Berberine Ameliorates Oxygen-glucose Deprivation/Reperfusion-induced Apoptosis by Inhibiting Endoplasmic Reticulum Stress and Autophagy in PC12 Cells*

Peng XIE[1, 2], Zhen-kui REN[1, 2, 3], Ju LV[1, 2], Yu-mei HU[1, 2], Zhi-zhong GUAN[2, 4], Wen-feng YU[1, 2#]
[1]*Department of Biochemistry and Molecular Biology, School of Basic Medical Science, Guizhou Medical University, Guiyang 550025, China*
[2]*Key Laboratory of Endemic and Ethnic Diseases, Ministry of Education, Guiyang 550004, China*
[3]*Department of Laboratory Medicine, the Second People's Hospital of Guizhou Province, Guiyang 550002, China*
[4]*Department of Pathology, Affiliated Hospital of Guizhou Medical University, Guiyang 550004, China*

Summary: This study aimed to elucidate the molecular mechanisms by which berberine protects against cerebral ischemia/reperfusion (I/R) injury. The oxygen-glucose deprivation/reperfusion (OGD/R) PC12 model was established. Cell counting kit-8 (CCK-8) was used to detect the toxicity of berberine and the viability of PC12 cells. Hoechst 33258 staining and flow cytometry were used to observe the nuclear morphology, and changes of apoptosis and reactive oxygen species (ROS), respectively. Western blotting and immunofluorescence assay were employed to detect autophagy-related proteins [microtubule-associated protein 1A/1B-light chain 3 (LC3), P62/SQSTM-1, Beclin-1] and endoplasmic reticulum (ER) stress-related markers [glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), Bcl-2-associated X (Bax) and cleaved caspase-3]. The GFP-RFP-LC3 adenovirus was used to assay the change of autophagic flux. Our results showed that berberine could increase the viability of PC12 cells, decrease the concentrations of ROS after OGD/R treatment, and suppress OGD/R-induced ER stress and autophagy. Moreover, the results revealed the involvement of the mammalian target of rapamycin (mTOR) pathway in the induction of autophagy, and berberine could activate the phosphorylation of mTOR and thus mitigate autophagy. In conclusion, our study suggested that berberine may protect against OGD/R-induced apoptosis by regulating ER stress and autophagy, and it holds promises in the treatment of cerebral I/R injury.
Key words: autophagy; berberine; endoplasmic reticulum (ER) stress; ischemia/reperfusion (I/R) injury; OGD/R

Cerebral ischemia/reperfusion (I/R) injury, a devastating event in ischemic stroke, is a primary cause of cerebral apoplexy. Early thrombolytic therapy and neuronal cell protection are important therapeutic and preventive strategies for ischemic stroke[1–3]. However, the thrombolytic agents, such as tissue plasminogen activator (tPA)[4, 5], fail to resolve the secondary effects of ischemic stroke, such as delayed neuronal necrosis and apoptosis.

Oxidative stress is caused by an imbalance between the production and clearance of reactive oxygen species (ROS) under stressful conditions, and it has been identified as an important driver of multiple pathological processes[6, 7]. An increasing number of studies have confirmed that excessive ROS and oxidative stress are generated in rat models of middle cerebral artery occlusion (MCAO) and the cell oxygen-glucose deprivation/reperfusion (OGD/R) model[8, 9]. Reportedly, the administration of antioxidants, such as α-lipoic acid (α-LA), can decrease infarction volume and oxidative stress levels in the rat MCAO model, and ROS in the OGD/R model[10]. Oxidative stress has thus become an additional therapeutic target for ischemic stroke.

Endoplasmic reticulum (ER) is an organelle that is involved in multiple vital processes, including lipid and protein biosynthesis and folding, post-translational

Peng XIE, E-mail: 1911839928@qq.com
#Corresponding author, E-mail: wenfengyu2013@126.com
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modification, and ROS release[11–14]. The ER stress response can be induced by unfolded/misfolded protein, excessive ROS, and disturbances in the intracellular environment, and it contributes to the maintenance of intracellular homeostasis[15, 16]. Excessive ER stress response, however, is closely related to pathological processes such as neurodegenerative diseases and cerebral I/R injury[17–19], and was confirmed to activate downstream signals of the apoptosis protein C/EBP homologous protein (CHOP) and caspase-3.

Recently, neuronal death or loss induced by excessive autophagy is reported to be responsible for the pathogenesis of cerebral I/R injury in a highly conserved process that reuses necrotic cytoplasmic components[20, 21]. More research reveals that autophagy plays a crucial role in maintaining the homeostasis of the intracellular environment. Nevertheless, when it upregulates apoptotic caspase cascades in excess, autophagic death results[22, 23].

