV-Vis spectrophotometer (Guangzhou Weijia Technology Co., Ltd., China). The IL-1β, IL-6, IL-4, and IL-10 kits were bought from Shanghai Jianglai Biotechnology Co., Ltd., China, and GPx, SOD, LDH, and MDA kits were bought from Dongguan Spectrum Lab Equipment Technology Co., Ltd., China.

Nrf2/HO-1/NQO1 pathway and neuronal apoptosis-related proteins: The obtained homogenate was centrifuged (5415D high-speed centrifuge, Eppendorf, Germany) at high speed and low temperature to remove the deposit, followed by protein quantification using the bicinchoninic acid (BCA, kit supplied by Beijing Solarbio Science & Technology Co., Ltd., China) method. The equal amount of protein samples was extracted, denaturized at 100°C for 5 min, and loaded onto 12% SDS-polypropylene gel electrophoresis. After being wet-transferred on PVDF membrane and blocked with 5% bovine serum albumin at room temperature for 2 h, proteins were incubated in primary antibodies (1:500), including Nrf2 diluent (1:500), HO-1 diluent (1:500), Bax diluent (1:500), and NQO1 diluent (1:2000) (all purchased from CST Biotechnology Co., Ltd., USA). On the following day after flushing with Tris-buffered saline with Tween 20 (TBST), the secondary antibody (1:2000), horseradish peroxidase labeled goat anti-rabbit IgG (Beijing Solarbio Science & Technology Co., Ltd.) was added for incubation at indoor temperature for an hour, followed by rinsing the membrane with TBST. ECL luminescent solution was adopted for color development, and quantitative analysis was performed using Image J software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000) was taken as internal reference, with the ratio result indicating the relative content of the target protein.

Statistical analysis

SPSS 24.0 statistical analysis software was used for data analyses. The measurement data conforming to normal distribution were described by mean ± standard deviation (mean ± SD), and one-way analysis of variance was performed for comparisons among multiple groups, and further pairwise comparison was made using the LSD t-test, with *P* value less than 0.05 denoting marked difference.

Results

PD content in Huhuang Shaoshang Liniment

The PD content in three batches of Huhuang Shaoshang Liniment was determined by HPLC, and the average result showed that the product contained 0.73 mg PD per 1 mL. This indicated that PD was one of the main components of Huhuang Shaoshang Liniment.

HE staining

The structure and morphology of the neurons in the Sham group were intact, and the nuclei were round and neatly arranged. The neurons in CI/R group had obvious loose cytoplasm, darkly stained nuclei, deformed cells, and small cytoplasmic volume. Compared with CI/R group, PD 25 and 50 mg/kg groups had orderly-arranged neurons and less pathological damage, and the nuclei were solidly and darkly stained. PD 50 mg/kg group had the most obvious improvement. The pathological quantification score was lowest in sham group, followed by PD 50 mg/kg group, PD 25 mg/kg group, and highest in CI/R group ($P < 0.05$). This indicates that cortical neuron of the rats with CI/R was obviously damaged, while PD could reduce the degree of neuropathological damage, with 50 mg/kg dosage showing the best effect (**Figure 1**).

Neurological impairment score

CI/R group exhibited significantly higher scores of neurological impairment than Sham group at 24, 48, and 72 h postoperatively ($P < 0.05$). For

