Received: 13 May 2021 | Revised: 11 September 2021 | Accepted: 18 September 2021

DOI: 10.1111/cbdd.13956

RESEARCH ARTICLE

WILEY

Pinoresinol diglucoside alleviates ischemia/reperfusion-induced brain injury by modulating neuroinflammation and oxidative stress

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Funding information
Application of Continuous Lumbar Drainage in Patients with Severe Spontaneous SAH after Operation

Abstract

Brain ischemia/reperfusion (I/R) injury is a common pathological process after ischemic stroke. Pinoresinol diglucoside (PDG) has antioxidation and anti-inflammation activities. However, whether PDG ameliorates brain I/R injury is still unclear. In this study, middle cerebral artery occlusion (MCAO) model was established with male C57BL/6 mice, and the mice were treated with 5 and 10 mg/kg PDG via intravenous injection, respectively. The neurological deficit, infarct volume, and brain water content were then evaluated. HE staining and Nissl staining were used to analyze neuron injury. Besides, enzyme-linked immunosorbent assay and colorimetry assay were used to examine the level of inflammatory markers and oxidative stress markers, and Western blot was used to detect the expressions of p-p65, Nrf2, and HO-1. It was revealed that PDG could significantly alleviate the MCAO-induced neurological dysfunction of the mice and reduce the infarct volume, brain water content, and neuron injury. PDG treatment decreased the levels of TNF-α, IL-1β, IL-6, NO, ROS, and MDA, and significantly increased the activities of SOD, GSH, and GSH-Px in the brain tissue of the mice. Additionally, PDG could repress the activation of p65 and promote Nrf2 and HO-1 expressions. In conclusion, PDG exerts anti-inflammatory and antioxidation effects via regulating the NF-κB pathway and Nrf2/HO-1 pathway, thereby reducing the I/R-induced brain injury of mice.

KEYWORDS

brain injury, ischemia, neuroinflammation, oxidative stress, pinoresinol diglucoside, reperfusion

1 | INTRODUCTION

Stroke, a common cerebrovascular disease, leads to neurological impairment, and it is one of the leading causes of disability and death worldwide (Hu et al., 2017; Lundberg & Volgman, 2016). Ischemic stroke is caused by the decreased blood supply, and thrombolytic therapy is still a crucial treatment strategy (Fisher & Saver, 2015;

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Muth, 2020). However, vascular recanalization and restoration of blood supply after thrombolysis often lead to secondary brain injury, which is called ischemia/reperfusion (I/R) injury, accompanied by diverse pathological processes, including neuroinflammation, neuron apoptosis, production of reactive oxygen species (ROS), oxidative stress (OS), excitatory toxicity, and mitochondrial dysfunction, ultimately aggravating neurological injury and dysfunction (Corrigan et al., 2016; Guadagno et al., 2015; Hankey, 2017; Kettenmann et al., 2011; Lucas et al., 2006; Neher et al., 2013; Tremblay et al., 2011; Wang, Mao, et al., 2020; Wang, Higashikawa, et al., 2020; Wu et al., 2020; Xu et al., 2018). Therefore, it is necessary to search for effective neuroprotective agents to prevent/ameliorate I/R-induced injury during the treatment of ischemic stroke.

Considering the mechanisms of I/R-induced brain injury mentioned above, in the early stage of ischemic stroke, inhibiting the excessive neuroinflammation and OS is a promising treatment strategy to inhibit brain injury and improve the prognosis of patients (Boutin et al., 2001). In recent years, many bioactive components extracted from traditional Chinese herbal medicine, which are with characteristics of antioxidation and anti-inflammation, have shown the potential to treat I/R-induced brain injury after ischemic stroke, such as theaflavin, kaempferol, curcumin, and astragalin (Chen, Cheg, et al., 2020; Li et al., 2019; Sun et al., 2015; Wang, Mao, et al., 2020; Zhou et al., 2020).

Pinoresinol diglucoside (PDG) is one of the main lignans isolated from the bark of *Eucommia ulmoides*, a kind of traditional Chinese herbal medicine. Previous studies show that PDG has many pharmacological effects, such as anti-inflammation, anti-hypertension, prevention of osteoporosis, and tumor-suppressive properties (During et al., 2012; Horn-Ross et al., 2003; Li et al., 2017; Luo et al., 2010; Saleem et al., 2005; Yao et al., 2016). However, the neuroprotective function of PDG on cerebral I/R injury is still unclear. In the present study, middle cerebral artery occlusion/reperfusion (MCAO/R) model in mice was established, and PDG was injected into mice through the tail vein to observe the therapeutic effects. We demonstrated that PDG was a promising drug to attenuate the neuroinflammation and OS during the pathogenesis of cerebral I/R injury.

2 | MATERIALS AND METHODS

2.1 | Animal grouping and drug interventions

The animal experiments in this study were approved by the Ethics Review Board of Chenzhou No. 1 People's Hospital (SYXK 2019-0011), and the procedures of experiments were designed according to the Guidelines for the National Institutes of Health on Animal Care and Use. The Animal Experimental Center of Wuhan University (Wuhan, China) was the provider of male C57BL/6 mice (7 to 8 weeks old, 20–23 g). The above mice were fed at 22 ± 2℃ and in 50 ± 10% humidity with a light/dark cycle of 12 h/12 h, with sufficient food and water. The mice were randomly grouped into four groups ($n = 9$ in each group): (1) Sham group; (2) MCAO group; (3) MCAO + 5 mg/kg PDG group; (4) MCAO + 10 mg/kg PDG group. PDG (Figure 1, purity ≥99%; Jieshikang) was dissolved with normal saline. Mice were respectively administered with 5 mg/kg PDG, 10 mg/kg PDG, or an equivalent volume of normal saline 1 h before MCAO, via the caudal vein.