Berberine is an isoquinoline alkaloid used in traditional Chinese medicine[24]. It has been effective for the treatment of cerebral I/R injury and the mechanisms involve its anti-inflammation, and anti-apoptotic and antioxidant activities[25], and a great number of studies have reported that berberine confers neuroprotective properties[26–28]. However, the specific mechanisms underlying the protective effects of berberine against cerebral I/R injury still need investigation. In the present study, we established the OGD/R PC12 model to examine whether berberine could protect against cerebral I/R injury by regulating oxidative stress, autophagy, and ER stress response.

1 MATERIALS AND METHODS

1.1 Chemicals and Reagents

Berberine, 3-methyladenine (3-MA), and 4-phenylbutyrate (4-PBA) were purchased from Sigma Aldrich (USA), with a purity of greater than 95%. Dimethyl sulfoxide (DMSO), cell counting kit-8 (CCK8), Dulbecco's Modified Eagle Medium (DMEM) high glucose medium, DMEM no glucose medium, phosphate-buffered saline (PBS), penicillin-streptomycin (PS), and fetal bovine serum (FBS) were obtained from Gibco (USA). Primary antibodies against β-actin (#3700), phospho-mTOR (Ser2448) (#2971), mTOR (#2972), cleaved-caspase-3 (#9661), LC3A/B (#12741), Beclin-1 (#3495) and SQSTM1/p62 (#39749) were purchased from Cell Signaling Technology (CST, USA). Bax (ab32503), GRP78 (ab108613), and CHOP (ab11419) were obtained from Abcam (UK).

1.2 Cell Culture and Establishment of the OGD/R Model

The PC12 cells were obtained from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (China). Cells were cultured in DMEM containing 10% FBS and 1% PS, and were incubated in a humidified atmosphere of 5% CO_2 at 37°C. To establish cerebral I/R injury model, 70% confluent PC12 cells were cultured in glucose-free medium (Invitrogen, USA) and incubated at 5% CO_2 and 1% O_2 at 37° C for 4 h. This was followed by reoxygenation for 18 h. Control cells were continuously incubated under normal conditions and not exposed to OGD/R.

1.3 Cell Viability Assay

CCK8 was used to assay the viability of PC12 cells according to the manufacturer's instructions. Briefly, 5×10^4 cells/well were seeded in 96-well plates and treated with different conditions of berberine (0.1, 0.25, 0.5, 1, 2, 5, 10, 25, 50, 100 μmol/L). The CCK-8 working solution (10 μL) was subsequently added to each well, and the cells were cultured for another 2 h at 37°C. The absorbance (*A*) value of each well was measured with enzyme labeling instrument (Elx800) at a wavelength of 450 nm.

1.4 Western Blot Analysis

After different interventions, cells were harvested and lysed with RIPA buffer (CST, USA) containing 1 mmol/L of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). BCA Protein Assay Kit was used to measure protein concentrations. Equal proteins were electrophoresed on 12% SDS-PAGE gels (Beyotime, China) and transferred to PVDF membranes. The membranes were blocked using 5% nonfat milk and the primary antibodies: anti-GRP78 (1:800), anti-SQSTM-1/P62 (1:800), anti-Beclin-1 (1:800), anti-CHOP (1:1000), anti-LC3 (1:1000), anti-cleaved-caspase3 (1:500) and anti-β-actin (1:2000). Dilution buffer (P0023A, Beyotime, China) was used to dilute primary antibodies to the indicated concentrations at 4°C overnight. Incubation with the HRP-conjugated secondary antibody (1:8000; CST) was performed for 2 h at room temperature. An ECL chemiluminescence kit was used to detect the protein band.

1.5 Hoechst 33258 Staining

After the intervention with berberine or OGD/R treatment, the cell medium was discarded, and 4% paraformaldehyde was used to fix the cells for 15 min at room temperature. The cells were washed once with PBS, incubated with Hoechst 33258 (C1011, Beyotime, China) at 37°C for 10 min, and observed under a fluorescence microscope (Olympus, Japan).

1.6 Detection of Apoptosis and ROS in PC12 Cells with Flow Cytometry

Cell apoptosis was measured with Annexin V-FITC and PI in normal, OGD/R, and berberine-treated cells. Briefly, after adding 450 μL of binding buffer to each sample, all the cells were incubated in the dark with 50 μL of binding buffer containing 5 μL Annexin V-FITC and 5 μL PI (MultiSciences Biotechs, China) for 15 min at room temperature. Cell apoptosis was detected

with a flow cytometer. The ROS level of each sample was measured with DCFH-DA kit (Beyotime, China) according to the manufacturer's instructions. Cell suspension was incubated with DCFH-DA (1:1000) at 37°C for 30 min. Detection was performed once more with a flow cytometer.

