Article

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Gentiopicroside Ameliorates Cerebrovascular Angiogenesis, Neuronal Injury and Immune Disorder in Rats with Cerebral Ischemia/Reperfusion Injury via VEGF and Phosphorylated Nrf2 Elevation

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Background: Cerebral ischemia-reperfusion (CI/R) injury is induction of blood flow restoration after an ischemic stroke. Gentiopicroside (GPC) is the principal active secoiridoid glycoside of *Gentiana Manshurica Kitagawa*. This research aimed to illuminate the function of GPC and its mechanism in CI/R injury.

Methods: After CI/R injury models were constructed, GPC (25, 50 or 100 mg/kg) was then administered by gavage to rats. Rats were grouped into Sham, CI/R, CI/R+25 mg/kg GPC, CI/R+50 mg/kg GPC, and CI/R+100 mg/kg GPC. Neuronal cells were exposed to oxygen-glucose deprivation and reperfusion (OGD/R) injury to establish ischemic-like conditions *in vitro*, and cells were further treated with 25, 50, or 100 µM GPC. Cells were grouped into control, OGD/R, OGD/R+25 µM GPC, OGD/R+50 µM GPC, and OGD/R+100 µM GPC. GPC's function on rat cerebral injury, angiogenesis, oxidative stress, neuronal injury and immune dysfunction *in vivo* was estimated using hematoxylin-eosin staining, Western blot, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, commercial kits and enzyme linked-immunosorbent assay. Meanwhile, GPC's mechanism in CI/R injury was examined via Western blot. GPC's function *in vitro* was estimated via Cell Counting Kit-8 assay, 5-ethynyl-2'-deoxyuridine (EdU) staining, flow cytometry.

Results: GPC alleviated cerebral injury through decreasing cerebral infarction volume, cerebral indexes, brain water contents ($p < 0.05$). GPC reduced oxidative stress and boosted cerebral angiogenesis in CI/R rats ($p < 0.05$). Meanwhile, GPC weakened neuronal cell apoptosis, and decreased neuron-specific enolase and S100beta protein levels in CI/R rats. GPC reduced inflammatory cytokines contents in serum and brain tissues of CI/R rats ($p < 0.05$). Moreover, GPC increased the viability and proliferation in OGD/R-treated neuronal cells, but decreased cell apoptosis ($p < 0.05$). Mechanistically, GPC upregulated vascular endothelial growth factor (VEGF) and phosphorylated nuclear factor E2-related factor 2 (p-Nrf2) levels in CI/R rat brain tissues ($p < 0.05$).

Conclusions: GPC reduced cerebrovascular angiogenesis, neuronal injury and immune disorder in CI/R injury through elevating VEGF and p-Nrf2.

Keywords: cerebral ischemia-reperfusion injury; gentiopicroside; cerebrovascular angiogenesis; neuronal injury, immune disorder

Introduction

The brain possesses a complex function, poor tolerance to ischemia and hypoxia, and requires a constant supply of oxygen and glucose from blood circulation [1]. Cerebral vessels exert a vital self-regulation function, but when cerebral blood flow is reduced to meet metabolic needs, ischemic brain injury will occur [2]. Cerebrovascular disease is a lethal disease with high morbidity, mortality and disability, and seriously threatens public health [3]. Cerebral ischemia-reperfusion (CI/R) injury is normally caused by ischemic stroke, and neurodegenerative disorders induced by CI/R injury impair human memory and learning abilities [4,5]. In light of relevant reports, CI/R injury is interrelated to oxidative stress, cerebrovascular angiogenesis, neuronal injury and immune disorder [6–8]. Currently, there is still a lack of effective therapeutic strategies to alleviate CI/R injury. Thus, investigating mechanism of CI/R injury and exploring drugs to alleviate CI/R injury has become the focus of cerebral ischemia therapy.

Gentiopicroside (GPC) is a kind of iridoid glycoside separated from the roots of perennial herbs in the Gentianaceae family [9]. Accumulated studies expound that GPC has extensive pharmacological effects, consisting of

anti-inflammatory, antioxidant [10]. GPC is highly hydrophilic due to the presence of sugar fragments in its structure, which reduces its oral bioavailability and limits its efficacy [11]. The bioavailability of oral GPC is 39.6%, and the area under concentration-time curve of all tissues after intravenous injection was kidney > serum > liver > brain [12]. As has been reported, GPC relieves osteoarthritis, hepatitis, osteoclast generation, and alcoholic hepatic bone hyperplasia [13]. With the continuous deepening of research, the therapeutic function of GPC on the central nervous system has been authenticated. For instance, Deng *et al*. [14] verified that GPC protects against lipopolysaccharide (LPS)-induced depression-like behavior by regulating tryptophan degradation pathway. Yao *et al*. [15] enunciated that GPC weakens hippocampus cell apoptosis and enhances cell proliferation in depressed animals by mediating metabolites, providing a theoretical basis for depression clinical treatment. Nuclear factor E2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) axis is involved in mediating inflammation in CI/R injury [16]. As far as we know, whether GPC alleviates CI/R injury and whether it is related to Nrf2/HO-1 axis is undisclosed.

We aimed to clarify GPC's mechanism in CI/R injury. We provided preliminary confirmation that cerebral infarction volume, cerebral indexes, brain water content were decreased, elucidating that GPC might possess a protective function in CI/R injury. Based on these findings, we further illuminated underlying mechanisms by which GPC exerted its protective function in CI/R injury, aiming to provide novel insights and promising drugs for CI/R injury.

Materials and Methods

Animals

Sprague-Dawley (SD) rats (200 ± 10 g, 8 weeks) were provided by Shulaibao Wuhan Biotechnology (Wuhan, China). Animal protocol was approved by Institutional Animal Care and Use Committee of the First Affiliated Hospital of Soochow University (2020-SCU-05).

Construction of an MCAO/IR Rat Model

Thirty rats were group: Sham, CI/R, CI/R+25 mg/kg GPC, CI/R+50 mg/kg GPC, CI/R+100 mg/kg GPC.