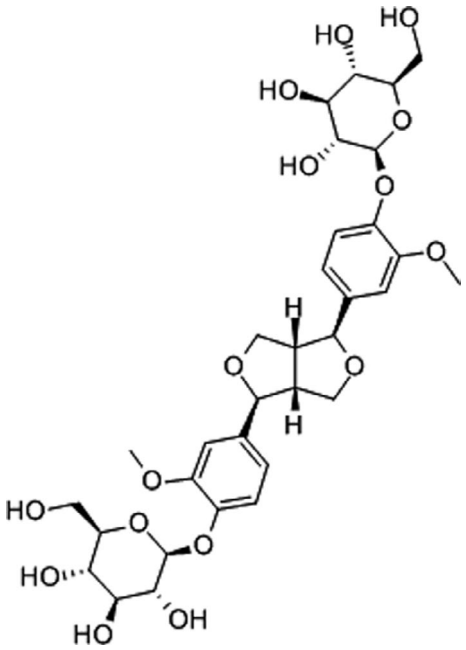

FIGURE 1 The molecular structure of PDG

2.2 | MCAO model

To establish the MCAO model, briefly, the mice were anesthetized with the intraperitoneal injection of 5% chloral hydrate (0.01 ml/g), and 1.5% isoflurane was used to maintain the anesthesia. The skin was incised in the middle of the neck, the left common carotid artery was gently isolated from the muscle and nerve fibers, and the external and internal carotid arteries were then carefully separated. A 6-0 nylon suture with silicon was inserted into the internal carotid artery and gently advanced to occlude the middle cerebral artery (MCA). After 40 min, the filaments were removed to allow reperfusion. After that, the MCAO model was established. The same surgical procedures were applied on mice in the Sham group, but without the occlusion of the MCA.

2.3 | Neurological deficit score

Twenty-four hour after MCAO, the neurological functions of the mice were evaluated by Modified Neurological Severity Score (mNSS) test according to a previous report (Yang et al., 2015), and the motor, sensory ability, reflex, and balance of the mice were scored, ranging from 0 to 18 (normal score, 0; maximal deficit score, 18). The higher the scores, the more severe the injury.

2.4 | Evaluation of brain infarct volume

Briefly, after mNSS test, the mice were anesthetized and decapitated. The brains of the mice were obtained and placed at −20℃ for 120 min and evenly cut into slices with a thickness of about 2 mm. The brain slices were incubated in 10 ml of phosphoric acid buffer containing 2% 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma) for 30 min at 37℃ and flipped every 5 min. Subsequently, the slices were fixed with 4% paraformaldehyde, and 3 days later, the slices were photographed. The brain infarct volumes were calculated with Image-proPlus 6.0 (Media Cybernetics).

2.5 | Brain water content

The water content of mice brain tissue was measured by dry-wet weight method. After the mice were decapitated, the injured cerebral hemisphere of each mouse was quickly weighed (wet weight). Then, it was placed in an oven at 105℃ and dried for 36 h, and subsequently, the brain tissue was weighed again (dry weight). Brain water content (%) = (wet weight − dry weight)/wet weight × 100%.

2.6 | Hematoxylin-eosin (HE) staining

The brain tissues were fixed with 4% paraformaldehyde, dehydrated, embedded in paraffin, and subsequently cut into serial slices with a thickness of 5 μm. Then, the slices were dewaxed, rehydrated, and stained using hematoxylin staining solution for 3 min, followed by the wash for 3 min with 1% hydrochloric acid ethanol. Next, the slices were washed with tap water until the nucleus turned blue. After being stained with eosin staining solution for 1 min, the slices were washed with tap water again, dehydrated by ethanol, and then immersed in xylene. The slices were sealed with the neutral gum after drying before being observed under a microscope.

2.7 | Nissl staining

After 24 h of reperfusion, the brain tissues were fixed with 4% paraformaldehyde, embedded in paraffin, dewaxed in xylene, and rehydrated with graded ethanol (95, 85, and 75%), and the brains were coronally cut with a thickness of 4 μm. The sections were stained in 1% toluidine blue (Beyotime) for 30 min at 45℃, and then immersed in 75% ethanol for several seconds, and then rinsed in distilled water. After that, the sections were observed under a microscope, and the number of intact neurons was counted.

2.8 | Immunohistochemistry

Heat-mediated antigen retrieval with sodium citrate buffer was performed. Then, the brain sections were incubated with anti-Iba1 antibody (1:200, ab178846, Abcam) in a wet box for 15 min at room temperature and then incubated with the secondary antibody (1:500, Beyotime) for 30 min at room temperature. After the brain sections were washed by phosphate buffer saline, DAB was used as the chromogen to develop the color. After being counterstained with hematoxylin and mounted with DPX, the sections were observed under a microscope.

2.9 | Evaluation of oxidative stress

The brain tissue was washed with normal saline and prepared to homogenate. The homogenate was centrifuged at 4℃ for 10 min, and the supernatant was obtained. Then, the activities of ROS (CA1410, U/mg prot), nitric oxide (NO, BC1470, nmol/mg prot), malondialdehyde (MDA, BC0020, nmol/mg prot), glutathione (GSH, BC1170, μg/mg prot), glutathione peroxidase (GSH-Px, BC1190, U/mg prot), and superoxide dismutase (SOD, BC0170, U/mg prot) were detected by different commercial detection kits (Solarbio) according to the manufacturer's instructions.