1.7 Immunofluorescence Assay

Cells in normal, OGD/R, berberine, and 4-PBA groups were seeded on coverslips and cultured. They were washed thrice with pre-chilled PBS, fixed with 4% formaldehyde for 30 min at room temperature, permeabilized with 0.5% Triton X-100 for 30 min, and blocked with 5% goat serum for 1 h at room temperature. The cells were then incubated overnight at 4°C with a primary antibody against GRP78 (1:200) and CHOP (1:200) followed by Alexa-FluorTM 568 goat anti-rabbit IgG (H+L) antibody (1:100; Invitrogen, USA) for 1 h at room temperature. The cells were then labeled with DAPI (Beyotime, China) for 5 min. Finally, 6 fields of each coverslip were randomly chosen for the fluorescence assay with a Laser confocal microscope (Olympus FV12-IXCOV, Japan).

1.8 GFP-RFP-LC3 Adenoviral Transfer

After the PC12 cells grew to approximately 60% confluence in the 6-well plates, the medium was discarded. The GFP-RFP-LC3 (HanBio Technology, Shanghai, China) adenovirus (4.5 μL) was added to the 0.5 mL medium containing cells. The cells were cultured in the normal incubator for 8 h, and 0.5 mL of complete medium was subsequently added and kept for 12 h. After transfection, the cells were switched to a glucose-free and serum-free medium and placed in a 1% O_2 incubator for 4 h, then treated with berberine or 3-MA and cultured in a normal oxygen incubator for an additional 18 h. The cells were washed thrice with PBS and fixed at room temperature for 15 min. The plates were sealed and photographed under a Laser confocal microscope (Olympus FV12-IXCOV, Japan).

1.9 Statistical Analysis

The data were presented as the mean ± standard deviation (SD). To compare statistical difference, one-way ANOVE was used with Tukey's post hoc test for multiple comparisons. Data results were analyzed with SPSS 22 software (IBM Corp., USA). *P* values of <0.05 were considered to be statistically significant.

2 RESULTS

2.1 ER Stress and Autophagic Death Induced by OGD/R in PC12 Cells

After OGD/R treatment, the levels of the ER stress sensors GRP78 and CHOP, and autophagy-related marker molecules Beclin-1 and LC3Ⅱ/LC3Ⅰ were significantly increased, which was accompanied by exacerbated apoptosis (fig. 1). These findings indicated that OGD/R promotes the ER stress and autophagy, and that the continuous increases of ER stress and autophagy aggravate apoptosis, thus stimulating cell stress and autophagy death.

2.2 Berberine Protects against OGD/R-induced Cell Injury in PC12 Cells

CCK-8 assay showed that the viability of PC12 cells after treatment with different concentrations of berberine for 24 h followed a normal distribution (fig. 2A). The IC_{50} value of berberine was about 50 μmol/L. The activity of cells treated with 1 μmol/L berberine was the highest and was significantly different from that of cells treated with other concentrations of berberine (fig. 2A). After treatment of cells with OGD for 4 h and then reoxygenation with berberine for 18 h, the CCK8 showed that berberine at concentrations of 2, 5, and 10 μmol/L could reverse OGD/R-induced decrease of cell activities (P<0.05, fig. 2B). Hoechst 33258 staining and flow cytometry showed that berberine (5 μmol/L) could decrease the cells apoptosis induced by OGD/R (fig. 2D and 2E), as evidenced by mitigated nuclear shrinkage and chromatin condensation, and decreased number of apoptotic cells (fig. 2C). Moreover, berberine was found to lower the concentrations of ROS after exposure to OGD/R (fig. 2F and 2G).

2.3 Berberine Attenuates OGD/R-induced Injury by Downregulating ER Stress in PC12 Cells

Cells were treated with berberine (5 μmol/L) or 4-PBA (an ER stress-specific inhibitor, 2 mmol/L) when reoxygenating, and they were subsequently cultured in normal oxygen for 18 h. It was found that berberine could diminish OGD/R-induced ER stress, as evidenced by decreased levels of ER stress-related molecules, such as GRP78, CHOP, Bax and cleaved caspase-3 (fig. 3A–3E). Moreover, 4-PBA was also found to significantly attenuate the protein levels of GRP78, CHOP, Bax, and cleaved caspase-3 (fig. 3F and 3G). Taken together, our data implicated the suppression of ER stress is involved in the berberine-mediated protection of PC12 cells against oxidative stress.

2.4 Berberine Attenuates OGD/R-induced Injury by Downregulating Excessive Autophagy in PC12 Cells

Our results showed that OGD/R markedly suppressed the expression levels of LC3Ⅱ/LC3Ⅰ and Beclin-1 but not those of SQSTM-1/P62, a specific substrate for autophagy degradation, suggesting abnormal autophagy in PC12 cells after OGD/R treatment. Cells were treated with 5 μmol/L berberine or 2.5 mmol/L 3-MA, an autophagy specific inhibitor, during reoxygenation. The results showed that both berberine and 3-MA could significantly decrease the levels of autophagy and apoptosis caused by OGD/R (fig. 4A–4C). The levels of autophagic markers LC3Ⅱ/LC3Ⅰ, Beclin-1, and autophagy-specific substrate P62 were profoundly decreased (fig. 4A–4C).