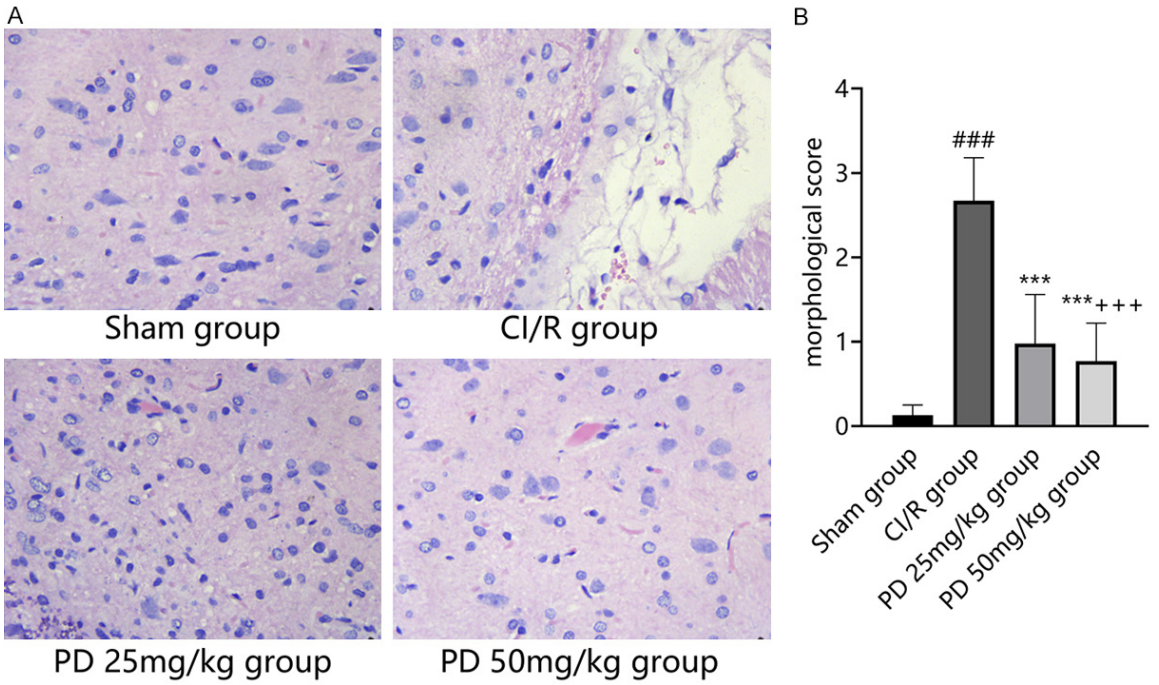

Figure 1. Effect of PD on pathological changes in brain tissue of rats (× 400). A: The HE staining shows that the morphological structure of cortical neurons in the Sham group was intact, with round nuclei and neatly arranged; the neurons in the CI/R group had significantly loose cytoplasm, darkly stained nuclei, deformed cells, and small cytoplasmic volume; the cortical neurons in the PD 25 mg/kg and 50 mg/kg groups were more neatly arranged, the degree of pathological damage was reduced compared with the CI/R group, and the nuclei were solidly and darkly stained. B: The quantitative score of HE pathological injury. CI/R: Cerebral Ischemia-Reperfusion; PD: polydatin. $^{###}P < 0.001$ compared with Sham group; $^{***}P < 0.001$ compared with CI/R group; $^{+++}P < 0.001$ compared with PD 25 mg/kg group.

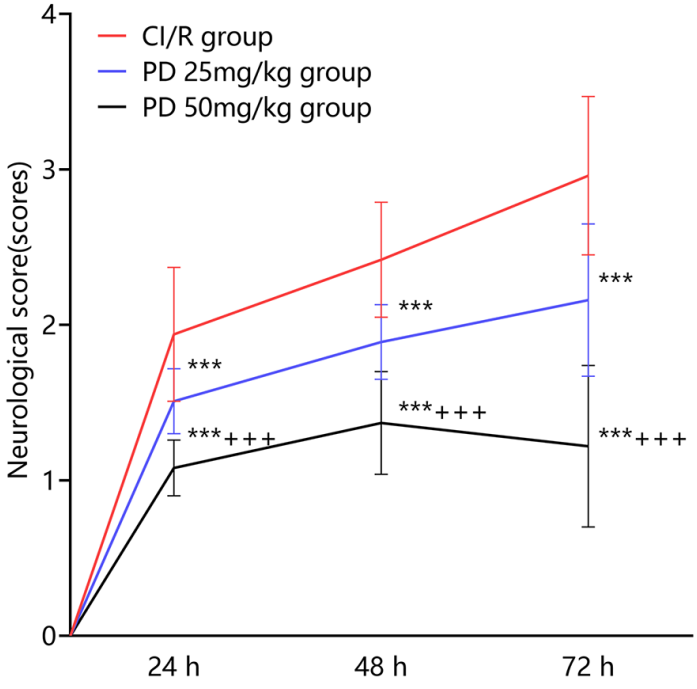

Figure 2. Effect of PD on neurological impairment scores. Note: CI/R: Cerebral Ischemia-Reperfusion; PD: polydatin. $^{***}P < 0.001$ compared with CI/R group; $^{+++}P < 0.001$ compared with PD 25 mg/kg group.

the three groups received CI/R establishment, the neurological impairment scores at 24, 48 and 72 h postoperatively were lowest in PD 50 mg/kg group, followed by PD 25 mg/kg group, and highest in CI/R group ($P < 0.05$). This shows that the neurological function of the rats with CI/R was significantly impaired, and PD could reduce the degree of neurological function, with 50 mg/kg dosage exhibiting the best effect (**Figure 2**).

