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ORIGINAL RESEARCH

Polygonatum cyrtonema Hua Polysaccharides regulates NF-κB and Nrf2 pathways to alleviates oxidative stress and neuroinflammation in cerebral ischemia/reperfusion injury

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Abstract

Cerebral ischemia/reperfusion (I/R) injury is a leading cause of death and disability globally, particularly in China, and presents significant neurotoxic challenges despite timely revascularization. *Polygonatum cyrtonema* Hua polysaccharides (PCP) are well-known for their antioxidative, anti-inflammatory and immunomodulatory properties. This study aimed to address an unknown mechanism that regulates oxidative stress and neuroinflammation in cerebral ischemia/reperfusion injury induced by PCP. Our findings demonstrated that cell viability improved significantly when PCP was applied at concentrations of 20, 40 and 80 g/mL for 24 h in Oxygen and Glucose Deprivation/Reperfusion (OGD/R)-stimulated HT22 cells. PCP also markedly decreased Reactive Oxygen Species (ROS) and Malondialdehyde (MDA) levels by over 40% while enhancing Superoxide Dismutase (SOD) activity by approximately 35%. Additionally, PCP significantly reduced pro-inflammatory cytokines production, such as Tumor Necrosis Factor-alpha (TNF-α), Interleukin (IL)-1β and IL-6, by more than 50%. PCP activated the Nuclear factor erythroid 2-related factor 2 (Nrf2) axis and inhibited the Nuclear Factor kappa B (NF-κB) axis in HT22 cells under OGD/R conditions. Essentially, PCP modulates the oxidative stress and inflammation pathways to prevent injury induced by I/R. PCP may therefore be a promising treatment for ischemic stroke.

Keywords

Polygonatum cyrtonema Hua polysaccharides; Ischemia/reperfusion injury; Oxidative stress; Neuroinflammation; Neuroprotection

1. Introduction

Cerebral ischemia/reperfusion (I/R) injury remains a significant challenge in neurology, contributing to high morbidity and mortality rates worldwide [1]. Among the deadliest diseases in China, stroke is the most prevalent [2]. Neuronal damage can be minimized by revascularization within 4.5 hours after an ischemic stroke. While various reperfusion therapies are available, neurotoxicity caused by I/R is inevitable, often resulting in irreversible neuronal damage and long-term disability [3]. Several studies have demonstrated that plant-derived compounds can be used to treat ischemic brain injury, although their clinical applications require further study [4].

Polysaccharides from medicinal plants exhibit antioxidative, anti-inflammatory and immunomodulatory properties [5, 6]. *Polygonatum cyrtonema* Hua Polysaccharides (PCP), a member of the Liliaceae family, is traditionally used in Chinese medicine for its tonic properties, including qi replenishment, yin nourishment, spleen fortification, lung moistening and kidney tonification [5]. Several diseases could be suppressed by PCP. PCP modulates the Nrf2 and NOD-like Receptor Family Pyrin Domain Containing 3 (NLRP3) signaling pathways, alleviating depressive-like behaviors [7]. In Non-Alcoholic Fatty Liver Disease (NAFLD) models, it may also mitigate liver pathology and balance gut microbiota composition [8]. PCP reduces oxidative stress-stimulated ROS accumulation in microglial cells, attenuating neuroinflammation [7, 9]. Furthermore, PCP prevents Post-Traumatic Stress Disorder (PTSD)-like behaviors and synaptic injury by regulating oxidative stress and NLRP3-mediated inflammation. PCP also inhibits pulmonary inflammation and reduces oxidative stress [9].

NF-κB is a transcription factor involved in multiple cellular functions, including hematopoiesis, inflammation, immune response and apoptosis [10]. NF-κB regulates several pro-inflammatory cytokines in neuronal cells [10]. Similarly, the transcription factor Nrf2 regulates cellular antioxidant responses [11, 12]. Nrf2 binds to the ARE in the nucleus under oxidative stress, modulating downstream factors to exert anti-apoptotic, anti-inflammatory and antioxidative effects, thereby maintaining redox balance in brain tissues and cells [12].

However, PCP's effects and mechanisms on cerebral I/R injury remain unclear despite these promising findings. While current reperfusion therapies minimize neuronal damage following ischemic stroke, the oxidative stress and neuroinflammation that accompany them can cause irreversible damage to neurons. These underlying mechanisms remain urgently unaddressed despite advancements. This study aimed to investigate the neuroprotective effects of PCP on cerebral I/R injury by specifically focusing on its regulation of oxidative stress and inflammatory cytokines such as TNF-α, IL-1β and IL-6, thus revealing new therapeutic strategies for ischemic stroke.