Middle cerebral artery occlusion/ischemia-reperfusion (MCAO/IR) was implemented following previously reported methods [17]. After rats were anesthetized with isoflurane (26675-46-7, MACKLIN, Shanghai, China), common carotid artery (CCA) was then separated from adjacent muscles to expose external carotid artery (ECA). Thereafter, internal carotid artery (ICA) was inserted from CCA and then turned to ECA origin until it blocked middle cerebral artery (MCA) origin. Blood flow to right MCA was blocked. After one hour of occlusion, suture was detached from vessel and reperfused for 24 h. In sham, right MCA was isolated without suture insertion. Meanwhile, 25, 50 or 100 mg/kg GPC (high-performance liquid chromatography (HPLC) ≥98%, 356.32, $C_{16}H_{20}O_9$, B20763, Shanghai Yuanye, Shanghai, China) was administered by gavage to rats [18,19]. Ultimately, rats were killed with phenobarbital intraperitoneal injection (57-30-7, Sigma-Aldrich, Shanghai, China). Rat brain tissues were isolated for subsequent studies.

Cell Culture and Treatment

Neuronal cells were provided by SUNNCELL (Wuhan, China). The purity of neuronal cells was identified using neuron-specific enolase (NSE) immunofluorescence and reached more than 90%. Neuronal cells did not contain bacteria, fungi, mycoplasma, infectious viruses, etc. Meanwhile, neuronal cells did not contain mycoplasma and referred to the website of ExPASy Cellosaurus databases (https://www.cellosaurus.org) for identification information. Neuronal cells were grown in Dulbecco's modified Eagle medium (DMEM, D5030, Sigma-Aldrich) with 10% fetal bovine serum (FBS, 12103C, Sigma-Aldrich). Cells were cultured at 37 °C, 5% CO_2.

Neuronal cells were maintained in glucose-free DMEM before inducing oxygen-glucose deprivation and reperfusion (OGD/R) injury. Cells were then placed in hypoxic incubator (CB260, Binder, Tuttlinger, Baden, Germany) containing 1% O_2, 5% CO_2, and 94% N_2 for an appropriate time to simulate OGD injury. Culture was then restored with glucose in DMEM and under normal oxygen (37 °C, 5% CO_2) for 12 h (OGD recovery) [20]. Meanwhile, neuronal cells were treated with 25, 50, or 100 μM GPC [21].

Detection of Infarction Volume, Cerebral Indexes, and Brain Water Content

Rat brain tissues were fixed using 4% paraformaldehyde (PN4410, G-CLONE, Beijing, China), embedded in paraffin, and sliced into 2 mm thick sections. Sections were stained with 1% triphenyl tetrazolium chloride solution (T8170, Solarbio, Beijing, China) for 25 min at room temperature (RT). Infarction volume was tested by Image J (Version 1.8.0, NIH, Bethesda, MD, USA).

Wet weight of brain tissues and body weight of rats were tested. Cerebral indexes = wet brain weight/body weight [8,22]. Brain tissues of rats were immediately weighed for wet weight. Then, rat brain tissues were placed in 100 °C oven and baked for 72 h to gain dry weight. Brain water contents were: (wet weight – dry weight)/wet weight × 100%.

Hematoxylin-Eosin Staining

Rat brain tissues were collected and made into 5 μm sections. Then, sections were exposed to hematoxylin (Solarbio). Thereafter, sections were further stained with 1% eosin solution (G1100, Solarbio). Images were acquired with microscope (DM500, Leica, Shanghai, China). Rats'