Cerebral infarction area and apoptotic index in rats of the ischemic side

CI/R group showed significantly higher percentage of cerebral infarction area on the ischemic side and the apoptotic index than Sham group (all $P < 0.05$). For the three groups with CI/R model, the percentage of cerebral infarction area

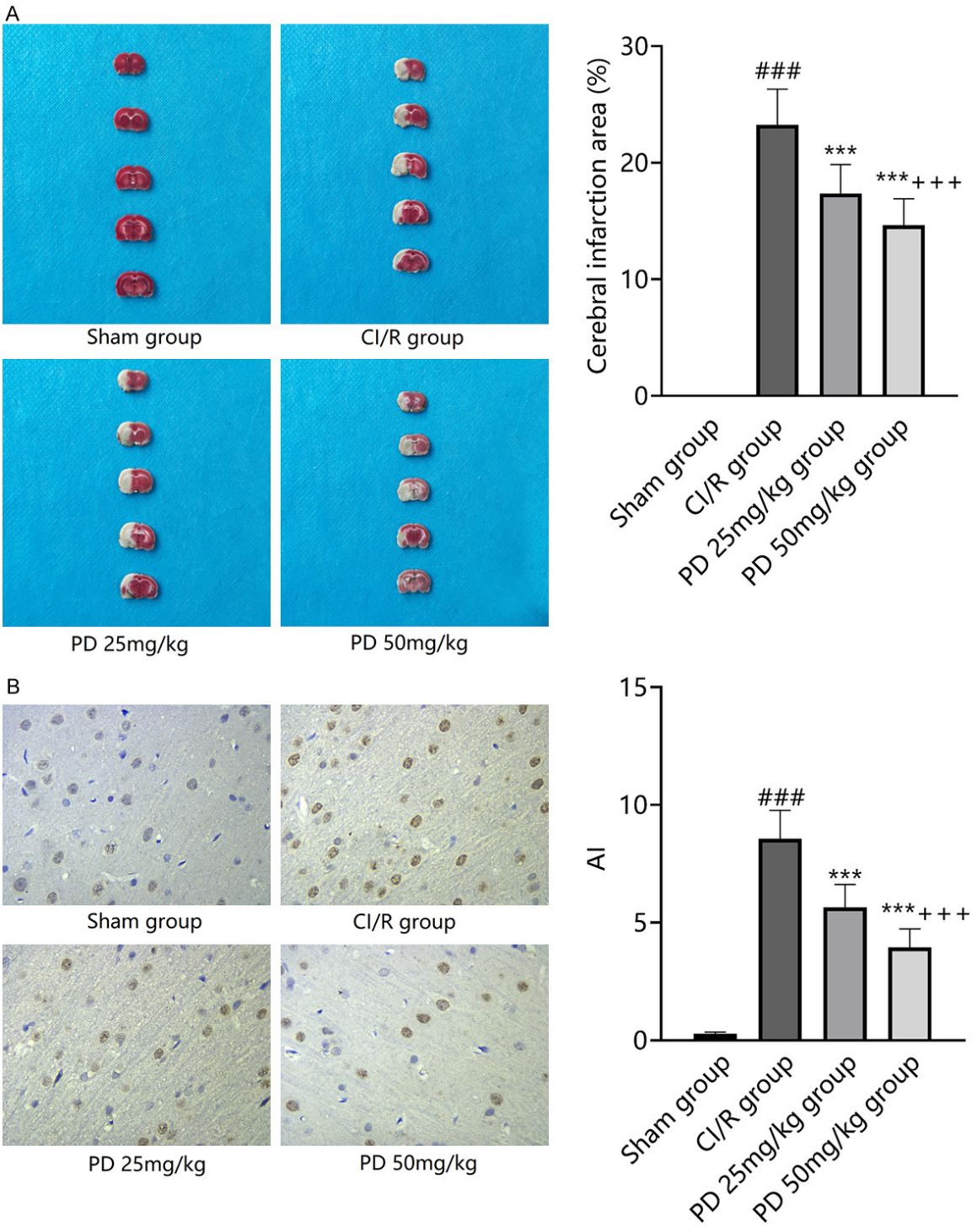

Figure 3. Effect of PD on the percentage of cerebral infarction area and apoptotic index. A: Cerebral infarction area; B: Apoptotic index. Note: CI/R: Cerebral Ischemia-Reperfusion; PD: polydatin. $^{###}P < 0.001$ compared with Sham group; $^{***}P < 0.001$ compared with CI/R group; $^{+++}P < 0.001$ compared with PD 25 mg/kg group.