2. Materials and methods

2.1 Cell culture and treatment

HT22 cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA, 11995065) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Grand Island, NY, USA, 16000044). Cells were incubated at 37 °C with 5% Carbon Dioxide (CO_2). Treatment groups for HT22 cells were: Control, OGD/R, and OGD/R combined with different PCP (G6396, Sigma, St. Louis, MO, USA) concentrations (0, 20, 40, 80, 160 μg/mL).

2.2 Oxygen-glucose deprivation/reperfusion (OGD/R) model

For the simulation of ischemia/reperfusion injury, HT22 cells were exposed to OGD for 6 h followed by reperfused for 24 h. To achieve OGD, cells were incubated in glucose-free DMEM (Gibco, Grand Island, NY, USA, 11966025) in a hypoxia chamber (27310, Stemcell Technologies, Inc, Vancouver, BC, Canada) with 1% Oxygen (O_2), 5% CO_2 and 94% Nitrogen (N_2). Cells were returned to normoxic conditions after 6 h of OGD.

2.3 Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay (Beyotime, Beijing, China, C0038).

2.4 Flow cytometry analysis of apoptosis

Cell apoptosis was analyzed by the Annexin V-FITC/PI Kit (Beyotime, Beijing, China, C1062L). HT22 cells were collected, washed with PBS and resuspended in 1× binding buffer. Annexin V-FITC and Propidium Iodide (PI) were added, and the mixture was incubated for 15 min in the dark. A flow cytometer (BD FACSCanto™ II, BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze the cells, recording at least 10,000 events per sample. The percentage of cells that are apoptotic was calculated using FlowJo software (8.0, BD Biosciences, Franklin Lakes, NJ, USA).

2.5 ROS measurement

2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe (Beyotime, Beijing, China, S0033S) were used to measure intracellular reactive oxygen species (ROS) according to the manufacturer's protocol. HT22 cells were seeded in 6-well plates overnight and allowed to adhere. After treatment, cells were washed twice with PBS and incubated with 10 μM 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) in serum-free medium at 37 °C for 30 min in the dark to prevent probe photoactivation. A three-time PBS wash was used to remove any excess DCFH-DA after incubation. Excitation at 488 nm and emission at 525 nm were used for measuring fluorescence using a fluorescence microscope (LSM710, Zeiss, Oberkochen, BW, Germany).

2.6 Malondialdehyde (MDA) assay

MDA content was determined using the Lipid Peroxidation MDA Assay Kit (Beyotime, Beijing, China, S0131S). Absorbance was measured at 532 nm using a microplate reader (SpectraMax® M5, Thermo, Waltham, MA, USA).

2.7 Superoxide dismutase (SOD) activity assay

SOD activity was measured using the Total Superoxide Dismutase Assay Kit (Beyotime, Beijing, China, S0101S) according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA, Multiskan FC).

2.8 ELISA

TNF-α, IL-1β and IL-6 levels were measured using specific enzyme-linked immunosorbent assay (ELISA) kits (Beyotime, Beijing, China, PT516 for TNF-α, PI302 for IL-1β, PI330 for IL-6) according to the manufacturer's instructions. Samples and standards were added to 96-well plates pre-coated with the corresponding antibodies and incubated at 37 °C for 2 h. After washing, a biotin-conjugated secondary antibody was added, followed by streptavidin-Horseradish Peroxidase (HRP). Plates were incubated with substrate solution and stopped with stop solution. Absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) and concentrations were calculated based on a standard curve. For accuracy and reliability, samples were measured in triplicate.

2.9 Quantitative polymerase chain reaction (qPCR)

TRIzol reagent (Thermo, Waltham, MA, USA, 15596026) was used to extract total RNA. cDNA was synthesized using the RevertAid cDNA Synthesis Kit (Thermo, Waltham, MA, USA, K1622). Quantitative polymerase chain reaction (qPCR) was performed by the PowerUp SYBR Green Master Mix (Thermo, Waltham, MA, USA, A25742) on a QuantStudio 3 Real-Time PCR System (3.0, Thermo, Waltham, MA, USA). The primer sequences used were as follows: TNF-α: Forward 5′-ATGAGCACAGAAAGCATGATC-3′, Reverse 5′-TACAGGCTTGTCACTCGAATT-3′; IL-1β: Forward 5′-GCAACTGTTCCTGAACTCAACT-3′, Reverse 5′-ATCTTTTGGGGTCCGTCAACT-3′; IL-6: Forward 5′-TAGTCCTTCCTACCCCAATTTCC-3′, Reverse 5′-TTGGTCCTTAGCCACTCCTTC-3′; β-actin: Forward 5′-AGAGGGAAATCGTGCGTGAC-3′, Reverse

5′-CAATAGTGATGACCTGGCCGT-3′.