2.10 | Enzyme-linked immunosorbent assay (ELISA)

Brain tissues and normal saline were fully mixed and homogenized in a homogenizer, and then the mixture was centrifuged at 6000 r/min (2500×*g*) at 4℃ for 15 min. Subsequently, the supernatant was collected, and TNF-α, IL-1β, and IL-6 levels were recorded by the ELISA reader (Bio-Rad Laboratories) with corresponding ELISA kits (TNF-α, ab208348; IL-1β, ab197742; IL-6, ab222503; Abcam) according to the manufacturer's instructions.

2.11 | Western blot

Total proteins in the brain tissues were extracted in RIPA lysis buffer (Beyotime), and the protein concentration was measured by a BCA protein concentration kit (Beyotime). The proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro-transferred onto the polyvinylidene difluoride membrane (Millipore). After being blocked with 5% skimmed milk for 1 h at room temperature, the membranes were firstly incubated with primary antibodies, including anti-p-IKKβ (1:500; ab194519; Abcam), anti-IKKβ (1:1000; ab124957; Abcam), anti-p-IkBα (1:3000; ab133462; Abcam), anti-IkBα (1:1000; ab32518; Abcam), anti-NF-κB p65 (1:1000; ab16502; Abcam), anti-NF-κB p-p65 (S536) (1:500; ab86299; Abcam), anti-Nrf2 (1:1000; ab62352; Abcam), anti-HO-1 (1:500; ab13248; Abcam), anti-NQO1 (1:500; ab80588; Abcam), anti-Histone 3 (1:1000; ab1791; Abcam), and anti-β-actin (1:1000; ab8226; Abcam), at 4℃ overnight and then with corresponding secondary antibodies (1:3000; Beyotime) at room temperature for 1 h. The blotted protein bands were visualized by an enhanced chemiluminescence kit (Beyotime, Shanghai, China). Image J software (NIH Image) was used to analyze the gray level of the bands.

2.12 | Statistical analysis

SPSS 24.0 (SPSS Inc.) was adopted to analyze the experimental data, which were expressed as "mean ± standard deviation". Student's *t*-test was employed to compare the data of two groups. GraphPad prism 6.0 (GraphPad software) was employed for plotting the figures. Statistically, $p < .05$ was meaningful.

3 | RESULTS

3.1 | PDG could ameliorate the neurological dysfunction of mice with MCAO/R

To pinpoint whether PDG had a neuroprotective effect on cerebral I/R injury, firstly, the neurological functions of the mice were evaluated by mNSS method, and it was revealed that the neurological function of mice with MCAO/R was seriously damaged compared with the mice in the Sham group; however, PDG treatment attenuated the neurological deficit, and the neurological function score decreased with the increase of PDG concentration (Figure 2a). Additionally, PDG treatment significantly decreased brain infarct volume and brain water content after MCAO/R (Figure 2b–c). These data highlighted that PDG has protective effects against I/R-induced cerebral injury.

3.2 | PDG could reduce the pathological changes of neurons of mice with MCAO/R

Next, HE staining was performed to examine the morphological changes of neurons in brain tissues of the mice, and the results showed that in the Sham group, the