on the ischemic side and the apoptotic index were lowest in PD 50 mg/kg group, followed by PD 25 mg/kg group, and highest in CI/R group ($P < 0.05$). It shows that the cerebral infarction area on the ischemic side was larger in rats with CI/R, and PD could decrease the cerebral

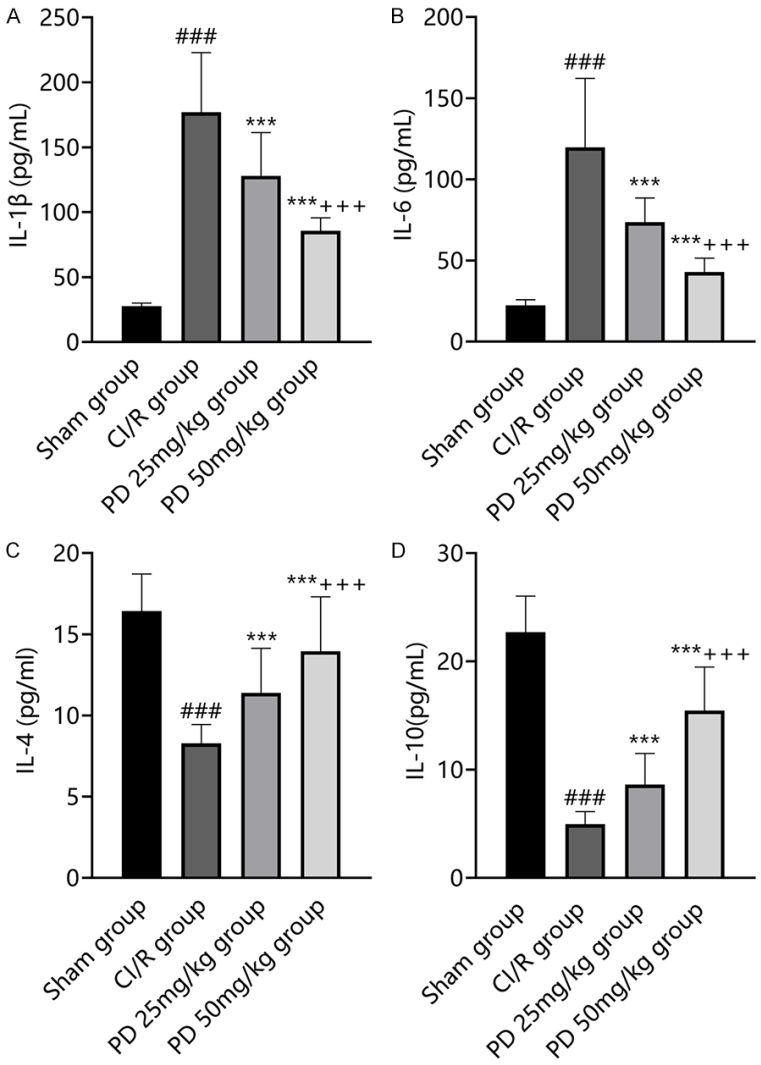

Figure 4. Effect of PD on inflammatory factor levels in brain tissue of rats. A: IL-1β; B: IL-6; C: IL-4; D: IL-10. Note: CI/R: Cerebral Ischemia-Reperfusion; PD: polydatin; IL-1β: Interleukin-1β; IL-6: Interleukin-6; IL-4: Interleukin-4; IL-10: Interleukin-10. Compared with Sham group, $^{###}P < 0.001$; compared with CI/R group, $^{***}P < 0.001$; compared with PD 25 mg/kg group, $^{+++}P < 0.001$.

infarction area and suppress the apoptosis of brain tissue, with 50 mg/kg dosage exhibiting the best effect (**Figure 3**).

Inflammatory factors in brain tissues

CI/R group showed significantly elevated IL-6 and IL-1β levels but reduced IL-4 and IL-10 levels in the brain tissue than Sham group (all $P < 0.05$). For the three groups with CI/R model, the IL-6 and IL-1β levels were lowest in PD 50 mg/kg group, followed by PD 25 mg/kg group, and highest in CI/R group ($P < 0.05$). The IL-4 and IL-10 levels were highest in PD 50 mg/kg group, followed by PD 25 mg/kg group, and lowest in CI/R group ($P < 0.05$), revealing that PD could reduce the inflammatory response in CI/R rats, with 50 mg/kg dosage exhibiting the best effect (**Figure 4**).