2.10 Immunoblot analysis

Proteins were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to Polyvinylidene Fluoride (PVDF) membranes (Millipore). Membranes were blocked with 5% non-fat milk for 1 h at room temperature to ensure thorough blocking and minimize non-specific binding. Membranes were then incubated with primary antibodies against Nuclear factor erythroid 2-related factor 2 (Nrf2, Cambridge, UK, Abcam, ab62352, 1:1000), p65 (Abcam, Cambridge, UK, ab16502, 1:1000), p-p65 (Abcam, Cambridge, UK, ab76302, 1:1000), and β-actin (Abcam, Cambridge, UK, ab8226, 1:3000) overnight at 4 °C. After washing, membranes were incubated with HRP-conjugated secondary antibodies (Abcam, Cambridge, UK, ab6721, 1:2000) and visualized using Enhanced Chemiluminescence (ECL) detection reagent (Thermo, Waltham, MA, USA, 32106).

2.11 Statistical analysis

For normally distributed data, we used one-way Analysis of Variance (ANOVA) followed by Tukey's *post hoc* test. Normality of the data distribution was assessed using the Shapiro-Wilk test. Non-parametric tests, such as the Kruskal-Wallis test, were used for data without a normal distribution. Data analysis was performed using GraphPad Prism (9.0, GraphPad Software, San Diego, CA, USA). $p < 0.05$ indicates statistically significant differences.

3. Results

3.1 Effects of PCP on OGD/R-induced HT22 cell viability

In an OGD/R-stimulated injury model, we performed a CCK-8 assay and flow cytometry analysis to determine the effect of PCP on cell viability. Based on the CCK-8 assay, PCP had modest effect on HT22 cells at low concentration (20, 40 and 80 μg/mL) but suppressed the viability at high concentration (160 μg/mL, Fig. 1A). PCP was therefore used at a low concentration in the next experiments. PCP significantly improved the viability of HT22 cells subjected to OGD/R, PCP significantly improved viability, with notable increases observed at 20, 40 and 80 μg/mL (Fig. 1B). In the OGD/R + PCP group, PCP reduced the percentage of apoptotic cells (Fig. 1C,D). PCP enhances cell viability and reduces cytotoxicity in HT22 cells under OGD/R conditions.

3.2 Effects of PCP on ROS levels in OGD/R-induced HT22 cells

In HT22 cells subjected to OGD/R, we measured ROS levels, MDA content, and SOD activity to determine the antioxidative effects of PCP. The DCFH-DA assay showed that PCP significantly decreased ROS levels in the OGD/R + PCP group (Fig. 2A). PCP significantly reduced MDA content, indicating decreased lipid peroxidation (Fig. 2B). PCP treatment significantly increased SOD activity in the OGD/R + PCP group (Fig. 2C). PCP is effective in reducing oxidative stress in HT22 cells under OGD/R conditions.

3.3 Effects of PCP on inflammatory cytokines production in OGD/R-induced HT22 cells

By using ELISA and qPCR, we assessed PCP's anti-inflammatory effects on pro-inflammatory cytokines. ELISA results showed that PCP significantly reduced TNF-α, IL-1β and IL-6 levels in the OGD/R + PCP group (Fig. 3A). qPCR analysis revealed that PCP significantly downregulated the mRNA expression of these factors in the OGD/R + PCP group (Fig. 3B). PCP exerts significant anti-inflammatory effects in HT22 cells under OGD/R conditions.

3.4 PCP activates the Nrf2 pathway and inhibits the NF-κB pathway in OGD/R-induced HT22 cells

To elucidate the molecular mechanisms underlying PCP protective effects, we examined the Nrf2 pathway and NF-κB pathway using immunoblot analysis. Immunoblot analysis showed that PCP inhibited p-p65 phosphorylation levels in the OGD/R + PCP group, indicating NF-κB pathway suppression (Fig. 4A). Immunoblot analysis demonstrated that PCP significantly increased Nrf2 protein expression in the OGD/R + PCP group, indicating Nrf2 pathway activation (Fig. 4B). PCP neuroprotective effects involve Nrf2 pathway activation and NF-κB pathway inhibition in HT22 cells under OGD/R conditions.

4. Discussion

Cerebral ischemia/reperfusion (I/R) injury is a pathological process that significantly contributes to morbidity and mortality [13]. Ischemic stroke is primarily caused by a disruption in blood flow, followed by a recovery of circulation, which paradoxically worsens neuronal damage [1, 14]. Inducing oxidative stress through ROS is vital to this process. Neuroinflammation, exacerbated by microglial activation and pro-inflammatory cytokines, is also a critical factor in ischemic injury [15, 16]. Therefore, new therapeutic agents that target oxidative stress and neuroinflammation are crucial for improving stroke outcomes.