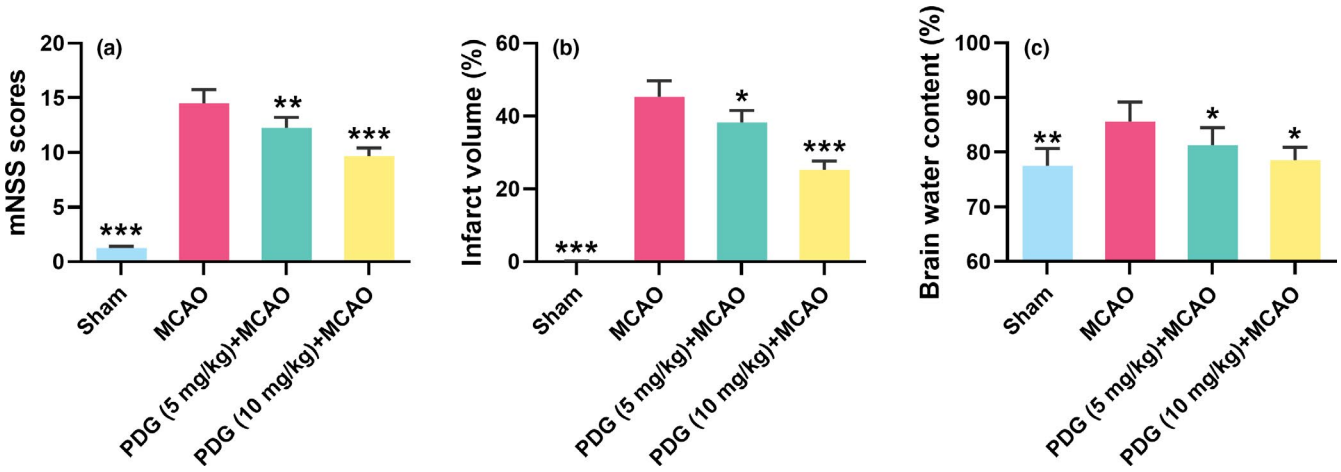

FIGURE 2 PDG could ameliorate the neurological deficit of mice with MCAO/R. Mice were randomly grouped into four groups: Sham group; MCAO group; MCAO + 5 mg/kg PDG group; MCAO + 10 mg/kg PDG group. Mice of MCAO + 5 mg/kg PDG group and MCAO + 10 mg/kg PDG group were respectively administered with 5 mg/kg PDG, 10 mg/kg PDG via the caudal vein. The equivalent volume of normal saline was used as the control in the MCAO group. (a) The effects of PDG on neurological deficit in mice were evaluated by mNSs score; (b) The effects of PDG on brain infarct volumes were measured by TTC staining; (c) The effects of PDG on the brain water content in mice were measured by dry-wet method. *$p < .05$, **$p < .01$, and ***$p < .001$ versus the MCAO group [Colour figure can be viewed at wileyonlinelibrary.com]

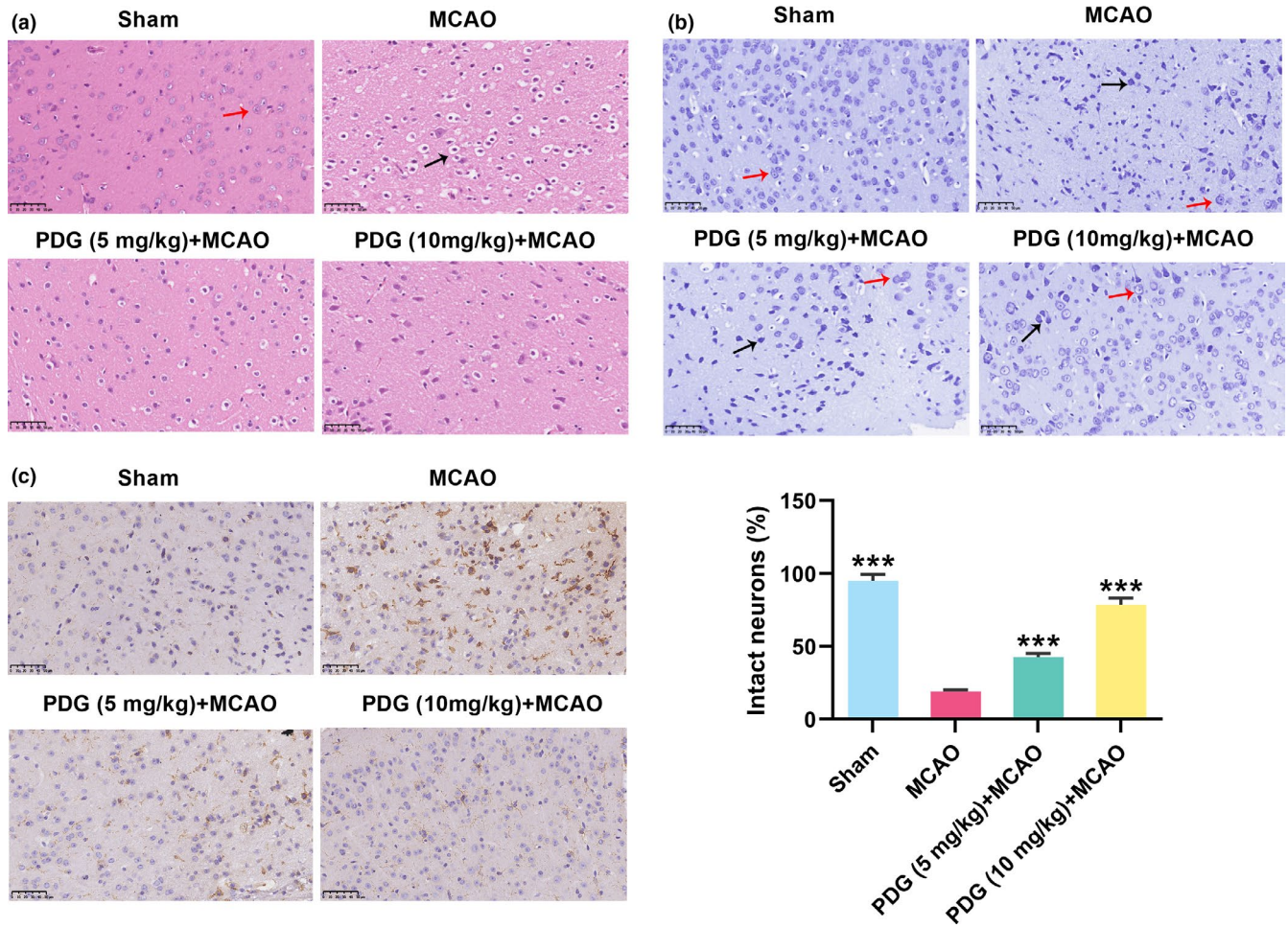

FIGURE 3 PDG could alleviate pathological changes of the neurons of mice with MCAO/R. (a) HE staining was used to evaluate the effects of PDG on the neuronal injury of the mice in the Sham group, MCAO group, MCAO + 5 mg/kg PDG group, and MCAO + 10 mg/kg PDG group. Black arrows represent injured neurons; Red arrows represent intact neurons; (b) Nissl staining method was used to evaluate the effects of PDG on the neuronal injury of the mice in Sham group, MCAO group, MCAO + 5 mg/kg PDG group, and MCAO + 10 mg/kg PDG group. Black arrows represent injured neurons; Red arrows represent intact neurons; the histogram showed the statistical results of Nissl staining; (c) Immunohistochemistry assay was used to detect the expression of microglia activation marker Iba1. ***$p < .001$ versus the MCAO group. Bar = 50 μm [Colour figure can be viewed at wileyonlinelibrary.com]