Oxidative stress in brain tissues

CI/R group exhibited significantly elevated MDA and LDH levels but decreased SOD and GPx levels in the brain tissue than Sham group (all $P < 0.05$). For the three groups with CI/R model, the MDA and LDH levels were lowest in PD 50 mg/kg group, followed by PD 25 mg/kg group, and highest in the CI/R group. The SOD and GPx levels were highest in CI/R group, followed by PD 25 mg/kg group, and lowest in CI/R group ($P < 0.05$). It suggests that PD could reduce the level of oxidative stress and reduce oxidative damage in CI/R rats, with 50 mg/kg dosage achieving the best effect (**Figure 5**).

Nrf2/HO-1/NQO1 pathway-related protein expression

CI/R group exhibited significantly lower protein expression of Nrf2, HO-1, and NQO1 in the brain tissue than the Sham group (all $P < 0.05$). Protein expression of Nrf2, HO-1, and NQO1 in the brain tissues of PD 25 mg/kg group was lower than that in PD 50 mg/kg group but was higher than that in CI/R group ($P < 0.05$). This reveals that PD could activate Nrf2/HO-1/NQO1 signaling pathway in CI/R rats, and with 50 mg/kg dosage achieving the best effect (**Figure 6**).

Neuronal apoptosis

CI/R group showed higher protein expression of Bax and lower protein expression of c-Myc in the brain tissues than Sham group (all $P < 0.05$). The protein expression of Bax was lowest in PD 50 mg/kg group, followed by PD 25 mg/kg group, and highest in CI/R group, while the protein expression of c-Myc was highest in

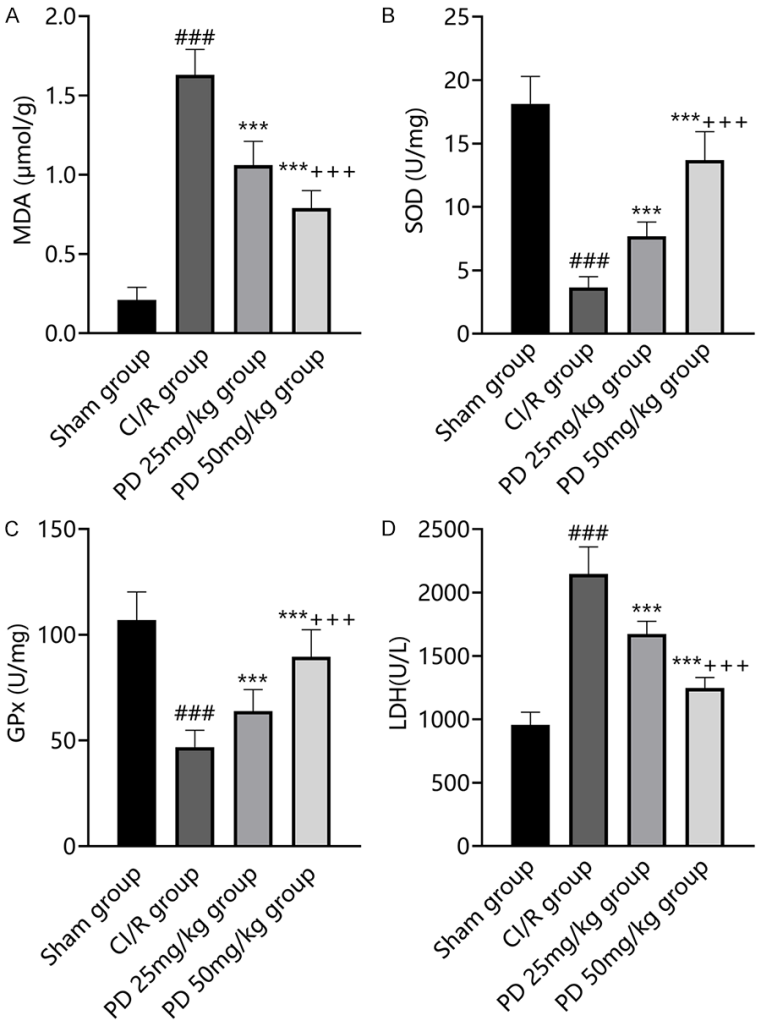

Figure 5. Effect of PD on oxidative stress levels in brain tissue of rats. A: MDA; B: SOD; C: GPx; D: LDH. Note: CI/R: Cerebral Ischemia-Reperfusion; PD: polydatin; MDA: malondialdehyde; SOD: superoxide dismutase; GPx: glutathione peroxidase; LDH: lactate dehydrogenase. $^{###}P < 0.001$ compared with Sham group; $^{***}P < 0.001$ compared with CI/R group; $^{+++}P < 0.001$ compared with PD 25 mg/kg group.