This study demonstrated that PCP exhibits significant neuroprotective effects in an OGD/R model using HT22 cells. PCP treatment significantly improved cell viability, Lactate Dehydrogenase (LDH) release, decreases ROS and MDA levels and enhances SOD activity. PCP may modulate these damaging processes by mitigating oxidative stress and neuroinflammation, which are known pathological factors of ischemic stroke.

PCP has antioxidative, anti-inflammatory, and immunomodulatory properties [7, 9]. PCP has been shown to regulate various signaling pathways and alleviate disease conditions [7]. By modulating Nrf2 and NLRP3 signaling pathways, PCP has been shown to reduce depressive-like behaviors [7]. In NAFLD models, it also improves lipid metabolism and reduces oxidative stress [8]. Additionally, PCP alleviates pulmonary inflammation through the NF-κB

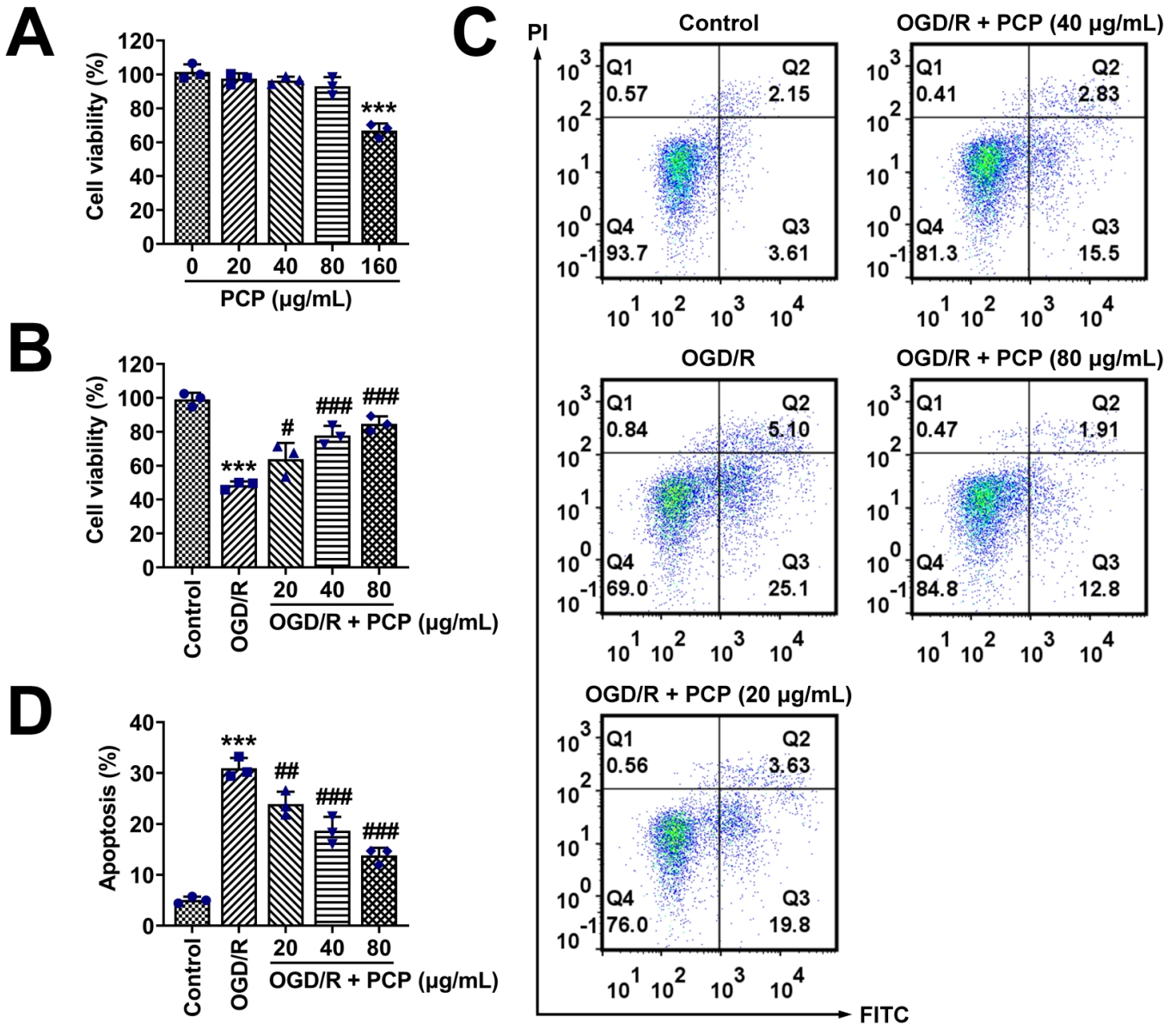

FIGURE 1. Effects of PCP on OGD/R-induced HT22 cell viability. (A) HT22 cells were assessed for viability using CCK-8 assay at different PCP concentrations (0, 20, 40, 80, 160 μg/mL). (B) OGD/R-induced HT22 cells were assessed for viability using CCK-8 assay at different PCP concentrations (0, 20, 40, 80, 160 g/mL). (C) FCM assays showed the apoptosis of HT22 cells in different groups (Control, OGD/R, OGD/R + PCP). (D) Quantification of panel C, quantitative analysis of apoptosis. Statistical analysis was performed using a one-way ANOVA. Significance was indicated as ***$p < 0.001$ compared to OGD/R group; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared to Control group. PCP: *Polygonatum cyrtonema* Hua Polysaccharides; OGD/R: Oxygen and Glucose Deprivation/Reperfusion; OGD/R + PCP: Oxygen and Glucose Deprivation/Reperfusion combined with *Polygonatum cyrtonema* Hua Polysaccharides; PI: Propidium Iodide; FITC: Fluorescein Isothiocyanate.

pathway and reduce oxidative stress via the AMP-Activated Protein Kinase (AMPK)-Nrf2 pathway [7]. It is evident from these findings, as well as our current study, that PCP has broad therapeutic potential for treating ischemic stroke and other oxidative stress-related conditions.

It is unique in that it investigates the effects of PCP on cerebral ischemia/reperfusion (I/R) injury in detail, specifically the modulation of oxidative stress and inflammatory cytokines. While previous studies have demonstrated that PCP reduces inflammation by targeting pathways such as NF-κB and AMPK-Nrf2, our study expands on these findings by showing that PCP significantly reduces the levels of key pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in the context of I/R injury. Despite their consistency with previous research, these results provide new insights into how PCP regulates cerebral I/R injury. Dual modulation of Nrf2 and NF-κB pathways further highlights PCP's comprehensive protective effects, contrasting our work with prior studies that focused only on isolated pathways.

This study also contributes innovatively by demonstrating these effects specifically after cerebral ischemia/reperfusion (I/R) injury. With an I/R model, we introduced unique