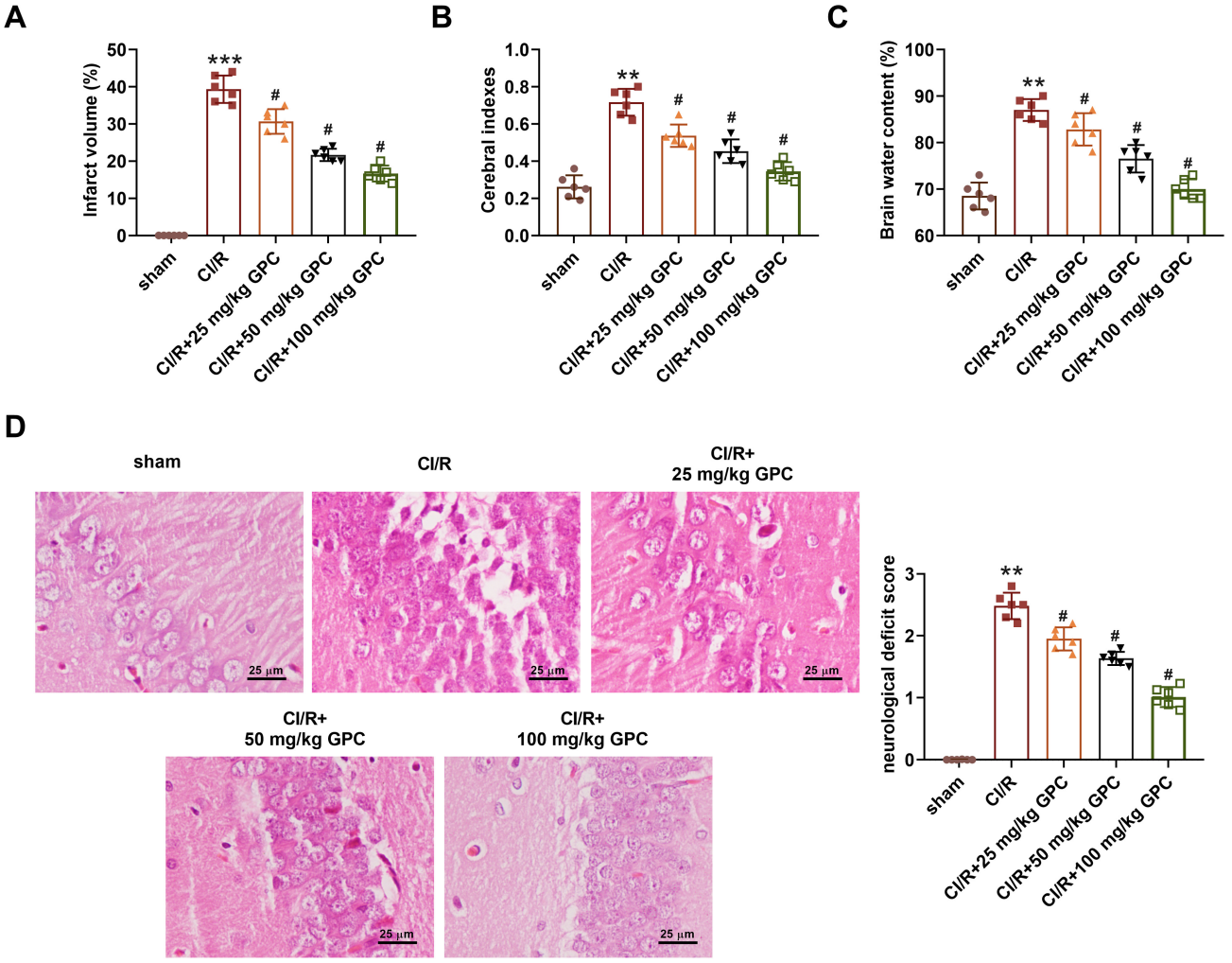

Fig. 1. Gentiopicroside mediates cerebral injury induced by cerebral ischemia-reperfusion in rats. After rat middle cerebral artery occlusion (MCAO) models were established, 25, 50, or 100 mg/kg gentiopicroside (GPC) was further administered by gavage to rats. (A–C) Cerebral infarction volume, cerebral indexes, brain water contents. (D) Rat cerebral injury was examined via hematoxylin-eosin (HE) staining (scale bar: 25 μm). $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. sham. $^{\#}p < 0.05$ *vs*. CI/R. CI/R, cerebral ischemia-reperfusion.

neurological deficit scores were displayed: 0 represents no motor disability, 1 represents limb weakness and limbs can not fully extend, 2 represents circling to one side, 3 represents being unconscious to ambulate spontaneously [8,23].

Glutathione, Superoxide Dismutase and Malondialdehyde Levels Detection

Rat brain tissues were placed in cold normal saline (1:10, w/v), homogenized by a homogenizer (Sinaekato, Shanghai, China), centrifuged (3000 r/min, 15 min), and supernatant was prepared. Glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) contents in supernatant were tested with GSH, SOD, and MDA Commercial Kits (ml077379 Mlbio, Shanghai, China).

Western Blot

After proteins were extracted with radioimmunoprecipitation assay (RIPA, R0010, Solarbio), protein concentrations were further quantified via Bicinchoninic Acid (BCA) Kits (BI-WB005-200T, Sbjbio, Nanjing, China). All proteins (25 μg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, BI-WB051, Sbjbio). Soon afterward, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (abs932, Absin, Shanghai, China). Membranes exposed to primary antibodies against platelet/endothelial cell adhesion molecule-1 (CD31, ab32457, 1 μg/mL, Abcam, Cambridge, UK), doublecortin (DCX, ab18723, 1 μg/mL, Abcam), cleaved caspase 3 (ab2302, 1/200, Abcam), phosphorylated Nrf2 (p-Nrf2, ab76026, 1/5000, Abcam), Nrf2 (ab137550, 1/1000, Abcam), heme oxygenase-1 (HO-1, ab305290, 1/1000, Abcam), vascular endothelial growth factor (VEGF, #2479, 1/1000), vascular endothelial growth factor receptor-2 (VEGFR2, ab39638, 1/500, Abcam), β-actin (ab8226, 1 μg/mL, Abcam). Membranes were washed and were then exposed to secondary antibodies (ab205719,

1/2000, Abcam) at RT for 2 h. All bands were examined with Gel-Pro-Analyzer software (Version 6.3, Media Cybernetics, MD, USA).

TUNEL Staining

Hippocampal neurons of rats were fixed with 4% formaldehyde (PN4410, G-CLONE). Cells were infiltrated with 0.2% TritonX-100 (T8787, Sigma-Aldrich). Cells were exposed to terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) solution (E-CK-A320, Elabscience, Wuhan, China) for 1 h as per manufacturer's instructions. TUNEL-positive cells were estimated with microscope.

Enzyme Linked-Immunosorbent Assay

Rat blood was centrifuged (3000 rpm, 10 min) to gain serum. NSE and S100beta (S100β) contents in serum were quantified using NSE ELISA Kit (ab233626, Abcam) and S100β ELISA Kit (ab234573, Abcam).

Interleukin-1beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interleukin-18 (IL-18) levels in rat serum and brain tissues were determined with ELISA Kits (Spbio, Wuhan, China) [24]. Absorbance was tested at 450 nm.

Cell Counting Kit-8 Analysis

Neuron cells (3×10^5 cells) were put in six-well plates with 2 mL of complete medium and cultured. Optical density (OD) at 450 nm was tested using microplate reader (Model 680, Bio-Rad, Hercules, CA, USA). Images were observed using microscope (Leica). All images were assessed using Image J.

EdU Staining

After neuron cells were exposed to 10 µmol/L 5-ethynyl-2'-deoxyuridine (EdU, ab219801, Abcam), cells were fixed by 4% paraformaldehyde. Cells were infiltrated with 0.3% Triton X-100 (T8787, Sigma-Aldrich), and cells were further co-stained with 5 µg/mL Apollo fluorescent dye (ab219801, Abcam) and 4',6-diamidino-2-phenylindole (DAPI, C1002, Beyotime, Shanghai, China). EdU-positive cells were counted with fluorescence microscope.

Flow Cytometry

Neuron cells were stained with 5 µL Fluorescein isothiocyanate (FITC) Annexin V and 10 µL propidium iodide with Annexin V-FITC Apoptosis Detection Kits (CBA059, Sigma-Aldrich) in the dark. Stained cells were examined with flow cytometry (FC500, Beckman Coulter, Shanghai, China).