morphology of neurons was normal, and cytoplasm and nucleus of the neurons were clear; however, in MCAO group, pyknotic nuclei could be observed, and the number of neurons decreased significantly. In PDG treatment groups, the structure of neurons was improved, the apoptotic morphology of neurons was significantly reversed (vs. MCAO/R group; Figure 3a). Toluidine blue staining suggested that in comparison with the Sham group, a large number of neurons in MCAO group were smaller, the space between cells was enlarged, the staining was deeper, and the number of Nissl's body was increased; however, these changes were reversed by PDG treatment (vs. MCAO/R group; Figure 3b). In addition, compared with MCAO group, the expression of Iba1 in PDG treatment groups was significantly decreased, which revealed that PDG could significantly inhibit the activation of microglia (Figure 3c).

3.3 | PDG inhibited the release of pro-inflammatory cytokines in brain tissue of mice with MCAO/R

We then investigated whether PDG modulated the inflammatory response induced by cerebral I/R. It was revealed that the contents of TNF-α, IL-6, and IL-1β in the brain tissues of mice with MCAO/R were significantly increased compared with the Sham group while those in PDG-treated mice were significantly decreased in a dose-dependent manner (vs. MCAO group; Figure 4a–c). Western blot was adopted to detect p-IKKβ, IKKβ, p-IkBα, IkBα, and NF-κB p65 protein expression in cytoplasm and nucleus, and it was revealed that nuclear p-p65 and the phosphorylation ratio of IKKβ and IkBα was significantly raised in MCAO group compared with Sham group, and PDG