PD 50 mg/kg group, followed by PD 25 mg/kg group, and lowest in CI/R group ($P < 0.05$), which showed that PD could inhibit neuronal cell apoptosis in CI/R rats, with 50 mg/kg achieving the best effect (**Figure 7**).

Discussion

Huhuang Shaoshang Liniment is effective in purging fire for removing toxin, relieving swelling and pain, and eliminating dampness and astringing sores. This preparation consists of eight traditional Chinese medicines, including Polygonum cuspidatum, Coptis chinensis, Phellodendri Chinensis Cortex, Angelica dahurica, Carthamus tinctorius, and Borneol. Among them, Polygonum cuspidatum is a monarch drug and has many pharmacological effects, including anti-inflammatory, antiviral, antibacterial, regulating blood lipids, anti-thrombosis, changing hemorheology, dilating blood vessels, protecting myocardium, antioxidant, antitumor, improving Alzheimer's disease, preventing AIDS, etc. A previous study has shown that PD is the main effective ingredient of Polygonum cuspidatum in Huhuang Shaoshang Liniment [10]. The results of this study showed that the product contained 0.73 mg PD per 1 mL, as one of the main components of Huhuang Shaoshang Liniment. This is consistent with the literature report mentioned above.

PD has the effects of anti-oxidative stress damage, neuronal protection, and inhibiting neuronal cell apoptosis [15, 16]. Gao et al. [17] found that PD improved mitochondrial dysfunction induced by ischemia-reperfusion injury, decreased infarct size, elevated neurological severity scores, and reduced caspase-3 and caspase-9 activities. Shah et al. [18] found that PD alleviated neurobehavioral deficits and reduced infarct size, thereby rescuing neuronal apoptosis. All the above findings have proved that PD can exert a protective role against CI/R injury in rats, but its mechanism has not been fully revealed. In this study, the CI/R model was built by the suture-occlusion method, and the results showed that the morphology of the cortical area, percentage of infarction area, neurological impairment score, and apoptosis-related protein expression in the ischemic side of the brain tissues were significantly improved in different doses of PD groups in contrast to CI/R group. The dose dependence was similar to the results of the above studies.

