Platelet rich plasma alleviates OGD/R injury in N2a cell by enhancing autophagy through the miR-223/PAQR3 pathway

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Our study constructed an *in vitro* model of cerebral ischemia/reperfusion (I/R) injury to evaluate the protective effect of platelet rich plasma (PRP) on I/R injury and uncover the mechanism behind it. Firstly, N2a cells were exposed in the condition of oxygen and glucose deprivation/reperfusion (OGD/R) to construct a model of cerebral I/R *in vitro*. MTT assay was employed to access the effects of PRP in N2a cell OGD/R injury. Then, we evaluated the role of the expression of miR-223, progestin and adipoQ receptors 3 (PAQR3) and autophagy markers in the neuroprotective effect of PRP by qPCR and western blot. And the effect of miR-223/PAQR3 axis regulated autophagy in the neuroprotection of PRP was verified by overexpressing miR-223 and PAQR3. Finally, the interaction between miR-223 and PAQR3 was analyzed by the luciferase reporter gene. The results showed that after OGD/R treatment of N2a cells, the expression of miR-223 increased and the expression of PAQR3 and autophagy decreased. PRP improved cells damage caused by OGD/R in N2a cell, and reduced the expression of miR-223 in cells, increased PAQR3 and autophagy. The luciferase reporter assay was used to prove that miR-223 could target PAQR3 directly. Overexpression of miR-223 could eliminate the improvement effect of PRP on OGD/R cells, but at the same time, overexpression of PAQR3 restored the protection of PRP from cell damage. Our research found that in the OGD/R injury in *vitro* model, PRP inhibited the expression of miR-223 and enhanced autophagy to attenuate the injury by increasing the expression of PAQR3.

Key words: cerebral ischemic injury, platelet rich plasma, miR-223, PAQR3, autophagy

INTRODUCTION

Ischemic stroke, characterized as primary hypoxic insults and secondary cascade reaction, such as excitotoxicity, reactive oxygen species, inflammation, and apoptosis finally, was the third leading cause of mortality and a main cause of permanent disability, cognitive and motor disorders all over the world (Wei et al., 2017; Offner et al., 2017). In the above context, accumulating evidence indicated the existence of autophagy in several types of cerebral ischemia (global ischemia (Wang et al., 2011), and focal ischemia (Zhang et al., 2013), hypoxia-ischemia (Zheng et al.,

2018). The activation of autophagy was thought to protect myocardial cells and intestinal cells against ischemia/reperfusion (I/R) injury (Hu et al., 2018; Li et al., 2018) and caused the neuroprotective effect induced by focal cerebral ischemic preconditioning (Singh et al., 2017). These investigations demonstrated that the activation of autophagy was benefit to resist ischemic injury.

Platelet rich plasma (PRP) was an autologous product included a high concentration of platelets derived from the human and animal blood that was rich in growth factors, cytokines, and hormones. PRP had been used to improve healing of wounds (Gupta et al., 2018), spinal injuries (de Castro et al., 2019) clinically

and ischemia injuries in animal models (Bakacak et al., 2016). Recently, a study also indicated that the PRP could increase the proliferation of chondrocytes by induction of autophagy in human osteoarthritic cartilage. Meanwhile, autophagy was associated with the survival of neuron in cerebral ischemia injury (Zhang et al., 2013). Whether the PRP affected the cerebral ischemia injury through the regulation of autophagy had not been investigated.

PAQR3 belongs to the family of the progestin and adipoQ receptors (PAQR), which was a 7-transmembrane protein located at the Golgi apparatus. As a negative regulator, PAQR3 could mediate multiple signaling pathways to regulate critical signaling molecules to the Golgi apparatus (Wang et al., 2013). Autophagic activity was significantly blunted under nutrient deprivation conditions in PAQR3-deficient cells (Xu et al., 2016). It was demonstrated that the PAQR3 was highly relative to autophagic activity in glucose-deprived condition and the potential role of PAQR3 in OGD/R insult had not been reported.

MicroRNAs (miRNAs) were a kind of small noncoding RNAs, which could bind target mRNAs to regulate gene expression. The miR-223 was a kind of miRNA and had an important role in many diseases, such as infection, inflammation and cancer (Haneklaus et al., 2013). While recent studies also had indicated that miR-223 was remarkably deregulated in mouse I/R hearts and could inhibit cell necroptosis and inflammatory in heart (Liu et al., 2018; Meng eta al., 2018). Meanwhile, a previous study had demonstrated that the expression of miR-223 could be downregulated by PRP extraction (Li et al., 2019). It had not been reported that whether PRP protected neurons from OGD/R injury by regulating miR-223.