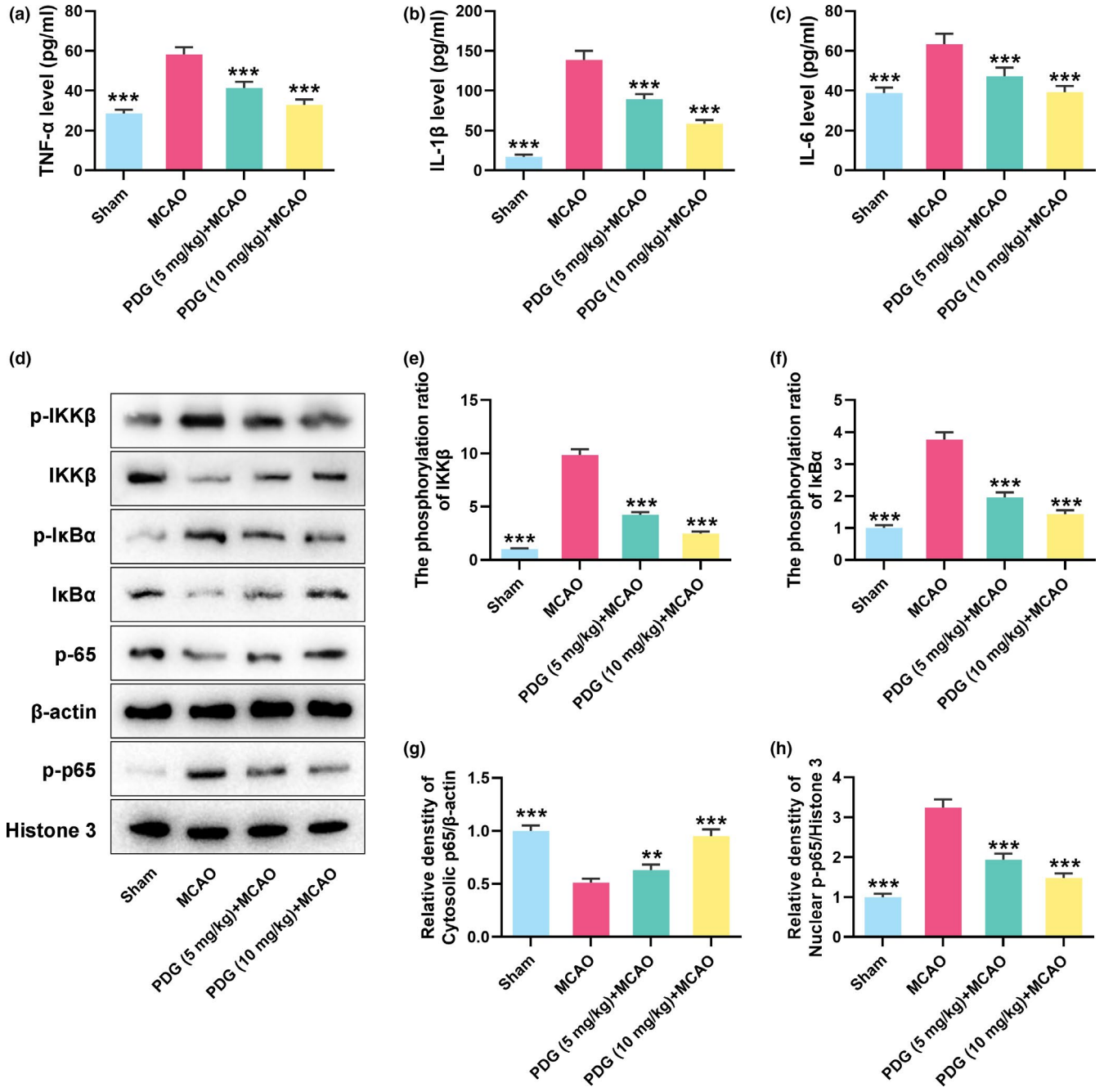

FIGURE 4 PDG inhibited the release of inflammatory cytokines from brain tissue of MCAO mice. (a–c) ELISA kit was used to detect the effects of PDG on the release of inflammatory cytokines TNF-α, IL-1 β, and IL-6 in brain tissues of the mice in the Sham group, MCAO group, MCAO + 5 mg/kg PDG group, and MCAO + 10 mg/kg PDG group. (d–h) Western blot was used to detect the effects of PDG on the expressions of p-IKKβ, IKKβ, p-IkBα, IkBα, NF-κB p65 and p-p65 in brain tissues of the mice in the Sham group, MCAO group, MCAO + 5 mg/kg PDG group, and MCAO + 10 mg/kg PDG group. $^{**}p < .01$, and $^{***}p < .001$ versus the MCAO group [Colour figure can be viewed at wileyonlinelibrary.com]

significantly restrained the phosphorylation ratio of IKKβ and IkBα and p65 activation, and inhibited the translocation of p65 protein into nucleus (Figure 4d–f). These data indicated that PDG could inhibit the release of inflammatory response during MCAO/R probably by inhibiting the activation of NF-κB signaling pathway.

3.4 | PDG reduced the oxidative stress in the brain tissues of the mice with MCAO/R

To further delve into the impacts of PDG on OS induced by I/R, the OS products in mice brain tissue were analyzed by colorimetry, and the findings revealed that the