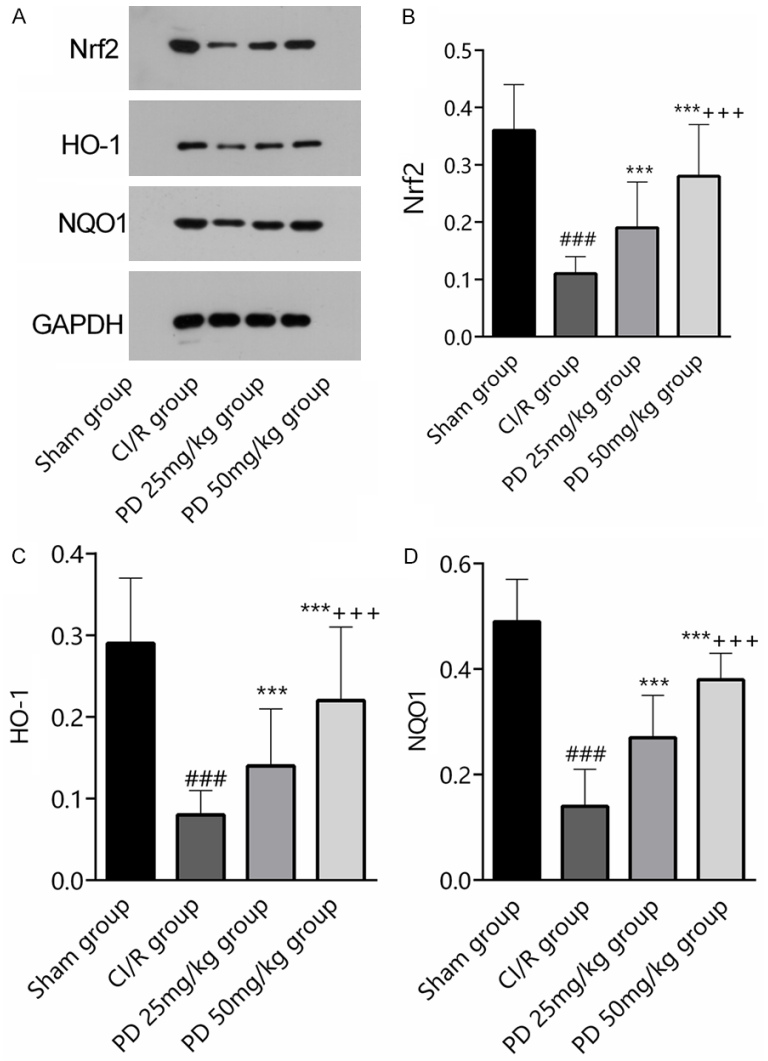

Figure 6. Comparison of Nrf2/HO-1/NQO1 pathway-related protein expression in brain tissue of rats in each group. A: The protein expressions of Nrf2, HO-1, NQO1 detected by Western blotting; B: Bar graph of Nrf2 protein expression; C: Bar graph of HO-1 protein expression; D: Bar graph of NQO1 protein expression. Note: Nrf2: NF-E2-related factor 2; HO-1: Heme Oxygenase-1; NQO1: NAD(P)H Quinone Oxidoreductase 1; CI/R: Cerebral Ischemia-Reperfusion; PD: polydatin. Compared with Sham group, $^{###}P < 0.001$; compared with CI/R group, $^{***}P < 0.001$; compared with PD 25 mg/kg group, $^{+++}P < 0.001$.

Oxidative stress is a primary mechanism of CI/R injury, which leads to brain tissue damage by mediating inflammation and immune responses, injuring endothelial cells, and stimulating the expression of cellular molecules and adhesion molecules [19]. In this study, CI/R group exhibited elevated contents of MDA and LDH, and reduced contents of SOD and GPx than Sham group, which showed that there was significant oxidative stress damage in CI/R rats. This may be due to the destruction of mitochondria in brain cells during the super-reperfusion time window; the oxygen brought by blood reperfusion converted alkyl radicals into lipid peroxide radicals on one hand, formed new oxygen ions under xanthine oxidase action on the other hand, and thus reignited the free radical chain and generated plenty of free radicals, resulting in oxidative damage. The Nrf2 pathway is one of the crucial cytotoxic defense and antioxidant mechanisms in cells. When oxidative stress damage occurs in the organism, Nrf2 activation can activate downstream antioxidant proteins levels including HO-1 and NQO1 as well as antioxidant products including SOD and GSH to protect organs and cells. Chen et al. [20] pointed out that Nrf2 activation could induce the expression of detoxifying enzymes and antioxidant enzymes, thus reducing the oxidative stress-induced brain tissue damage. Ding et al. [21] found that knockdown of the Nrf2 gene in CI/R rats aggravated neurological deficits and increased infarct size, while administration of the Nrf2 activator reduced infarct size and inhibited neuronal cell apoptosis. Wang et al. [22] reported that serum MDA and LDH levels were abnormally elevated in ischemia-reperfusion rats, and there was significant oxidative stress damage, while activation of Nrf2 ameliorated tissue/cell damage. Feng et al. [23] found that activation of Nrf2/HO-1/NQO1 signaling pathway inhibited endoplasmic reticulum stress, oxidative stress, and inflammatory responses. It shows that up-regulation of the activity of Nrf2/HO-1/NQO1 signaling pathway can reduce the oxidative stress response of CI/R injury and improve prognosis. In this study, the protein expression of Nrf2, HO-1 and NQO1 as well as the contents