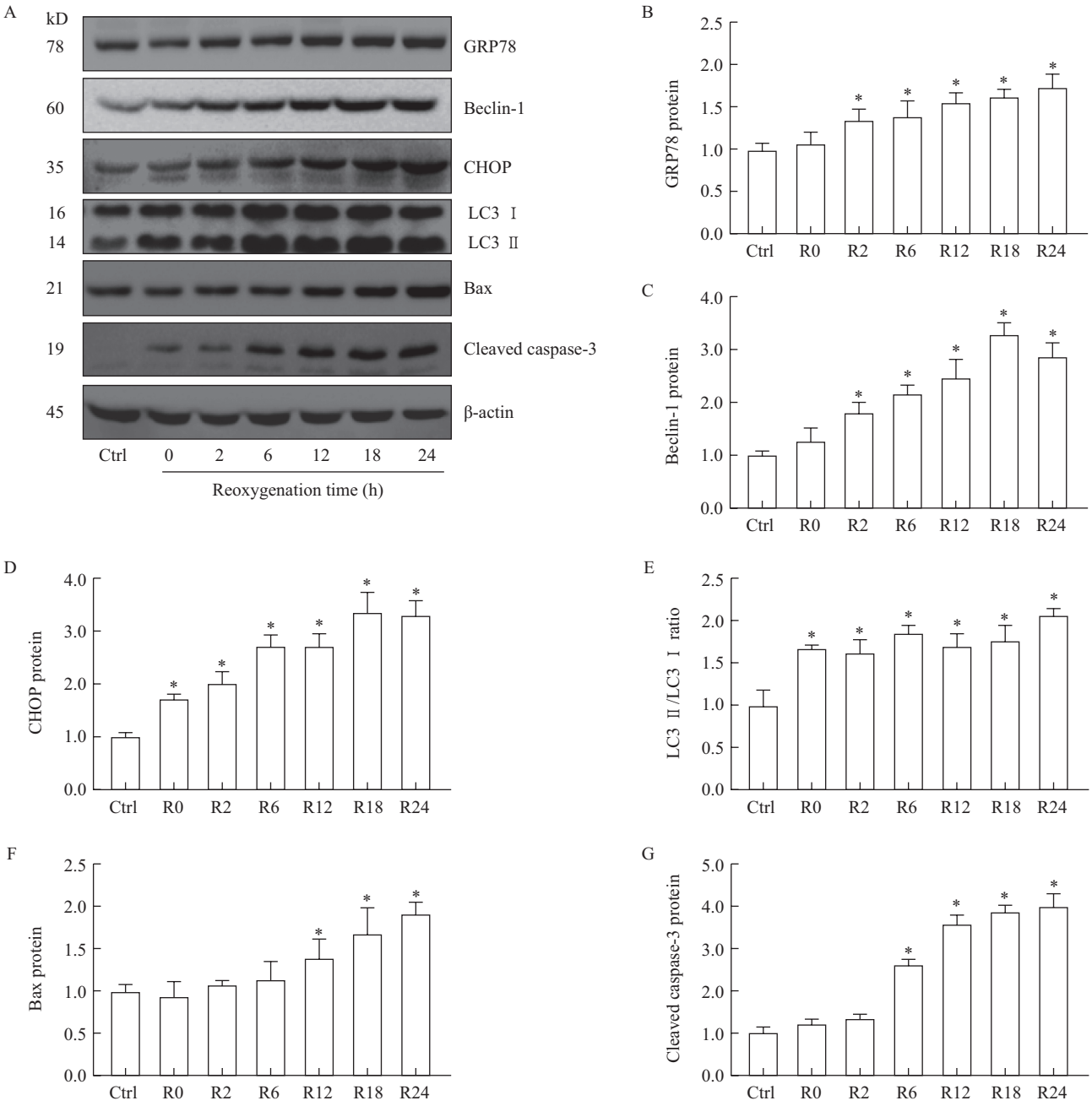

Fig. 1 ER stress and autophagic death induced by OGD/R

A: The confluent PC12 cells were exposed to OGD for 4 h followed by varying recovery periods (0, 2, 6, 12, 18, and 24 h). The expression of GRP78, Beclin-1, CHOP, LC3 Ⅱ/LC3 Ⅰ, Bax, and cleaved caspase-3 was detected by Western blotting. β-actin was used as the protein-loading control. B–G: densitometric analysis of the aforementioned proteins. Values are presented as the mean ± SD (n=3). $^{*}P<0.05$ *vs.* the control group. Ctrl: control; R0–R24: reoxygenation for 0, 2, 6, 12, 18 or 24 h

Concurrently, the levels of apoptosis-related molecules Bax and cleaved caspase-3 were diminished. The present study also examined the changes in mTOR/p-mTOR, a classical pathway related to autophagy. It was shown that OGD/R could lower the level of the phosphorylation of mTOR, which was reversed by berberine and 3-MA (fig. 4A–4C). Additionally, the results of autophagy double-labeled adenovirus (GFP-RFP-LC3 adenovirus) infection showed that OGD/R induced the accumulation of autophagosomes, which was also significantly reduced by berberine and 3-MA (fig. 4D).