Statistical Analysis

All data were shown as mean $\pm$ SD. Statistical differences between multiple groups were examined using one-way Analysis of variance (ANOVA) followed by Tukey's post-test. Statistical differences among groups were assessed using SPSS 19.0 (IBM, Chicago, IL, USA) and GraphPad Prism (Version 5.0, GraphPad Software, Inc., San Diego, CA, USA). $p < 0.05$ represented results were statistically significant.

Results

GPC Attenuates Rat Cerebral Injury Induced by CI/R

As emerged in Fig. 1A–C, cerebral infarction volume, cerebral indexes, brain water content were gradually decreased with increased GPC doses ($p < 0.05$ *vs.* sham or CI/R). Meanwhile, cerebral injury degree in GPC was evidently less severe than that in CI/R, and the higher the GPC dose, the lower the cerebral injury degree (Fig. 1D). Also, rat neurological deficit scores were presented in Fig. 1D ($p < 0.05$ *vs.* sham or CI/R). Our experimental data illustrated that GPC mitigated rat cerebral injury caused by CI/R.

GPC Relieves Oxidative Stress and Enhances Cerebral Angiogenesis in CI/R Rats

To elucidate GPC neuroprotective mechanism, we investigated whether GPC had an antioxidant function. As shown in Fig. 2A–C, the levels of GSH and SOD (antioxidant markers) in CI/R were decreased more than twofold, and MDA levels (oxidative marker) in CI/R were raised more than twofold in comparison with sham, while GPC abolished these impacts ($p < 0.05$ *vs.* sham or CI/R).

Meanwhile, cerebral angiogenesis markers CD31 and DCX protein levels in CI/R+GPC were higher than that in CI/R, and CD31, DCX expressions were up-regulated with the increased GPC doses ($p < 0.05$ *vs.* sham or CI/R, Fig. 2D). Summarily, GPC alleviated oxidative stress and strengthened cerebral angiogenesis in CI/R rats.

GPC Mitigates Rat Neuronal Injury Induced by CI/R

Subsequently, we further evaluated the GPC's impact on neuronal injury in CI/R rats. TUNEL staining clarified that neuronal cell apoptosis was enhanced in CI/R, but neuronal cell apoptosis was weakened in CI/R+GPC ($p < 0.05$ *vs.* sham or CI/R, Fig. 3A,B). Meanwhile, cleaved caspase 3 were increased in CI/R brain tissues, while cleaved caspase 3 was decreased with different GPC concentrations ($p < 0.05$ *vs.* sham or CI/R, Fig. 3C). As displayed in Fig. 3D, GPC partially reversed high protein levels of brain-relevant markers NSE and S100β caused by CI/R ($p < 0.05$ *vs.* sham or CI/R). To sum up, GPC reduced rat neuronal injury caused by CI/R.