In our present study, we used the OGD/R injury in N2a cell to construct the cerebral ischemia injury *in vitro* and the effect of PRP on OGD/R injury was investigated. Then the effects of PRP on autophagy and the expression of miR-223 and PAQR3 was explored. Furthermore, we investigated the potential mechanism underlying these roles of PRP on OGD/R injury.

METHODS

Cell culture and OGD/R model

Mouse N2a neuroblastoma cells were obtained from the American Type Culture Collection (ATCC). The cells were cultured in supplemented DMEM (10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin). The OGD/R was used here to mimic I/R insult *in vitro*, as previously described (Hu et al., 2016). First,

mouse N2a neuroblastoma cells were transferred into a temperature-controlled anaerobic chamber (Forma Scientific) with a humidified atmosphere of 5% CO_2 and 95% air. To perform OGD/R treatment, the culture medium was replaced by deoxygenated glucose-free Hanks' Balanced Salt Solution (Invitrogen) and cells were maintained in the hypoxic chamber. After 4 h of OGD, N2a cells were returned in DMEM supplemented with 10% FBS under normoxic culture conditions for corresponding time as reperfusion. Images of cells were observed by an Olympus fluorescence microscope (Olympus).

To evoke overexpression of PAQR3, the cDNA for PAQR3 was obtained from a plasmid containing the wild type PAQR3 gene through PCR amplification. The resulting PCR product was cloned into the pEGFP-N1 vector confirmed by DNA sequencing. Then, the pEGFP-N1 and pEGFP-PAQR3 were transfected into N2a cells using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). The miR-223 mimic, as well as their respective negative controls, were synthetized by GenePharma (Shanghai, China) for *in vitro* transfection. N2a cells were transiently transfected with miR-223 mimic or miRNA control using Lipofectamine 2000 as described by the manufacturer's instructions (Invitrogen). After incubation for 24 h, the transfected cells were used to do some biological assays. To co-overexpression of miR-223 and PAQR3, N2a cells were transfected with miR-223 mimic and pEGFP-PAQR3 vector together using Lipofectamine 2000.

The level of PARQ3 in saline, platelets, PRP was tested by ELISA detection kit (Abcam. MA, USA) according to the manufacturer's instruction.

PRP preparation

PRP was isolated from enriching whole blood platelets by using two centrifugation steps. In details, venous blood was collected from 12 randomly chosen 180-210 g male SD rats (6-8 weeks old) containing anticoagulant 3.8% sodium citrate solution. Each blood sample was centrifuged at 1500 rpm for 15 min to separate the whole blood into three typical layers at room temperature: platelets and white blood cells in upper layer, a buffy coat layer in between and red blood cells at bottom layer. Transferred the upper layer and buffy coat to an sterile centrifuge tube and centrifuged at 2800 rpm for 8 min for PRP collection, as previously described (11). Meanwhile, cells were treated PRP via adding different ratio (v/v) of PRP to DMEM (10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin). Moreover, cells were purified by centrifugating

and washing with PBS according to the previous reference (Xu et al., 2018). On the other hand, the purified platelets were collected by ultracentrifugation and washing with saline. Then, the collected platelets were used in ELISA. All experiments were approved by the ethics committee of the Second Xiangya Hospital of Central South University.