3 DISCUSSION

The excessive activation of ER stress and dysfunction of autophagy are significant contributors to the pathophysiology of cerebral I/R injury[29]. Our

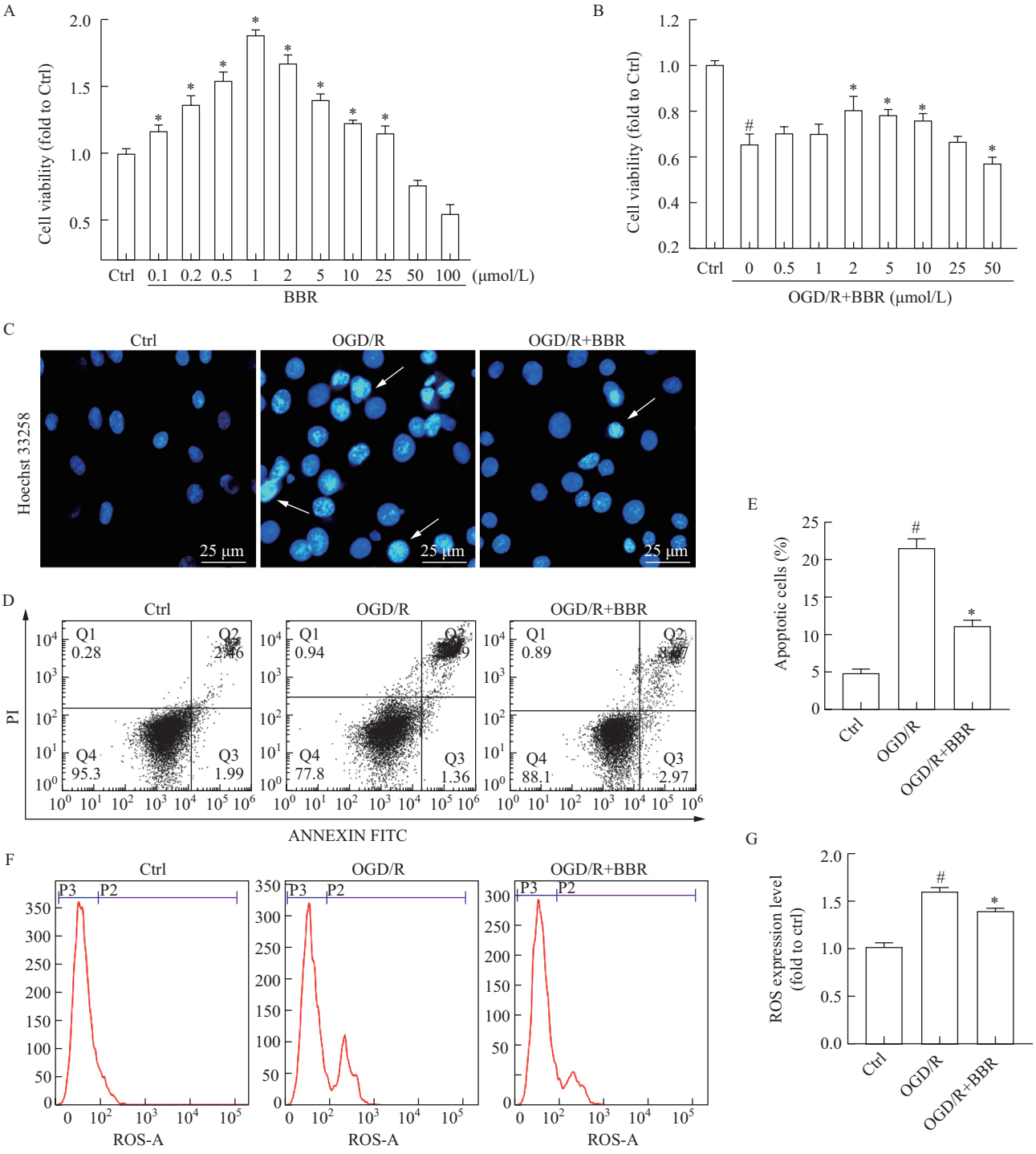

Fig. 2 Berberine exerted a protective effect against OGD/R-induced injury in PC12 cells