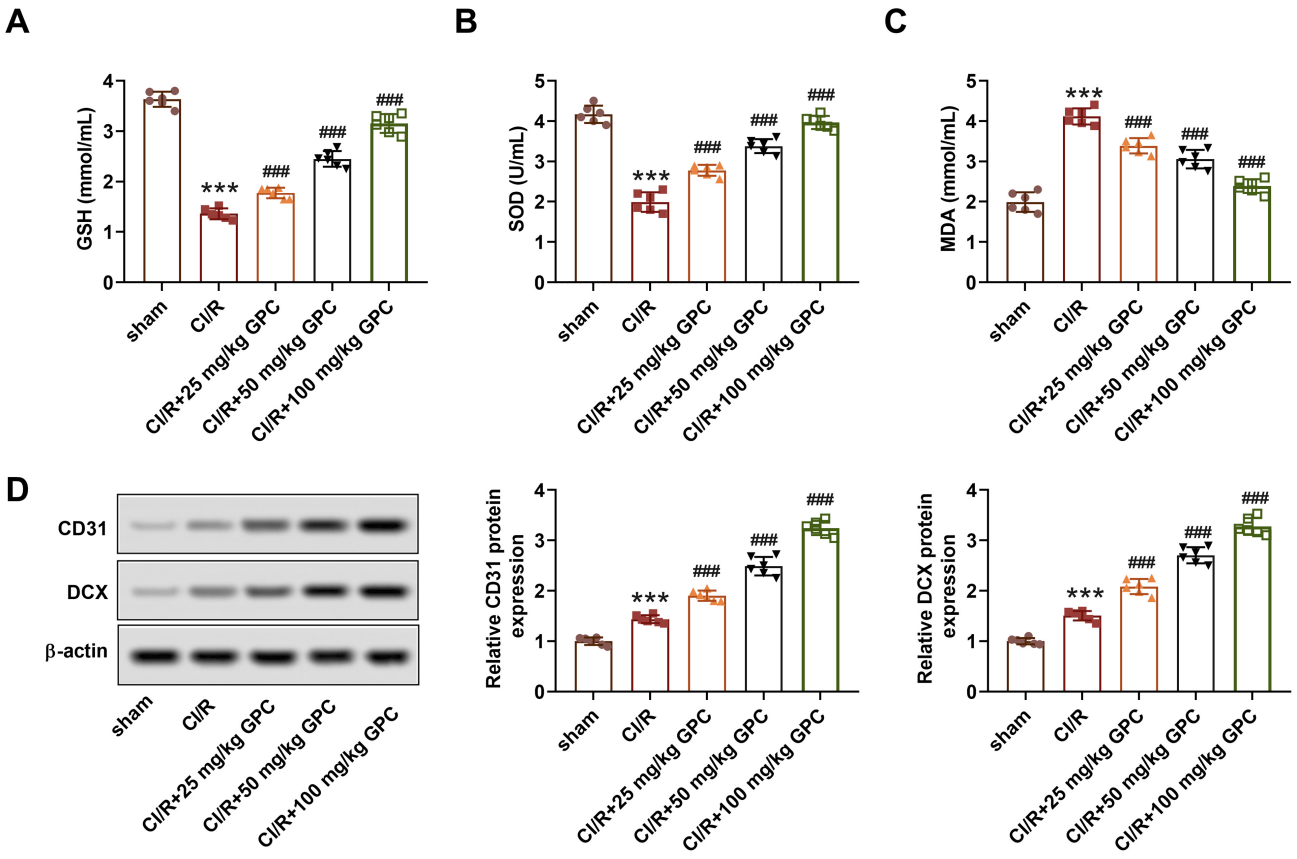

Fig. 2. GPC modulates oxidative stress and cerebral angiogenesis in CI/R rats. Followed by rat MCAO models were established, 25, 50, or 100 mg/kg GPC was then administered by gavage to rats. (A–C) Glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) levels were tested using Commercial Kits. (D) Distinction of platelet/endothelial cell adhesion molecule-1 (CD31) and doublecortin (DCX) protein levels. ***$p < 0.001$ *vs.* sham. $^{###}p < 0.001$ *vs.* CI/R.

GPC Reduces Immune Dysfunction of Brain Tissues Induced by CI/R in Rats

To further clarify GPC's impact on immune dysfunction of brain tissues of CI/R rats, inflammatory cytokines IL-1β, IL-6, TNF-α and IL-18 levels in serum and brain tissues were checked. As emerged in Fig. 4A,B, IL-1β, IL-6, TNF-α, IL-18 contents were raised in CI/R, while IL-1β, IL-6, TNF-α and IL-18 were lessened with increasing GPC doses ($p < 0.05$ *vs.* sham or CI/R). To sum up, GPC possessed the function of slowing down the immune dysfunction of CI/R rat brain tissues.

GPC Regulates VEGF and p-Nrf2 Levels in Brain Tissues of CI/R Rats

Nrf2-HO-1 axis exerts vital functions in CI/R [25]. Here, p-Nrf2, Nrf2, and HO-1 protein levels in rat brain tissues were further examined. Western blot confirmed that CI/R induced an increase in p-Nrf2 and HO-1 protein levels, and p-Nrf2 and HO-1 were further increased after GPC treatment, while Nrf2 levels remained unchanged among groups ($p < 0.05$ *vs.* sham or CI/R, Fig. 5A). VEGF is a pivotal mediator of angiogenesis and signals through VEGFR2 [26]. As emerged in Fig. 5B, VEGF and VEGFR2 expressions were raised in CI/R, and GPC further increased VEGF and VEGFR2 protein levels compared with CI/R ($p < 0.05$ *vs.* sham or CI/R). In summary, GPC increased VEGF and p-Nrf2 expressions in CI/R rat brain tissues.

GPC Mediates Neuronal Cell Viability, Proliferation, and Apoptosis

Subsequently, we further investigated GPC's impact on neuronal cell growth. As exhibited in Fig. 6A, neuronal cell viability in GPC was prominently higher than that in OGD/R ($p < 0.05$ *vs.* control or OGD/R). Western blot confirmed that VEGF protein levels were raised in OGD/R, and GPC further enhanced this raise ($p < 0.05$ *vs.* control or OGD/R, Fig. 6B). Meanwhile, EdU positive cell percent was lessened in OGD/R, yet GPC reversed this decrease ($p < 0.05$ *vs.* control or OGD/R, Fig. 6C). Neuronal cell apoptosis showed opposite trends ($p < 0.05$ *vs.* control or OGD/R, Fig. 6D). In short, GPC enhanced neuronal cell viability, proliferation, and weakened cell apoptosis.

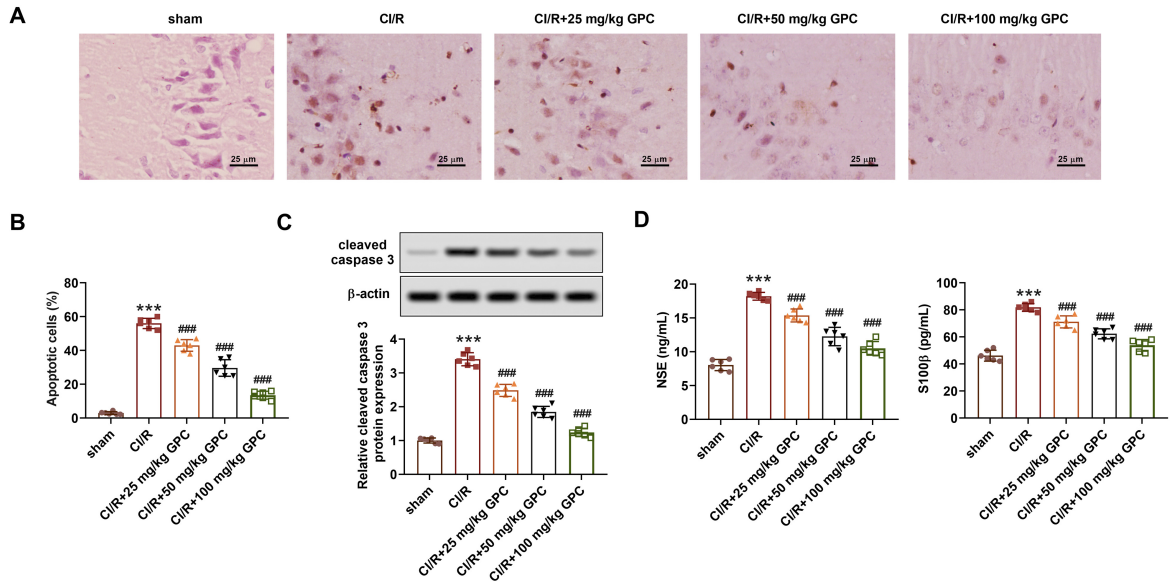

Fig. 3. Impact of GPC on rat neuronal injury caused by CI/R. After establishing rat MCAO models, 25, 50, or 100 mg/kg GPC was further administered by gavage to rats. (A,B) Neuronal cell apoptosis was checked via terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining (scale bar: 25 μm). (C) Cleaved caspase 3 protein levels. (D) Contents of neuron-specific enolase (NSE) and S100beta (S100β) were tested via enzyme linked-immunosorbent assay (ELISA). ***$p < 0.001$ *vs.* sham. $^{\#\#\#}p < 0.001$ *vs.* CI/R.