Cells (5×10^3/well) were seeded in 96-well plates and treated with different OGD/R procedures or with different concentrations of PRP. After the treatment interval, cell viability was evaluated using a tetrazolium (MTT) assay. The absorbance value was measured at 490 nm in triplicate using a spectrophotometer (Thermo Scientific, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA from different treatment group was isolated using TRIzol reagent. RNA was transcribed to cDNA using the One Step PrimeScript miRNA cDNA Synthesis Kit. qRT-PCR were performed with SYBR Premix Ex Taq™II by using the ABI 7500 Real Time PCR system (Applied Biosystems, USA). PCR parameters for miRNA quantification were used as follow: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The primer pairs were used for qRT-PCR as follows (Xu et al., 2019; Zhou et al., 2017):
miR-223-F: 5´-GCGGCGGGTTGAGTCGAACAG-3´;
miR-223-R: 5´-ATCCAGTGCAGGGTCCGAGG-3´;
U6-F: 5´-CTCGCTTCGGCAGCACA-3´;
U6-R: 5´-AACGCTTCACGAATTTGCGT-3´;
PAQR3-F: 5´-GCACCCCGAGTAATTGTGAT-3´;
PAQR3-R: 5´-CATACACCGTGGACTGATGC-3´;
GAPDH-F: 5´-CCAGGTGGTCTCCTCTGA-3´;
GAPDH-R: 5´-CTGTAGCCAAATCGTTGT-3´.
Three duplications were performed in all real-time PCR reactions, and analysis of relative gene expression data used the $2^{-\Delta\Delta Ct}$ method.

Western blot

Protein samples (20 μg) were loaded on a 12% SDS polyacrylamide gel for electrophoresis and then transferred to PVDF membranes. After blocking with 5% nonfat milk in TBS–Tween buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) for 1 h at room temperature (RT), the membrane was incubated with primary rabbit anti-PAQR3 antibody (1:500, Abcam), rabbit anti-p62 antibody (1:200, Abcam), goat anti-LC3I or anti-LC3II antibody (1:400, CST), and mouse anti-GAPDH antibody (1:800, Abcam) at 4°C overnight. The membrane was washed three times with

TBST and then HRP-conjugated secondary antibody was added at a dilution of 1:40,000 at RT for 1 h. After three times washing, the membrane was stained with an enhanced chemiluminescence (ECL) kit (Bio-Rad).

Luciferase reporter assay

The analysis of bioinformatics identified PAQR3 as a possible gene target of miR-223. The 3'-UTR of PAQR3 containing the putative binding sites of miR-223 were synthetized and obtained from Sangon Biotech (Shanghai, China), then were cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vectors (Promega, Madison, WI, USA) to construct reporter vectors PAQR3 (WT/MT). PAQR3 (WT/MT) were transfected into cells together with control, vector-control (NC), miR-223 mimics or miR-223 inhibitor using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. The relative luciferase activity was analyzed by the Dual-Glo Luciferase Assay System (Promega).

Meanwhile, normalizer means Renilla luciferase was considered as internal control in order to calculate the value of firefly luciferase/Renilla luciferase. Meanwhile, the value of control group was normalized as 1 for obtaining the luciferase activity in different groups.

Statistical analysis

All data were expressed as mean value ± SD. One-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* comparison test was performed for comparisons among multiple groups. *P* value < 0.05 was considered statistically significant.