A: PC12 cells were treated with different concentrations of berberine for 24 h, and their cell viability was measured with a CCK8 assay. $^{*}P<0.05$ *vs.* control group. B: PC12 cells were exposed to OGD for 4 h and treated with different concentrations of berberine during 18-h reoxygenation. C–E: PC12 cells were exposed to OGD/R and treated with 5 μmol/L berberine when reoxygenating for 18 h. Apoptosis was detected by Hoechst 33258 staining and flow cytometry. The apoptotic cells are indicated by arrows. F, G: Intracellular ROS levels were measured with the fluorescent probe DCFH-DA. The scale bar is 25 μm, and values are presented as the mean ± SD (n=3). BBR: berberine. $^{\#}P<0.05$ *vs.* the control group, $^{*}P<0.05$ *vs.* OGD/R group

study demonstrated that OGD/R could induce ER stress-related apoptosis and autophagic death in PC12 cells; berberine treatment followed by OGD/R injury could increase cell viability and mitigate the OGD/R-induced increase in ROS levels; berberine could reverse OGD/R-induced apoptosis, which was associated with the decreases in ERS-related markers (GRP78/CHOP); berberine could mitigate dysfunctional autophagy, as

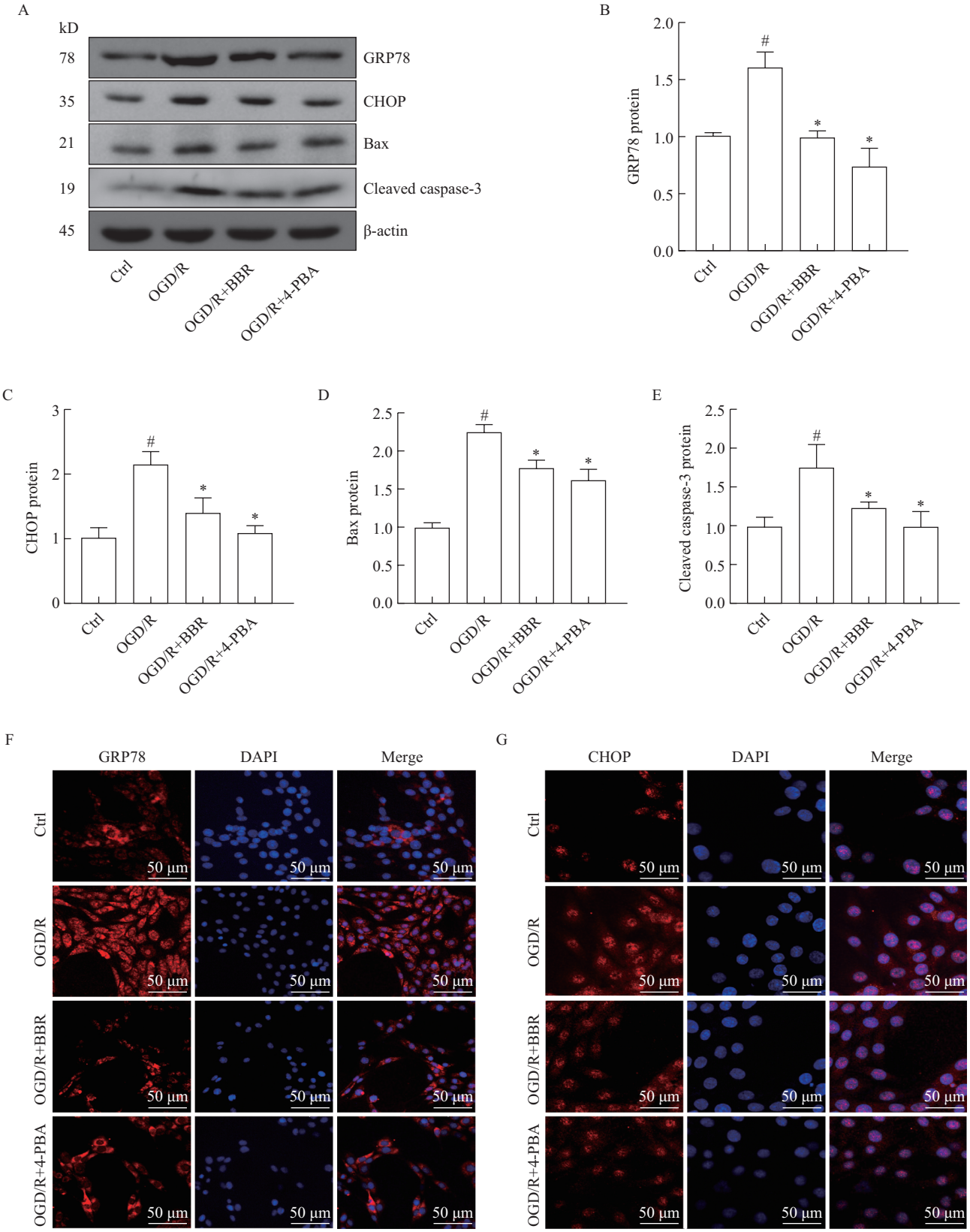

Fig. 3 Berberine repressed OGD/R-induced ER stress in PC12 cells

PC12 cells were exposed to OGD for 4 h and then reoxygenation for 18 h in the presence or absence of berberine (5 μmol/L) or 4-PBA (2 mmol/L). A–E: representative Western blot images (A) and quantitation of ER stress-related molecules GRP78 (B), CHOP (C), Bax (D), and cleaved caspase-3 (E). The expression of β-actin was used as the protein loading control. F, G: The fluorescence images of GRP78 (F) and CHOP (G) were detected with immunofluoroscopy and the DAPI-staining. The scale bar is 50 μm, and quantitative values are expressed as the mean ± SD (n=3). $^{\#}P$<0.05 *vs.* the control group, $^{*}P$<0.05 *vs.* the OGD/R group