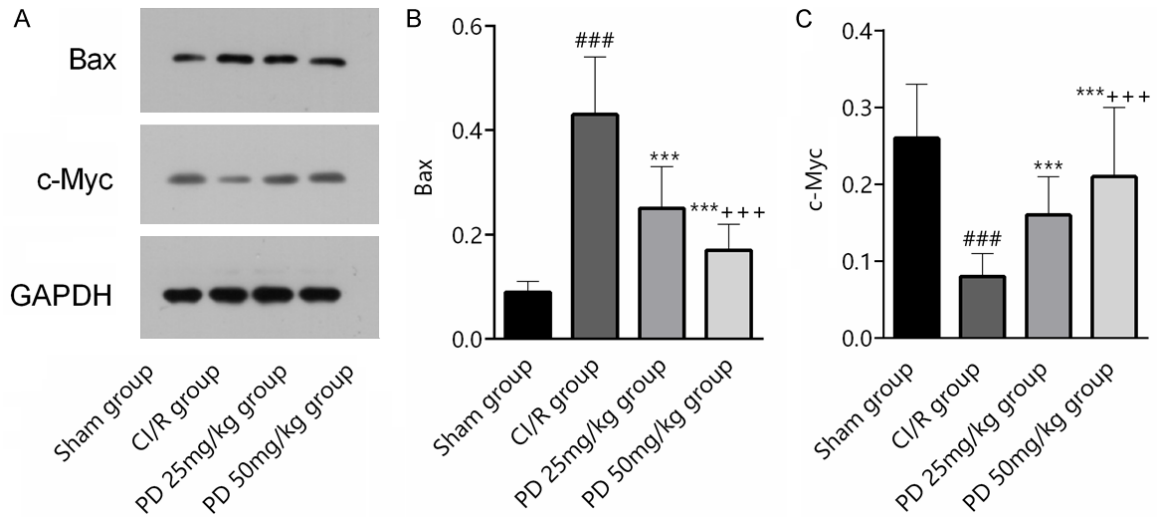

Figure 7. Comparison of apoptosis-related protein expression in brain tissue of rats in each group. A: Apoptosis-related protein expression detected by Western blotting; B: Bax protein expression; C: c-Myc protein expression. Note: CI/R: Cerebral Ischemia-Reperfusion; PD: polydatin. $^{###}P < 0.001$ compared with Sham group; $^{***}P < 0.001$ compared with CI/R group; $^{+++}P < 0.001$ compared with PD 25 mg/kg group.

of SOD and GPx in the brain tissues of CI/R rats treated with PD elevated significantly, the contents of MDA and LDH decreased, and the PD 50 mg/kg group showed the most obvious effects, indicating that PD can activate Nrf2/HO-1/NQO1 signaling pathway and reduce the oxidative stress injury in CI/R rats, and the effect shows a dose dependence. The mechanism may be that PD can facilitate nuclear translocation of Nrf2, induce downstream protein expression of HO-1 and NQO1, activate Nrf2/HO-1/NQO1 signaling pathway in rats, accelerate reduction of oxygen radicals, enhance antioxidant protein expression, and thus reduce oxidative damage to cell membranes [24].

Cerebral ischemia and hypoxia can disrupt the normal function and structure of mitochondria of glial cells and vascular endothelial cells, leading to dysfunction of cellular energy metabolism and calcium overload in tissue cells, which triggers the massive expression of adhesion molecules and cytokines related to inflammatory response such as IL-1β and IL-6, resulting in cascade activation of inflammatory response in brain tissues and aggravating neuronal damage in brain tissues. In the present study, CI/R group exhibited increased IL-1β and IL-6 levels and decreased IL-4 and IL-10 levels in the brain tissue than Sham group, indicating that CI/R exacerbated the inflammatory response, which was similar to the findings of Liu et al. [25] over the years, increasing evidence suggests that the upregulation of the Nrf2 pathway suppresses the expression of inflammatory factors and decrease the inflammatory cascade response in brain tissues. Tao et al. [26] pointed out that the total flavonoids of Rosa can suppress the expression of inflammatory factors like TNF-α, IL-1β and IL-6 via activation of Nrf2 signaling pathway, thus reducing the inflammatory response. Masuda et al. [27] demonstrated that myeloperoxidase activity and neutrophil infiltration were significantly reduced in the liver of ischemia-reperfusion injury rats treated with the Nrf2 agonist. In the present study, the levels of pro-inflammatory factors were decreased, while the levels of anti-inflammatory factors were elevated in the brain tissues of CI/R rats treated with PD, which again confirmed that PD may exert a role in reducing the inflammatory response and brain cell injury by upregulating the Nrf2/HO-1/NQO1 signaling pathway.

This experiment confirmed the effects of PD on neurological function, oxidative damage, and inflammatory response in CI/R rats, which provides a new idea for clinical treatment and new drug development, and preliminarily analyzed its mechanism of action. Nevertheless, there are still certain shortcoming. For example, the mechanism is only analyzed from the

perspective of Nrf2/HO-1/NQO1 signaling pathway, and only in vitro studies were performed to simulate the pathogenic process of CI/R injury. The *in vivo* experiments will be conducted later to further confirm the pharmacological mechanism of PD.

In conclusion, PD can reduce neurological damage, improve neuronal morphology and structure, reduce cerebral infarction area, alleviate oxidative damage and inflammatory response, and inhibit neuronal cell apoptosis in CI/R rats, and its mechanism may be connected with the upregulation of Nrf2/HO-1/NQO1 signaling pathway.

Disclosure of conflict of interest

None.

Address correspondence to: Wei Fang, Department of Pharmacy, Chongqing Sanxia Central Hospital, No. 165, Xincheng Road, Wanzhou District, Chongqing 404100, China. Tel: +86-023-58103181; E-mail: si2ypg@163.com; Jing Li, Department of Pharmacy, People's Hospital of Chongqing, Banan District, Chongqing 401320, China. Tel: +86-13996567009; E-mail: fangwei006688@163.com

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