RESULTS

The effects of OGD/R on the expression of miR-223 and PAQR3 in N2a cells

The results of cell viability assays showed that OGD/R treatment in N2a cell dramatically reduced the cell viability compared to the control group after reperfusion 12 h (Fig. 1A). The expression of miR-223 gradually increased in the 0 h after reperfusion, while the PAQR3 showed the opposite trend (Fig. 1B, C). Furtherly, the expression of autophagy-related protein, LC3I, LC3II and p62 were detected after reperfusion 24 h. It was demonstrated that the expression of LC3I and LC3II were significantly decreased and the expression of p62 increased significantly after OGD/R treat-
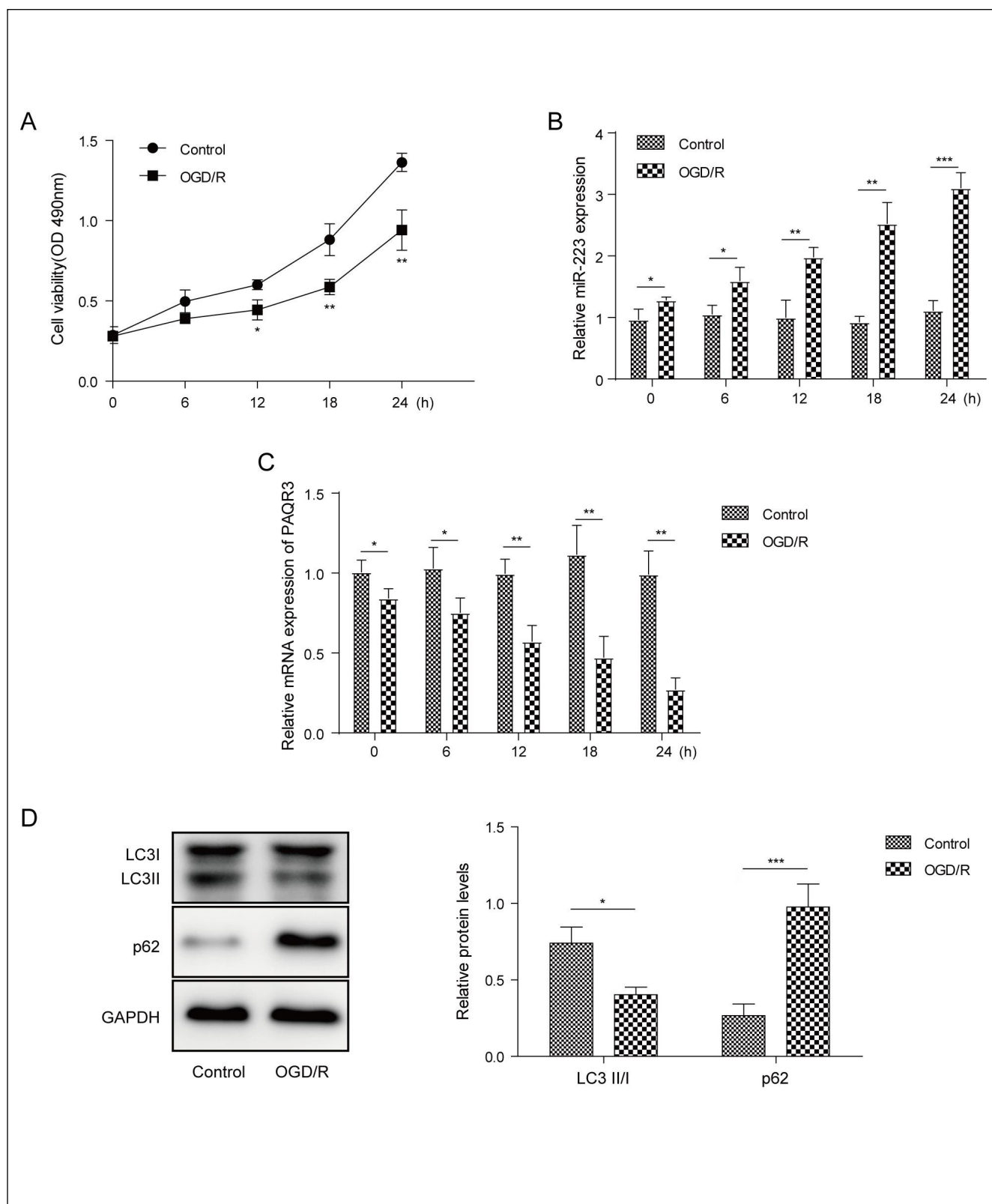
Fig. 1. The effects of OGD/R on the expression of miR-223 and PAQR3 in N2a cells. (A) MTT assay results of N2a cell followed treatment with different reperfusion times after OGD 4 h. (B and C) The expression of miR-223 and PAQR3 followed detection by qRT-PCR with different reperfusion times after OGD 4 h. (D) The proteins level of LC3I, LC3II and p62 followed detection by western blots after OGD 4 h. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Data were presented as mean ± SD. At least three independent experiments were performed.

ment (Fig. 1D). These results suggest that OGD/R treatment cause the change of miR-223 and PAQR3 and the decrease of autophagy.

The effects of PRP on autophagy and the expression of miR-223 and PAQR3 in OGD/R-induced N2a cells

In order to study the effect of PRP on autophagy and the expression of miR-223 and PAQR3, we added different dosage of PRP for 0 to 48 h after 12 h of reperfusion. As shown in Fig. 2A, compared with the control group, the cell viability of PRP-treated OGD/R cells increased significantly from 0 to 48 h in a dose dependent manner. After cells OGD/R treatment, the cell clumped and the outline of the cell body was blurred. After 48 h of PRP treatment, the cell morphology was improved in a dose-dependent manner (Fig. 2B). Therefore, we choose to use PRP to treat the cells under the condition of 48 h after reperfusion for 12 h for the next investigation. The expression of miR-223 was gradually reduced and the expression of PAQR3 was obviously increased compared to the OGD/R group (Fig. 2C-E). Finally, the expression of LC3I and LC3II were significantly increased compared to the OGD/R group and the expression of p62 decreased approximately 2-fold after PRP treatment (Fig. 2F). On the other hand, the level of miR-223 and PAQR3 in platelets or PRP were not expressed, compared with that in saline (Supplementary Fig. 1A, B), Consistently, PRP did not affect the proliferation and expressions of miR-223 and PAQR3 in N2a cells (Supplementary Fig. 1C, D). These results indicated that the administration of PRP regulated the expression of miR-223 and PAQR3 and the increase of autophagy after OGD/R injury.