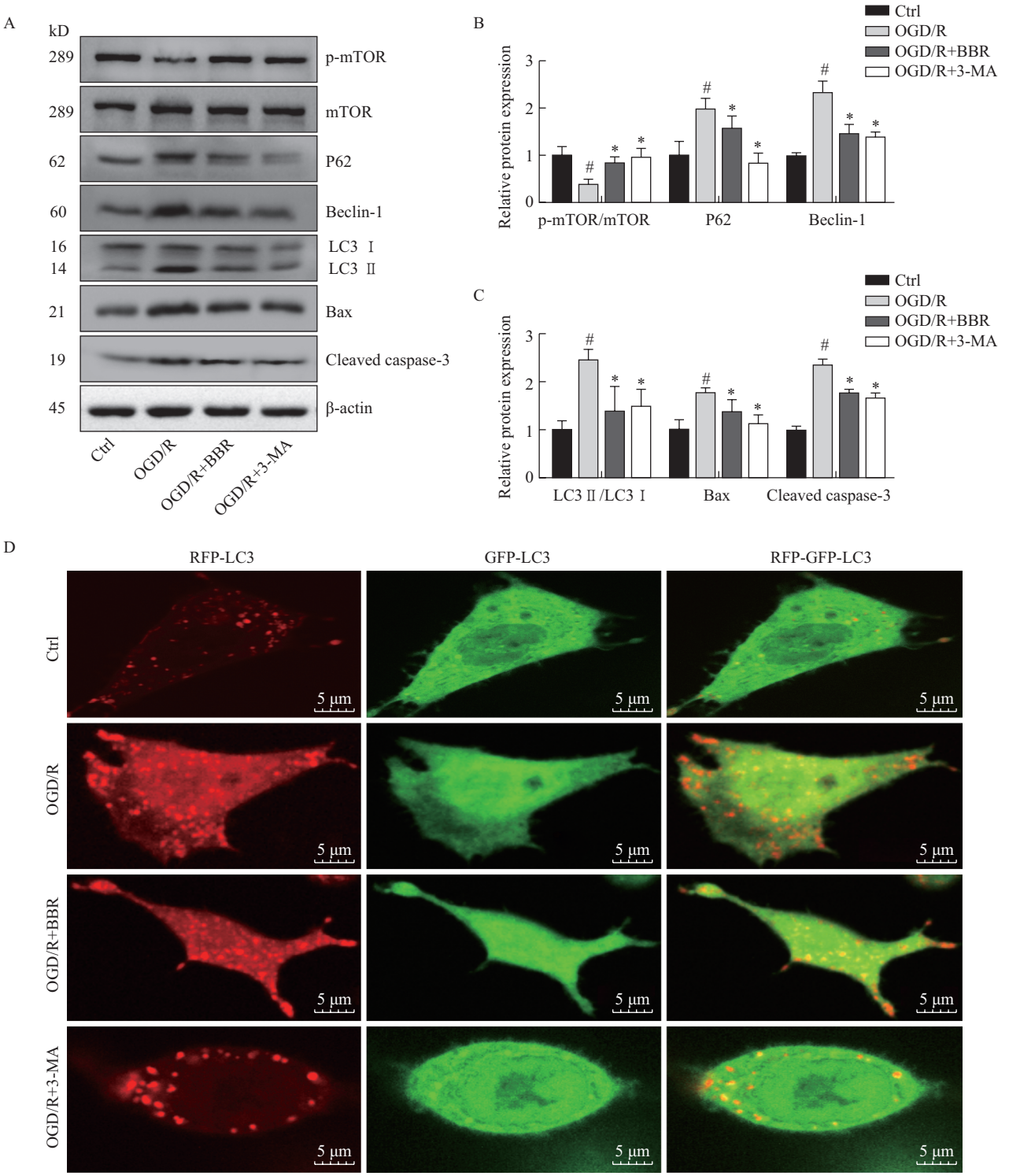

Fig. 4 Berberine attenuated excessive autophagy induced by OGD/R in PC12 cells

PC12 cells were exposed to OGD/R (OGD 4 h+R18 h) when co-cultured with berberine (5 μmol/L) or 3-MA (2.5 mmol/L). A–C: representative Western blot images (A) and quantitation of p-mTOR/mTOR, P62, Beclin-1 (B), LC3 Ⅱ/LC3 Ⅰ, Bax and cleaved caspase-3 (C); the expression of β-actin was used as the protein loading control. D: representative RFP-GFP-LC3 fluorescence images. Scale bar=5 μm. The quantitative values are expressed as the mean ± SD (n=3). $^{\#}P$<0.05 *vs.* the control group; $^{*}P$<0.05 *vs.* the OGD/R group

evidenced by decreased levels of autophagy-related markers (LC3 Ⅱ/LC3 Ⅰ, Beclin-1 and P62). We further found that berberine treatment activated the p-mTOR/mTOR pathway during OGD/R, which might account for the berberine-induced inhibition of autophagy. Therefore, our findings indicated that berberine ameliorates the OGD/R injury by inhibiting excessive ER stress and autophagy.

In eukaryotic cells, the ER is the most important organelle apart from the nucleus, as it is responsible for protein synthesis, folding, and transport[30, 31]. It is sensitive to ROS levels and intracellular redox states[32]. While the activation of ER stress by the accumulation of unfolded or misfolded proteins in the ER lumen helps to promote adequate protein folding and modification and reduce global mRNA translation, excessive and/or prolonged periods of ER stress promote apoptotic cell death rather than survival via the activation of downstream signals CHOP and caspase3[29, 33]. The ER-mediated promotion of the apoptosis pathway reportedly contributes to various degenerative diseases, such as Alzheimer's disease, Parkinsonism, and cerebral I/R injury (stroke)[34, 35]. Some study revealed that berberine for the treatment of multiple diseases involve its effects on ER stress[36]. The present study provides further evidence for the significant berberine-induced inhibition of ER stress and subsequent ER-stress dependent apoptosis in PC12 cells subjected to OGD/R.

Autophagy is a highly conserved process that degrades and recycles misfolded proteins and damaged cytoplasmic organelles under stressful conditions such as hypoxia, oxidative stress, ER stress, and nutrient deprivation[37, 38]. Autophagy plays a two-edged role during cerebral I/R injury. On the one hand, it predominantly mitigates the effects of damage to organelles and maintains cellular homeostasis. On the other hand, excessive or prolonged autophagy can cause autolysosome accumulation and autophagic blocking[20], eventually leading