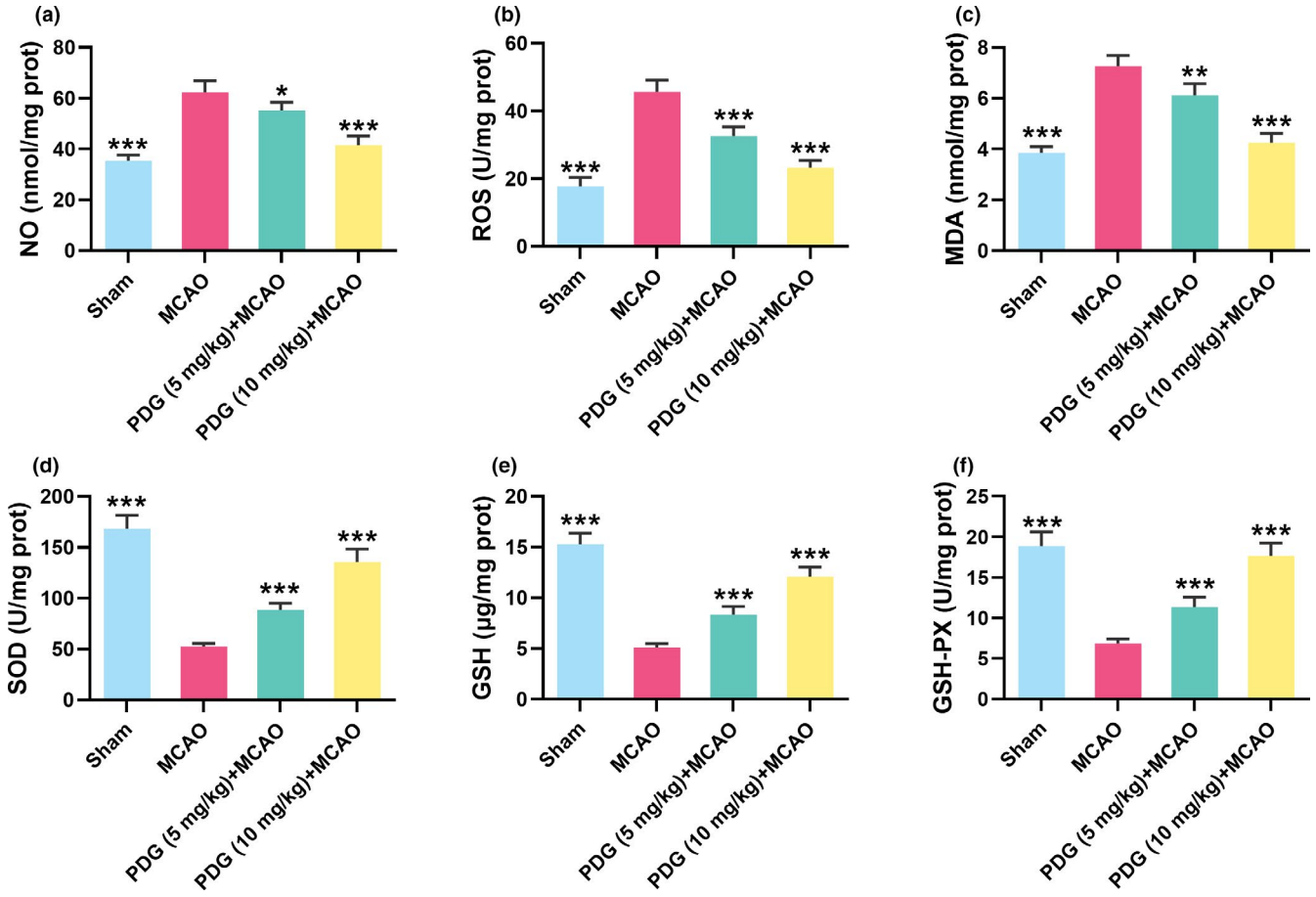

FIGURE 5 PDG reduced OS in brain tissues of the mice with MCAO/R. (a–c) The effects of PDG on the contents of NO, ROS, and MDA in brain tissues of mice in Sham group, MCAO group, MCAO + 5 mg/kg PDG group, and MCAO + 10 mg/kg PDG group were detected by colorimetry. (d–e) The effects of PDG on the activities of antioxidant enzymes SOD, GSH, and GSH-Px in brain tissues of the mice in Sham group, MCAO group, MCAO + 5 mg/kg PDG group, and MCAO + 10 mg/kg PDG group were detected. *$p < .05$, **$p < .01$, and ***$p < .001$ versus the MCAO group [Colour figure can be viewed at wileyonlinelibrary.com]

activities of NO, ROS, and MDA in mice brain tissue with MCAO/R were demonstrably higher than those in Sham group; however, PDG treatment reduced the activity of NO, ROS, and MDA (vs. MCAO group; Figure 5a–c). In addition, the activities of antioxidant and antioxidant enzyme were also detected, the results of which showed that the activities of SOD, GSH, and GSH-Px in MCAO group were markedly lower, compared with those in the Sham group, while PDG treatment increased the antioxidant ability (vs. MCAO group; Figure 5d–e).

3.5 | PDG played an antioxidant role via activating Nrf2/HO-1 signaling pathway

To detect whether PDG treatment could regulate Nrf2/HO-1 signaling pathway, we used Western blot to detect the expressions of Nrf2, HO-1 and NAD(P)H quinone dehydrogenase 1 (NQO1) in mice brain tissues. As shown, nuclear Nrf2 expression showed no significant change, but the expression levels of HO-1 protein and NQO1 protein were markedly decreased in MCAO group compared with those in the Sham group. Nrf2, HO-1 and NQO1 protein expressions in PDG treatment groups, compared with those in the MCAO group, were observably increased (Figure 6a–c). These data suggested that PDG could probably activate Nrf2/HO-1 signaling pathway to enhance the antioxidant ability of neurons, which ameliorated the injury induced by I/R.

4 | DISCUSSION

Ischemic stroke accounts for about 70%–80% of all cases of acute cerebrovascular diseases, with a high incidence and mortality, bringing a considerable burden to society (Homocysteine Studies Collaboration, 2002). Restoration of the blood supply, the main strategy for treating ischemic stroke, can cause a series of secondary pathological changes. Therefore, it is necessary to find new treatment

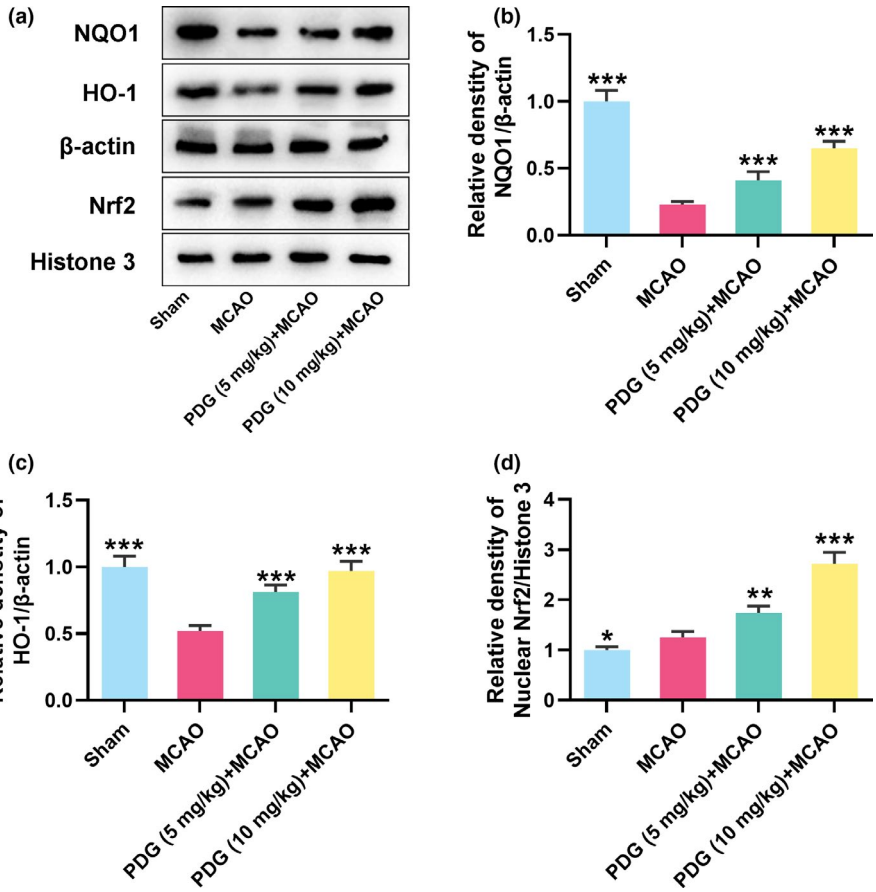

FIGURE 6 PDG played an antioxidant role by regulating the activation of Nrf2/HO-1 signaling pathway. (a–d) Western blot was used to detect the effects of PDG on Nrf2, HO-1 and NQO1 protein expressions in brain tissues of the mice in the Sham group, MCAO group, MCAO + 5 mg/kg PDG group, and MCAO + 10 mg/kg PDG group. *$p < .05$, **$p < .01$, and ***$p < .001$ versus the MCAO group [Colour figure can be viewed at wileyonlinelibrary.com]