PRP affected OGD/R cells autophagy and increased cell viability through down-regulating miR-223

Next, we studied the role of miR-223 in the process of cell autophagy increased by PRP after the OGD/R injury. The results showed the miR-223 mimic significantly increased the expression of miR-223 in 10% PRP treatment group after the process of OGD/R (Fig. 3A). Meanwhile the cell viability assays indicated that the miR-223 mimic treatment dramatically decreased the cell viability compared to the treatment of 10% PRP group (Fig. 3B). The cell autophagy of miR-223 mimic treatment group also decreased compared to the 10% PRP group. As shown in Fig. 3C, the expression of LC3I and LC3II in miR-223 mimic group were significantly

reduced after PRP treatment, while the protein level of p62 increased about 2 times. The results showed that PRP increased the viability of N2a cell treated with OGD/R by increasing autophagy of cells.

MiR-223 targeted OGD/R cells autophagy and reduced cell viability by regulating PAQR3

Furthermore, we studied the expression of PAQR3 in cells transfected with miR-223 mimic after OGD/R treatment. It was showed that the expression of PAQR3 increased in 10% PRP group compared to OGD/R group and the change was almost reversed by adding the miR-223 mimic in qRT-PCR and WB assay (Fig. 4A, B). Meanwhile we used a bioinformatics software and we found that PAQR3 was a possible target gene of miR-223 (Fig. 4C). The results of the luciferase reporter assay demonstrated that miR-223 mimic or inhibitor co-transfected cells with wild-type PAQR3 (wt-PAQR3), and it was found that wt-PAQR3 luciferase activity was inhibited by miR-223 mimic, the miR-223 inhibitor increased the fluorescence intensity. The mutant-type PAQR3 (mut-PAQR3) did not change the fluorescence intensity (Fig. 4D). In order to study the effects of PAQR3 on the autophagy of N2a cells treated with OGD/R, we increased the expression level of PAQR3 in N2a cells (Fig. 4E, F). Overexpression of PAQR3 significantly enhanced the cell viability and the expression of LC3I and LC3II compared to the OGD/R treatment (Fig. 4G, H). These results showed miR-223 affected OGD/R cell autophagy and cell viability through targeting PAQR3.

PRP increased cells autophagy through miR-223/PAQR3 and affected OGD/R cells autophagy and viability