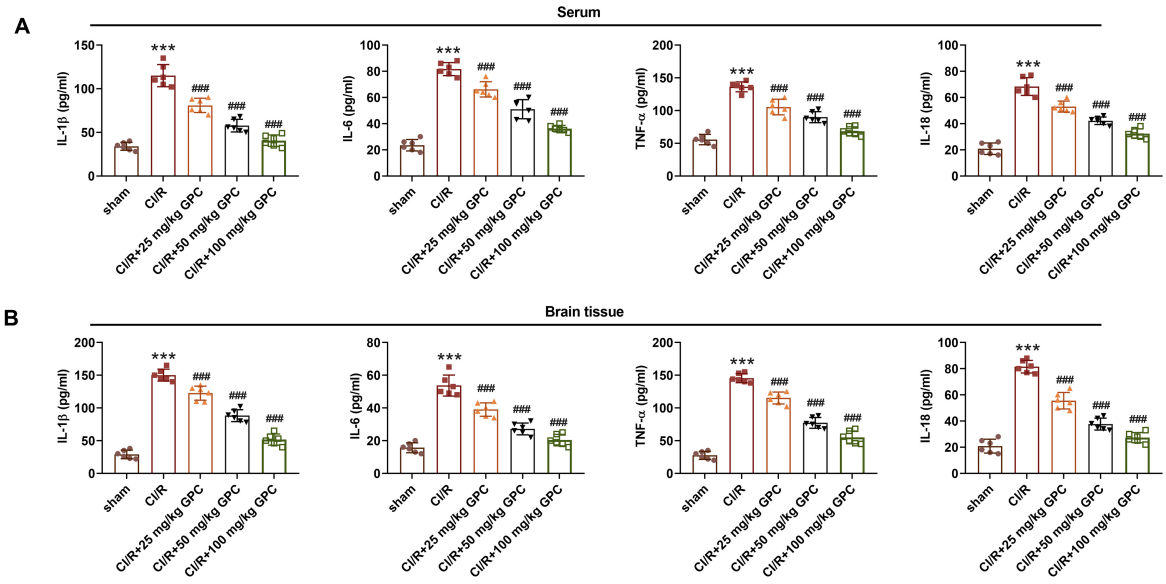

Fig. 4. GPC regulates immune dysfunction of brain tissues caused by CI/R in rats. Rat MCAO models were established, and 25, 50, or 100 mg/kg GPC was then administered by gavage to rats. (A,B) Comparison of interleukin-1beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interleukin-18 (IL-18) levels using ELISA. ***$p < 0.001$ *vs.* sham. $^{\#\#\#}p < 0.001$ *vs.* CI/R.

Discussion

Due to threat of CI/R injury to human health [27], it is urgent to excavate clinical therapeutic drugs that alleviate CI/R injury. GPC possesses protective functions in numerous human diseases [28,29]. Analogously, our study preliminarily confirmed that GPC attenuated rat cerebral injury induced by CI/R. Also, we further indicated that GPC mitigated oxidative stress and enhanced cerebral angiogenesis, and reduced neuronal injury in CI/R rats. Meanwhile, GPC possessed the function of relieving immune dysfunction of CI/R rat brain tissues. Also, neuronal cell viability and proliferation in GPC were higher than that in OGD/R, and neuronal cell apoptosis displayed an opposite trend. Furthermore, we illustrated that GPC increased the expressions of VEGF and p-Nrf2 in CI/R rat brain tissues.