to cellular dysfunction, such as apoptosis, even cell death and necrosis. Mounting evidence suggests that excessive autophagy in cardiomyocytes and neurons induced by OGD/R aggravates I/R injury[39]. Berberine, however, can alleviate cardiac I/R injury by inhibiting excessive autophagy in cardiomyocytes[40, 41]. Our work expanded this earlier finding and demonstrated that berberine significantly increased the cellular viability of PC12 cells after OGD/R by inhibiting excessive autophagy; berberine could reduce cellular apoptosis and enhance cellular survival in PC12 cells after OGD/R via inhibition of excessive activation of ER stress and autophagy.

Berberine has anti-inflammation, anti-oxidative and neuroprotection functions. Recently, a study reported that berberine could attenuate the I/R injury through inhibiting HMGB1 release and NF-κB nuclear translocation in tMCAO model of mice[42]. Their results mainly focused on anti-inflammation effect of berberine. Our results further revealed that berberine ameliorated OGD/R injury through suppression of ER stress and autophagy. Furthermore, the autophagy flux was also detected by GFP-RFP-LC3 adenovirus infection experiment in our study. These findings suggested that berberine plays a protective role in OGD/R injury via inhibiting excessive ER stress and autophagy.

ER stress and autophagy pathways are regularly considered as cell protective processes[43, 44]. Our research found that excessive ER stress and autophagy were harmful to the OGD/R cells. OGD/R-induced ER stress and increased release of apoptotic proteins resulted in activation of the autophagy and exacerbation of injury. Nevertheless, there are some limitations in our work. First, whether autophagy and ER stress activation at the early stage have a protective effect on the cells was not examined in our research model. Second, the mechanism through which berberine regulates ER-stress and autophagy to control OGD-induced cell injury of PC12 requires further elucidation. Finally, berberine protecting OGD-induced injury needs to be further investigated *in vivo*.

In conclusion, the present study demonstrated the beneficial effects of berberine against OGD/R injury in PC12 cells, and the mechanism involves inhibition of excessive ER stress and autophagy. In the future experimental research, we will examine the related mechanisms in animal models, and endeavor to clarify the specific molecular mechanism via sound research designs.

Conflict of Interest Statement

All authors confirm that they have no conflicts of interest related to the preset study.

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