Finally, we studied the effect of co-expression of PAQR3 and miR-223 mimic in N2a cell after OGD/R treatment. As shown in Fig. 5A-C, the overexpression of miR-223 alone decreased the expression of PAQR3, but the overexpression of PAQR3 alone did not affect miR-223. The co-expression of PAQR3 and miR-223 significantly reversed the change of the cell viability trend compared to both OGD/R group transfected with miR-223 mimic (Fig. 5D). Overexpression of miR-223 significantly reduced the autophagy of the PRP group, while overexpression of miR-223 and PAQR3 reversed the affect the overexpression of miR-223 alone and increased OGD/R cell autophagy (Fig. 5E). The results showed that PRP can increased the autophagy of cells by inhibiting miR-223/PAQR3 pathway and enhancing OGD/R cell viability.
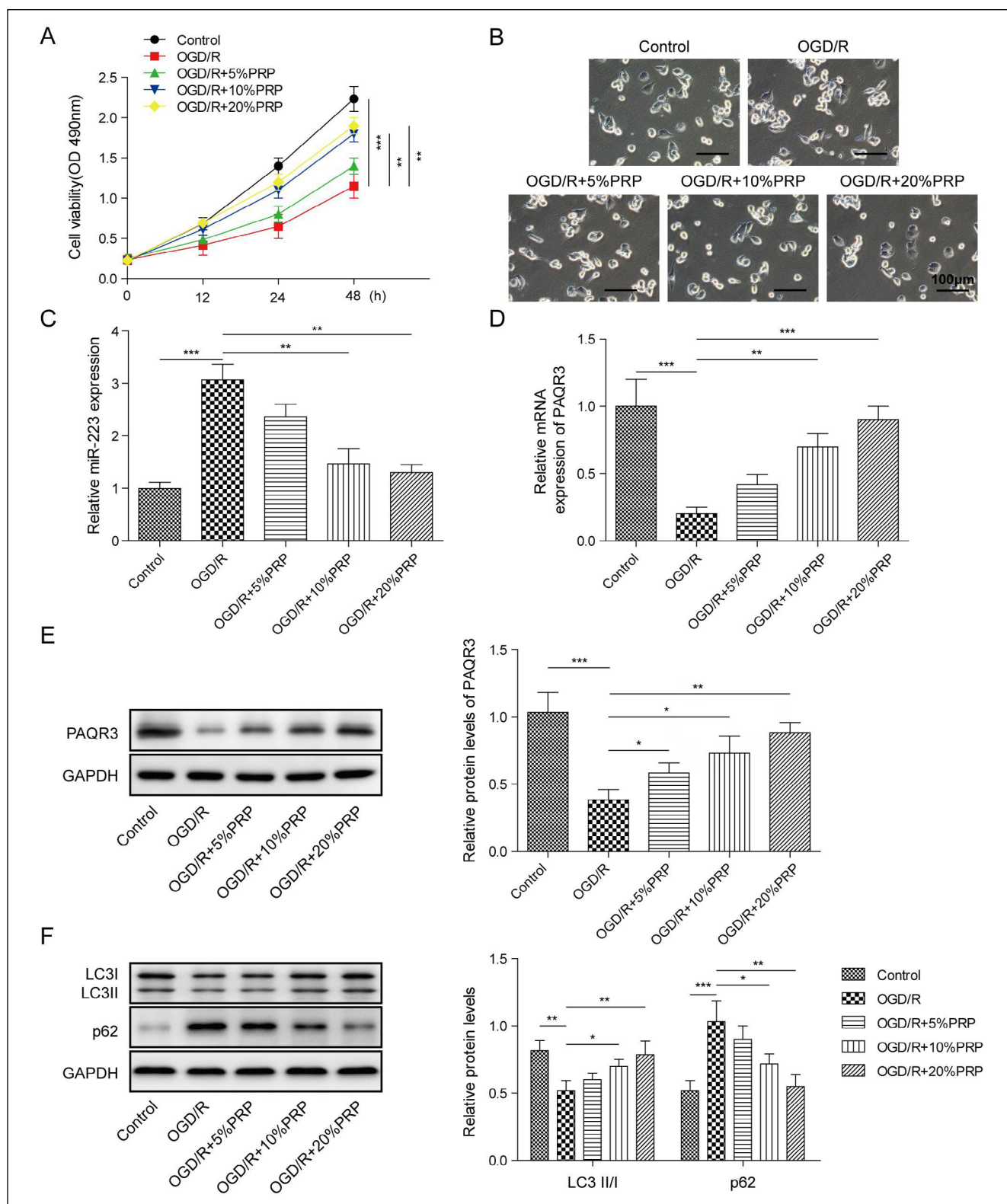
Fig. 2. The effects of PRP on autophagy and the expression of miR-223 and PAQR3 in OGD/R -induced N2a cells. (A) Photographs of N2a cell treatment with different concentration