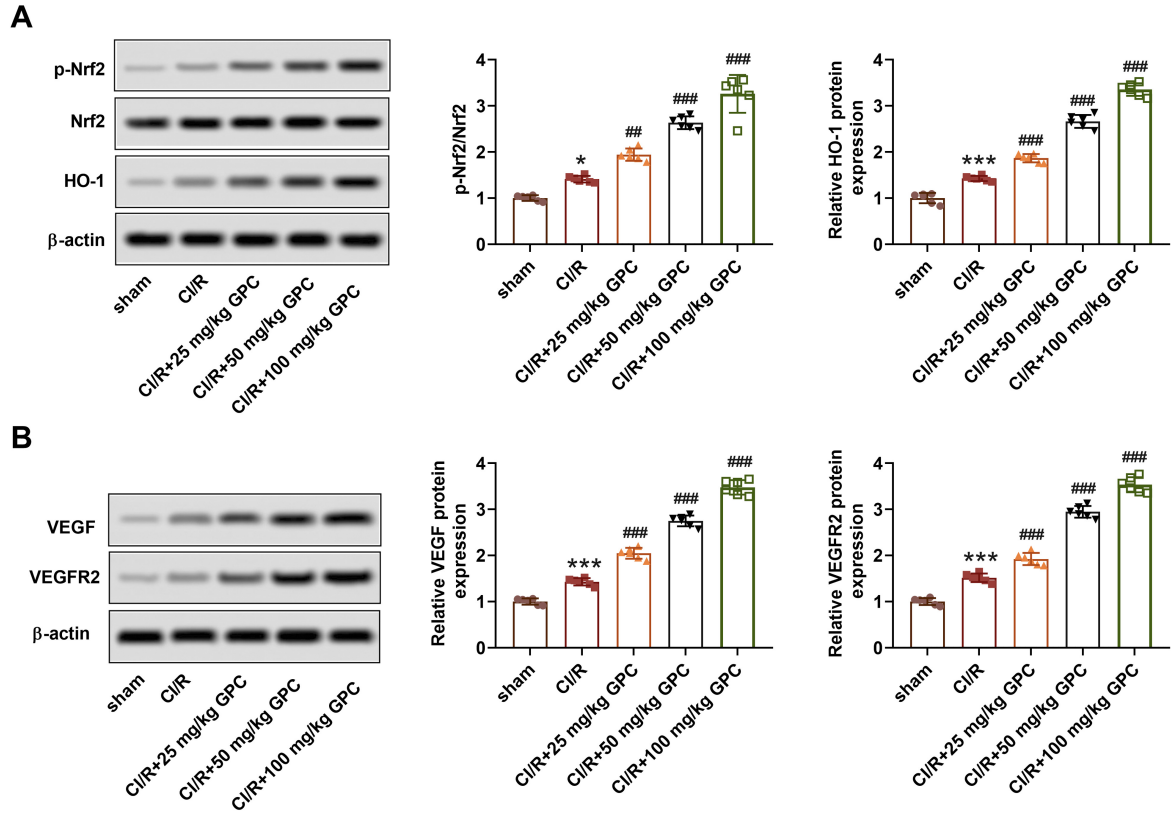

Fig. 5. GPC influences VEGF and phosphorylated Nrf2 expressions in CI/R rat brain tissues. After rat MCAO models were established, 25, 50, or 100 mg/kg GPC was administered by gavage to rats. (A) Detection of phosphorylated nuclear factor E2-related factor 2 (p-Nrf2), Nrf2 and heme oxygenase-1 (HO-1) protein levels. (B) Vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor-2 (VEGFR2) protein levels were determined. $^{*}p < 0.05$, $^{***}p < 0.01$ *vs.* sham. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* CI/R.

GPC is the major active secoiridoid glycoside of *Gentiana Manshurica Kitagawa* [30]. GPC represses the progress of various diseases by reducing the inflammatory signaling axes. For example, GPC prevents renal inflammation in diabetic mice by mediating the TGR5-β-arrestin2-NF-κB axis, and ultimately ameliorates the pathological process of diabetic renal fibrosis [29]. GPC reduces osteoclast genesis via restraining c-Jun N-terminal kinase (JNK) and nuclear factor-kappaB (NF-κB) axes, implying that GPC is an osteoporosis therapeutic drug [31]. Correspondingly, our research elucidated that GPC reduced cerebral infarction volume, cerebral indexes, brain water content, and GPC relieved cerebral injury caused by CI/R.

Oxidative stress is an inducement to aggravate injury after cerebral ischemia [32]. Antioxidant biomarkers GSH, SOD, and oxidative biomarker MDA are normally applied to indicate oxidative stress [33]. Crucially, the protective effect of GPC on adjuvant-induced arthritis rats is related to repressing oxidative stress [34]. In this research, we also confirmed that GPC raised the GSH, SOD levels, and lessened MDA contents, which implied that GPC restrained oxidative stress caused by CI/R. Meanwhile, the increase in cerebral angiogenesis is beneficial to reduce CI/R injury [35]. Considering that CD31 and DCX are cerebral angiogenesis markers [36], our study further revealed that GPC increased the protein levels of CD31 and DCX, suggesting that GPC enhanced cerebral angiogenesis in CI/R rats.

Excessive neuronal cell apoptosis is another pathological characteristic of CI/R injury [37]. Previous studies expound that propofol reduces CI/R injury by enhancing the viability of hippocampal neurons and reducing cell apoptosis [38]. Rutaecarpine alleviates CI/R injury by lessening caspase 3 activation and B cell lymphoma-2 associated protein X apoptosis regulator (Bax) expression to reduce neuronal cell apoptosis [16]. As expected, we confirmed that GPC repressed neuronal cell apoptosis in CI/R rats and reduced the cleaved caspase 3 protein levels in CI/R brain tissues. Furthermore, our experimental data illustrated that GPC reversed CI/R-induced high expressions of NSE and S100β (brain-related markers), which implied that GPC mitigated neuronal injury in CI/R rats.

Accumulating studies suggest that inflammatory mediators are pivotal stimulative factors of cerebral ischemic injury [39,40]. As we all know, IL-1β, IL-6, TNF-α and IL-18 are commonly used proinflammatory factors [41]. Peng *et al.* [42] indicated that interference with ATG3 relieves