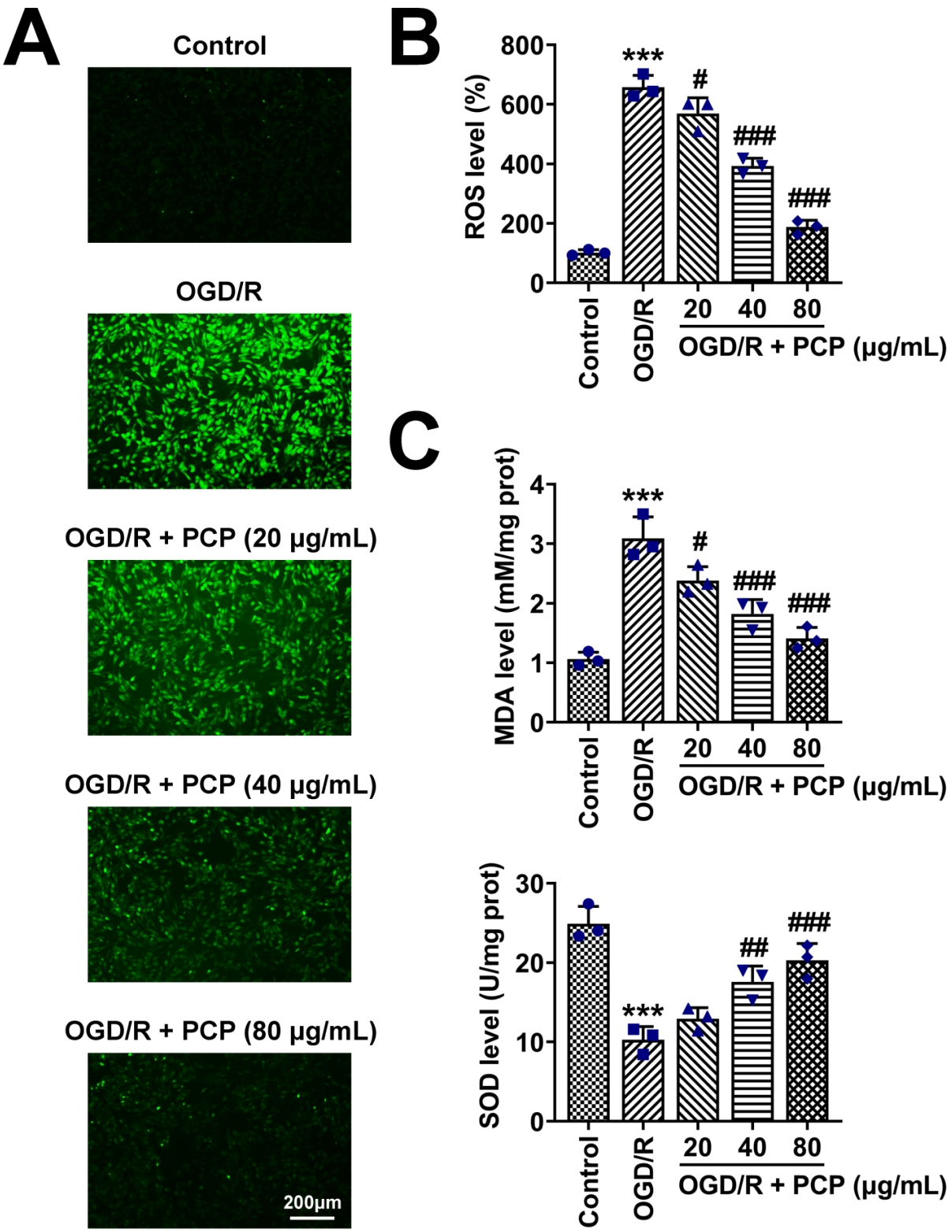

FIGURE 2. Effects of PCP on ROS levels in OGD/R-induced HT22 cells. (A) ROS levels in different groups (Control, OGD/R, OGD/R + PCP) were assessed by DCFH-DA assay. (B) MDA content in different groups (Control, OGD/R, OGD/R + PCP) was detected using MDA assay. (C) SOD activity assay evaluated SOD activity in different groups (Control, OGD/R, OGD/R + PCP). Statistical analysis was performed using a one-way ANOVA. Significance was indicated as ***$p < 0.001$ compared to OGD/R group; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared to Control group. OGD/R: Oxygen and Glucose Deprivation/Reperfusion; PCP: *Polygonatum cyrtonema* Hua Polysaccharides; ROS: Reactive Oxygen Species; MDA: Malondialdehyde; SOD: Superoxide Dismutase.