PRP 48 h after OGD/R. (B) MTT assay results of N2A cells treated with PRP at different concentrations after OGD/R. (C and D) The expression of miR-223 and PAQR3 were detected in cells by RT-qPCR which were treated with PRP at different concentrations after OGD/R. (E and F) Western blot detection of PAQR3, LC3I, LC3II and p62 proteins level after treating cells with different concentrations of PRP after OGD/R. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Data were presented as mean ± SD. At least three independent experiments were performed.
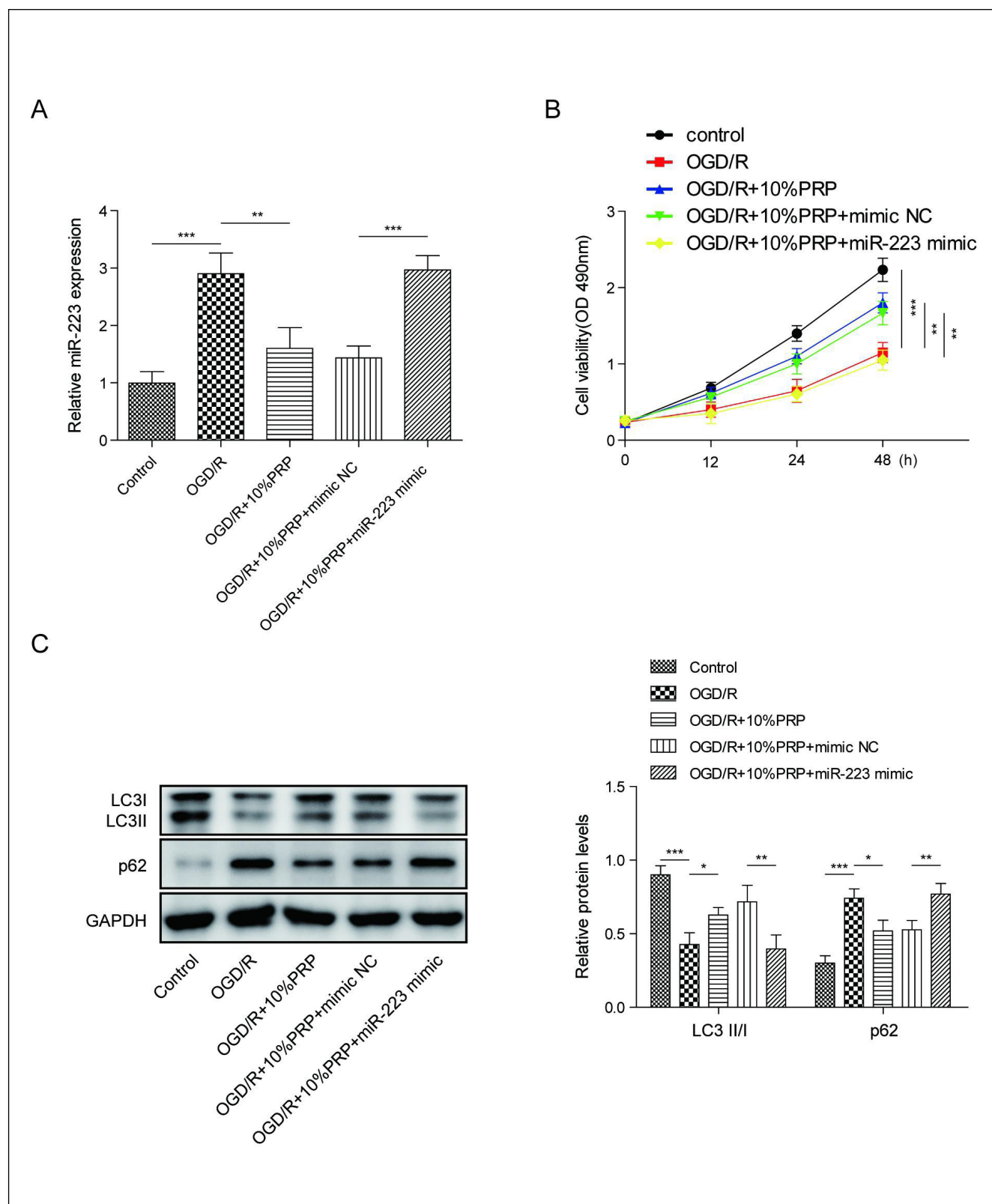
Fig. 3. PRP affected OGD/R cells autophagy and increased cell viability by down-regulating miR-223. (A) qRT-PCR detection of miR-223 expression in cells after overexpression of miR-223. (B) Detection of cell activity after overexpression of miR-223 by MTT. (C) Analysis of protein levels of LC3I, LC3II and p62 in cells after overexpression of miR-223 by western blot detection. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Data were presented as mean ± SD. At least three independent experiments were performed.