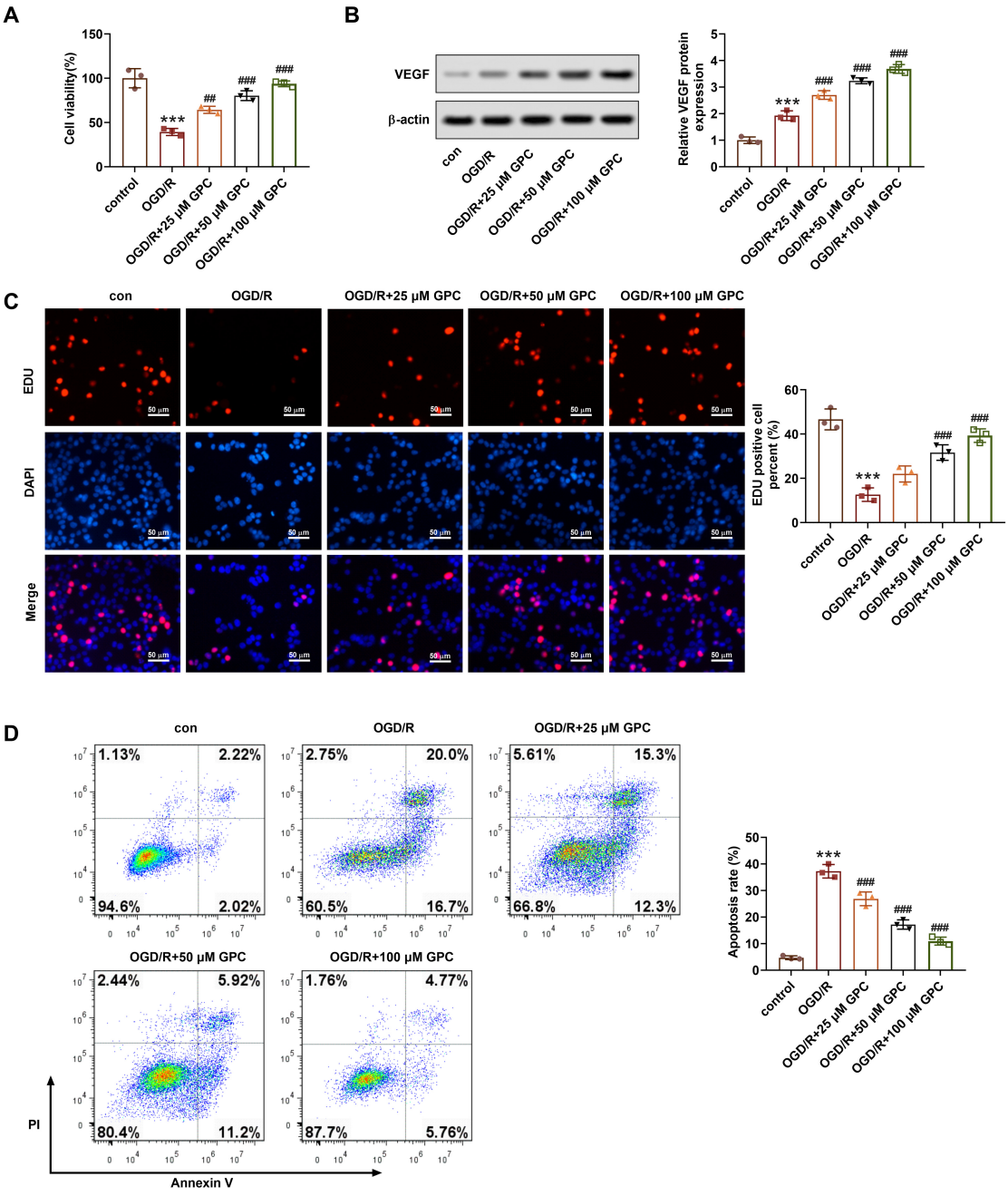

Fig. 6. GPC regulates neuronal cell viability, proliferation, and apoptosis. Neuronal cells were exposed to oxygen-glucose deprivation and reperfusion (OGD/R) injury to establish ischemic-like conditions *in vitro* models. Neuronal cells were further treated with 25, 50, or 100 μM GPC. (A) Neuronal cell viability was checked via Cell Counting Kit-8 (CCK-8). (B) Analysis of VEGF protein levels. (C) Neuron cell proliferation was detected with 5-ethynyl-2'-deoxyuridine (EdU) (scale bar: 50 μm). (D) Neuronal cell apoptosis was examined using flow cytometry. $^{***}p < 0.001$ *vs.* control. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* OGD/R. DAPI: 4',6-diamidino-2-phenylindole.

brain microvascular endothelial cell inflammation induced by OGD/R through reducing IL-1β, IL-6, TNF-α. Chen *et al.* [43] expounded that maraviroc alleviates the neuroinflammatory response after CI/R injury by decreasing proinflammatory factor levels. Crucially, our data also illustrated that GPC decreased IL-1 β, IL-6, TNF-α, IL-18 contents in CI/R rats, implying that GPC owned the functions of repressing immune dysfunction of CI/R rats.

A previous study has shown that a large amount of Nrf2 exists in an inactive form [44]. Under pathological conditions, active Nrf2 activates transcription of multiple antioxidant and detoxification genes, which aggravates the occurrence of diseases [45,46]. Accumulating evidence validates that Nrf2-HO-1 is a vital axis in CI/R injury [16,47]. Crucially, another research indicates that GPC reduces oxidative injury and lipid accumulation in nonal-

coholic fatty liver disease by increasing Nrf2 [10]. As expected, our data indicated that GPC increased p-Nrf2/Nrf2, HO-1 expressions in CI/R rats. Previous studies expound that VEGF activates cell proliferation and angiogenesis by binding to VEGFR2 [48]. Meanwhile, we further confirmed that GPC increased VEGF and VEGFR2 expressions in CI/R rats, implying that GPC reduced CI/R injury by increasing VEGF and p-Nrf2 levels.

Conclusions

This research enucleated function of GPC and its underlying mechanisms in CI/R rats. Our data demonstrated that GPC mitigated cerebrovascular angiogenesis, neuronal injury and immune disorder in CI/R rats through elevating VEGF and p-Nrf2. As a limitation of this research, we did not conduct *in vitro* studies to elucidate the protective function of GPC against CI/R injury, and we would enrich this research in the future.

Abbreviations

CI/R, cerebral ischemia-reperfusion; GPC, gentiopicroside; p-Nrf2, phosphorylated nuclear factor E2-related factor 2; AUC, area under the concentration-time curve; LPS, lipopolysaccharide; SD, Sprague-Dawley; MCAO/IR, middle cerebral artery occlusion/ischemia-reperfusion; CCA, common carotid artery; ECA, external carotid artery; MCA, middle cerebral artery; NSE, neuron-specific enolase; FBS, fetal bovine serum; TTC, triphenyl tetrazolium chloride; RT, room temperature; HE, hematoxylin-eosin; GSH, glutathione; SOD, superoxide dismutase; MDA, malondialdehyde; BCA, Bicinchoninic Acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; DCX, doublecortin; HO-1, heme oxygenase-1; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; ELISA, enzyme linked-immunosorbent assay; CCK-8, Cell Counting Kit-8; OD, optical density; EdU, 5-ethynyl-2'-deoxyuridine; PI, propidium iodide.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

LZ and XLC contributed to the conception of the study and wrote the paper; CX contributed significantly to analysis and manuscript preparation; GC made substantial contributions to conception and design of the study, and was involved in revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Animal procedures were approved by Institutional Animal Care and Use Committee of the First Affiliated Hospital of Soochow University (2020-SCU-05) and were implemented following ARRIVE guidelines (https://arriveguidelines.org).

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Conflict of Interest

The authors declare no conflict of interest.

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