methods to prevent I/R injury. In recent years, the neuroprotective effect of traditional Chinese medicine extracts on neurological function attracts a lot of attention (Chen, Chen, et al., 2020). PDG can regulate inflammation and OS (During et al., 2012; Yao et al., 2016). Specifically, for intestinal Caco-2 cells stimulated by IL-1β, pinoresinol shows stronger anti-inflammatory properties than some other plant lignans, including secoisolariciresinol diglucoside, secoisolariciresinol, lariciresinol, matairesinol, and hydroxymatairesinol (During et al., 2012); PDG alleviates oxLDL-induced dysfunction in human umbilical vein endothelial cells, accompanied by the inhibition of SOD activity, endothelial nitric oxide synthase expression, and NO production (Yao et al., 2016). Herein, for the first time, we confirmed that PDG could alleviate the neurological damage induced by I/R via repressing inflammation and OS.

Reportedly, neuroinflammation is one of the most vital pathological mechanisms of ischemic stroke (Khoshnam et al., 2017). In this process, microglia will be abnormally activated, which can secrete diverse pro-inflammatory cytokines and neurotoxic compounds, including TNF-α, IL-1 β, and IL-6, as well as NO, ROS, etc., causing damage to neurons (Lucas et al., 2006). In the present study, it was revealed that the contents of TNF-α, IL-1 β, and IL-6 in the brain tissues of the mice in MCAO group were significantly higher than those in the Sham group while PDG treatment could counteract those effects, suggesting that PDG could probably inhibit the activation of microglia. NF-κB is one of the most crucial pathways dominating the inflammatory response (Shih et al., 2015), and its activation is pivotal for the enhanced transcription of various inflammatory mediators, such as IL-1 β, TNF-α, and IL-6 (Takeda & Akira, 2004). Additionally, previous studies show that the inhibition of NF-κB signaling pathway can protect neurons from excitotoxicity and inflammation (Fann et al., 2018; Kaltschmidt et al., 1997; Sarnico et al., 2009; Zhai et al., 2020). For example, dexmedetomidine post-conditioning can alleviate cerebral I/R injury in rats via restraining the NF-κB signaling pathway (Zhai et al., 2020). In this work, it was demonstrated that PDG could inhibit NF-κB activation in the brain tissues of the mice after MACO/R, highlighting that PDG played an anti-inflammatory role by inhibiting the activation of NF-κB signal pathway.

In addition, OS features prominently in the pathogenesis of ischemic stroke, which mediates tissue injury and the deficits of neurological functions. With the continuous and excessive production of ROS, there is an imbalance in antioxidant defense system, leading to lipid peroxidation, protein and DNA damage, and the

accelerated apoptosis of neurons (Huang et al., 2018). Herein, we proved that PDG could markedly inhibit the production of NO, ROS, and MDA in brain tissue of the mice with MCAO/R and promote the activities of antioxidant enzymes, including SOD, GSH, and GSH-Px, proving that PDG could improve the antioxidant effect after I/R. Nrf2 is a member of the bZIP protein family; as a transcription factor, it modulates genes that contain antioxidant response elements (ARE) in their promoters, and many of these genes participate in regulating the production of free radicals (Gold et al., 2012). HO-1, also known as heat shock protein 32 (Hsp32), is a stress-inducible protein which regulates iron homeostasis, antioxidant defense, and anti-apoptosis (Wang et al., 2019). As reported, Nrf2/HO-1 signaling pathway plays a key role in fighting against oxidant stress induced by brain I/R injury (Chen, Cheng, et al., 2020; Guo et al., 2017; Zeng et al., 2019). For example, metformin can activate Nrf2/HO-1 signaling and inhibit OS induced by cerebral ischemia (Zeng et al., 2019); astragalin has a neuroprotective effect in cerebral ischemia-reperfusion injury via activating Nrf2/ARE/HO-1 signaling pathway (Chen, Cheng, et al., 2020). In this study, we further analyzed the molecular mechanism of PDG's effects on enhancing antioxidant effects and found that PDG could promote Nrf2, HO-1 and NQO1 expressions.

In conclusion, we substantiate the neuroprotective effects of PDG on brain I/R injury with a mice model. To be specific, PDG can alleviate inflammation and OS induced by I/R via regulating NF-κB and Nrf2/HO-1 pathways. Theoretically, PDG may be a promising drug to ameliorate neuronal injury, and improve the recovery of neurological function. However, its clinical application waits for more detailed and systemic pre-clinical data to evaluate its safety and efficacy on humans.

ACKNOWLEDGMENTS

This study was supported by grants from the Application of Continuous Lumbar Drainage in Patients with Severe Spontaneous SAH after Operation (Grant No. 2018XJ57). We thank Hubei Yican Health Industry Co., Ltd (Wuhan, China) for its linguistic assistance during the preparation of this manuscript.

CONFLICT OF INTEREST

None.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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How to cite this article: Zhang, Y., Lei, Y., Yao, X., Yi, J., & Feng, G. (2021). Pinoresinol diglucoside alleviates ischemia/reperfusion-induced brain injury by modulating neuroinflammation and oxidative stress. *Chemical Biology & Drug Design*, 98, 986–996. https://doi.org/10.1111/cbdd.13956