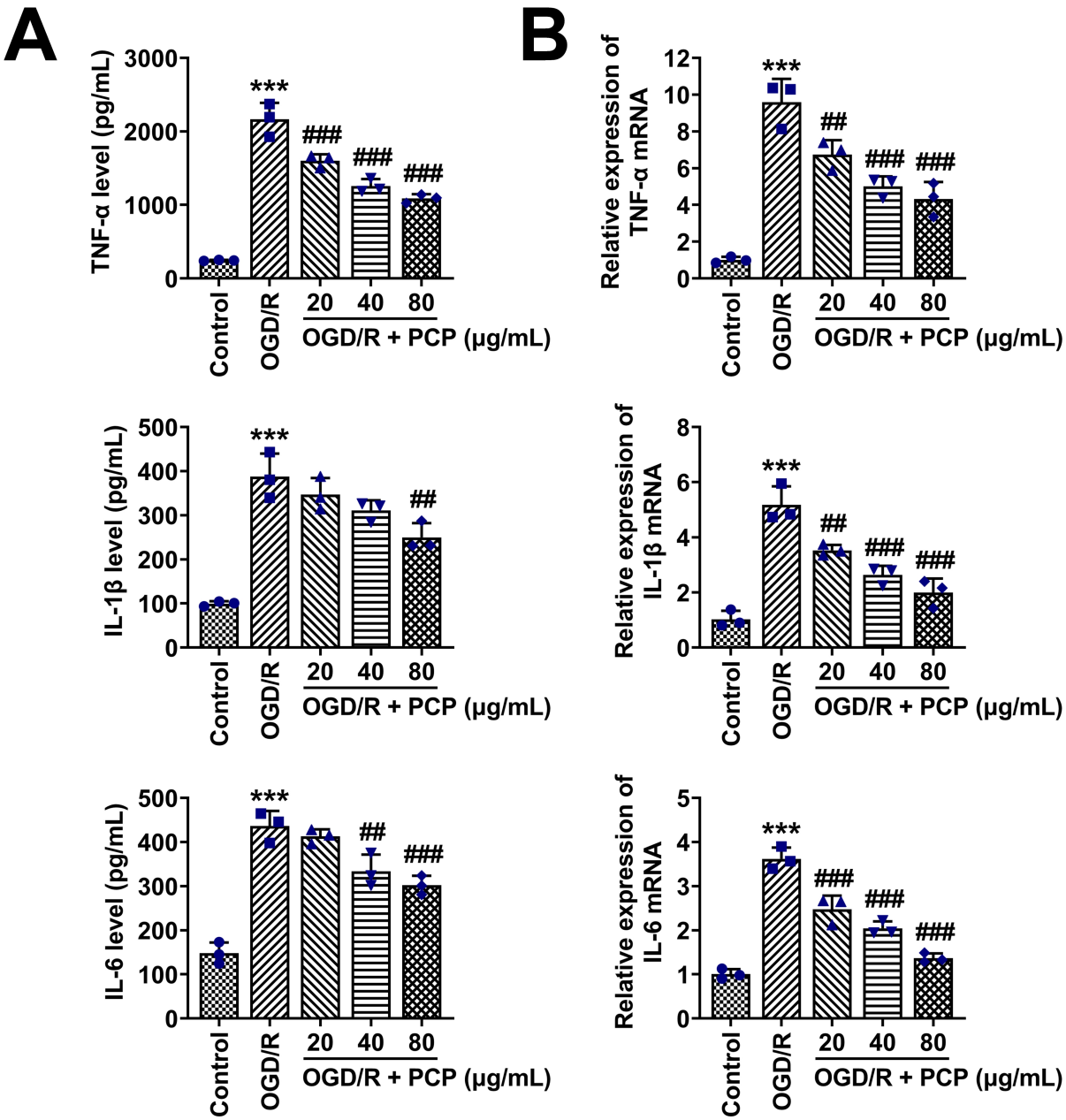

FIGURE 3. Effects of PCP on inflammatory cytokines production in OGD/R-induced HT22 cells. (A) TNF-α, IL-1β, and IL-6 levels in different groups (Control, OGD/R, OGD/R + PCP) were measured with ELISA. (B) qPCR analysis of TNF-α, IL-1β, and IL-6 mRNA expression in different groups (Control, OGD/R, OGD/R + PCP). Statistical analysis was performed using a one-way ANOVA. Significance was indicated as ***$p < 0.001$ compared to OGD/R group; ##$p < 0.01$, ###$p < 0.001$ compared to Control group. TNF: Tumor Necrosis Factor; mRNA: Messenger RNA; IL: Interleukin; OGD/R: Oxygen and Glucose Deprivation/Reperfusion; OGD/R + PCP: Oxygen and Glucose Deprivation/Reperfusion combined with *Polygonatum cyrtonema* Hua Polysaccharides.

stressors such as oxygen-glucose deprivation and reperfusion, thereby providing new insights into PCP's neuroprotective mechanisms under these specific pathological conditions. Our study further differentiates itself from previous studies by studying precise modulation of pro-inflammatory cytokines and oxidative stress markers.

This study indicates that PCP significantly reduces pro-inflammatory cytokine production. Neuroinflammation is mediated by these cytokines in ischemic stroke, contributing to further neuronal damage and exacerbating the injury. By lowering the levels of cytokines, PCP protects neuronal cells from secondary damage by attenuating the inflammatory response. PCP's anti-inflammatory properties as well as its antioxidative properties demonstrate its potential to treat both major pathological components of ischemic stroke.

The Nrf2 and NF-κB are vital in the regulation of oxidative stress and inflammation [10, 17]. Activation of Nrf2 leads to an increase in antioxidative enzyme activity, including SOD, which is a key regulator of antioxidant responses [18]. In contrast, NF-κB plays an important role in regulating inflammatory cytokines [19]. Nrf2 activation prevents oxidative damage and reduces ischemic injury [20]. Inhibition of the

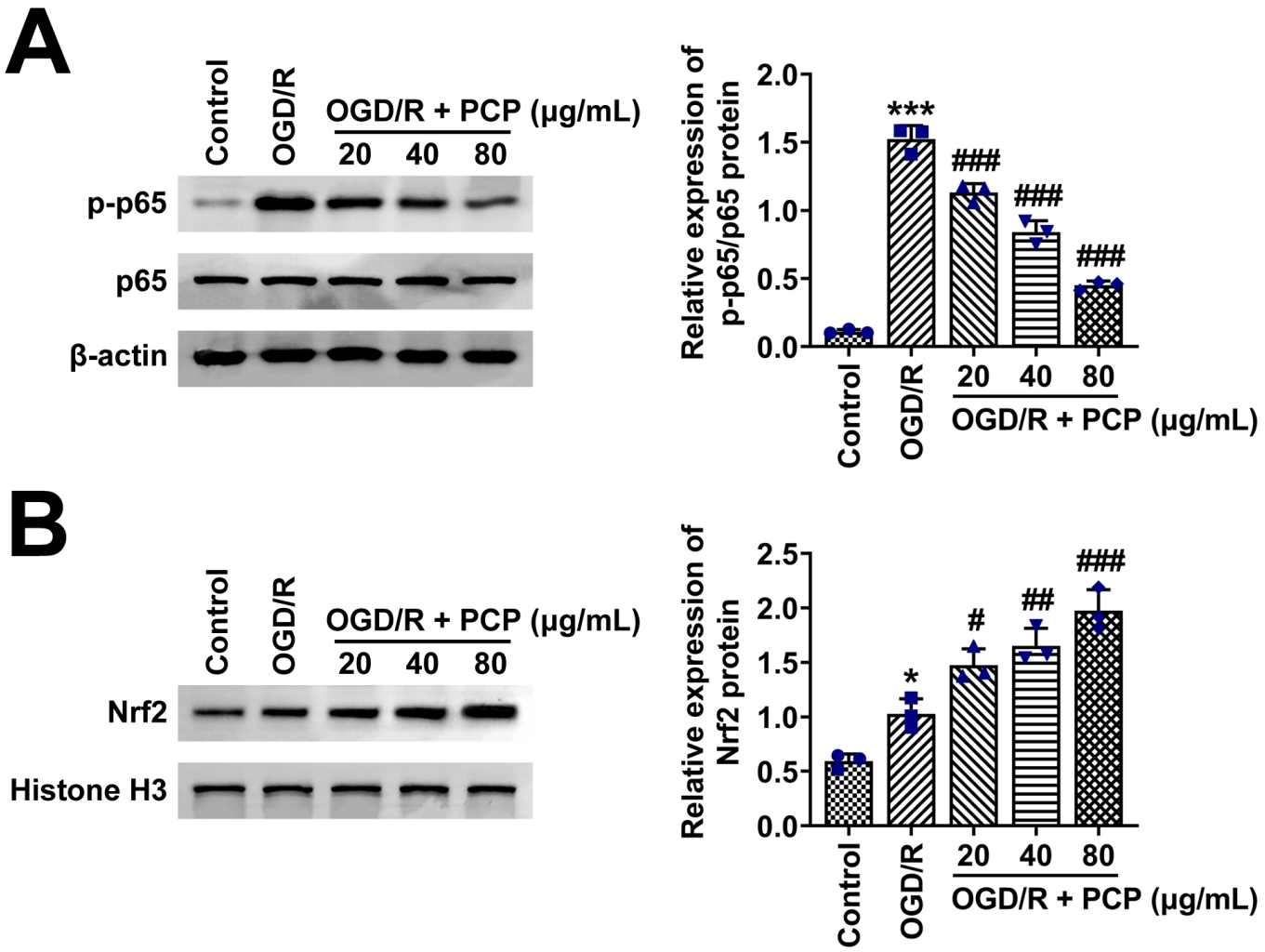

FIGURE 4. PCP activates the Nrf2 pathway and inhibits the NF-κB pathway in OGD/R-induced HT22 cells. (A) Immunoblot analysis of p65 and p-p65 protein expression in different groups (Control, OGD/R, OGD/R + PCP). (B) Immunoblot analysis of Nrf2 protein expression in different groups (Control, OGD/R, OGD/R + PCP). Statistical analysis was performed using a one-way ANOVA. Significance was indicated as $*p < 0.05$, $***p < 0.001$ compared to OGD/R group; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ compared to Control group. OGD/R: Oxygen and Glucose Deprivation/Reperfusion; OGD/R + PCP: Oxygen and Glucose Deprivation/Reperfusion combined with *Polygonatum cyrtonema* Hua Polysaccharides.

NF-κB axis decreases inflammation and improves outcomes in models of cerebral ischemia [21–23]. We found that PCP activated Nrf2 while inhibiting NF-κB in HT22 cells under OGD/R conditions. By enhancing cellular defense against oxidative stress as well as suppressing inflammation, PCP provides comprehensive protection against ischemic injury.

5. Conclusions

This study has limitations, despite its promising findings. As an *in vitro* model, the HT22 cell model cannot fully replicate the complexity of ischemic stroke conditions *in vivo*. Animal models and clinical trials are necessary to validate PCP's neuroprotective effects and elucidate its mechanisms in a more complex biological context. Further research is required to determine PCP's molecular interactions and potential side effects. In conclusion, PCP modulates oxidative stress and inflammatory pathways to confer neuroprotection against I/R-induced injury. Therefore, PCP could be a promising therapeutic agent for ischemic stroke, offering new avenues for effective neuroprotective strategies.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article. The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

DCP—performed material preparation and the experiments. XYR and MG—performed data collection and analysis. JZ—wrote the first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors contributed to the study conception and design. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

ACKNOWLEDGMENT

Not applicable.

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CONFLICT OF INTEREST

The authors state that there are no conflicts of interest to disclose.

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