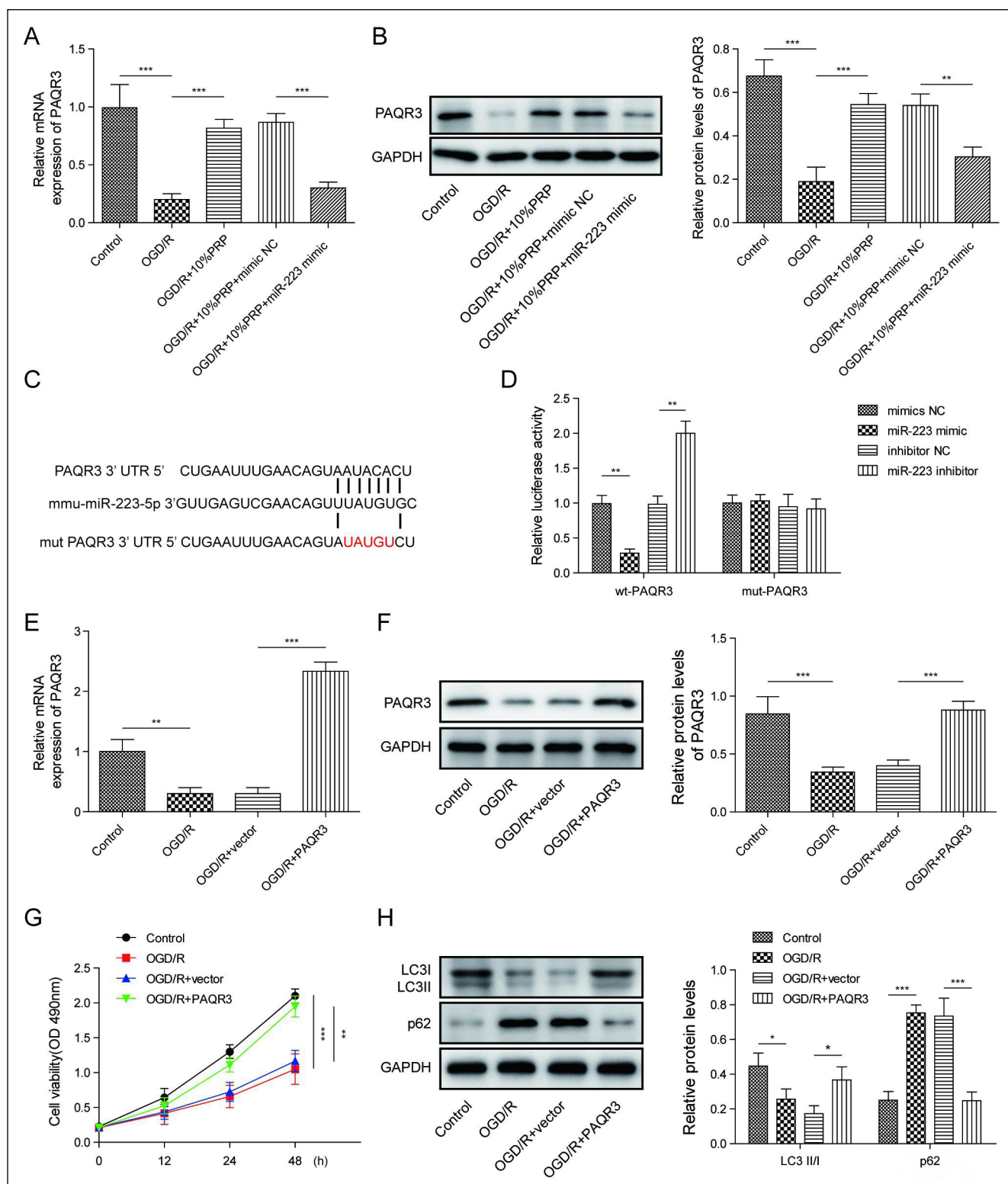
Fig. 4. MiR-223 affected OGD/R cells autophagy and increased cell viability by regulating PAQR3. (A) qRT-PCR detection of PAQR3 expression in cells after miR-223 overexpression. (B) Western blots detection of PAQR3 expression in cells after miR-223 overexpression. (C) The binding sites of PAQR3 and miR-223 were predicted by TargetScan online. (D) The binding between PAQR3 and miR-223 tested by the luciferase reporter assay. (E and F) qRT-PCR and Western blot analysis of PAQR3 in cells after overexpression of PAQR3 using an overexpression vector. (G) Cells activity after overexpression of PAQR3 by MTT. (H) Western blots were used to detect the protein levels of LC3I, LC3II and p62 in cells after overexpression of PAQR3. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Data were presented as mean ± SD. At least three independent experiments were performed.
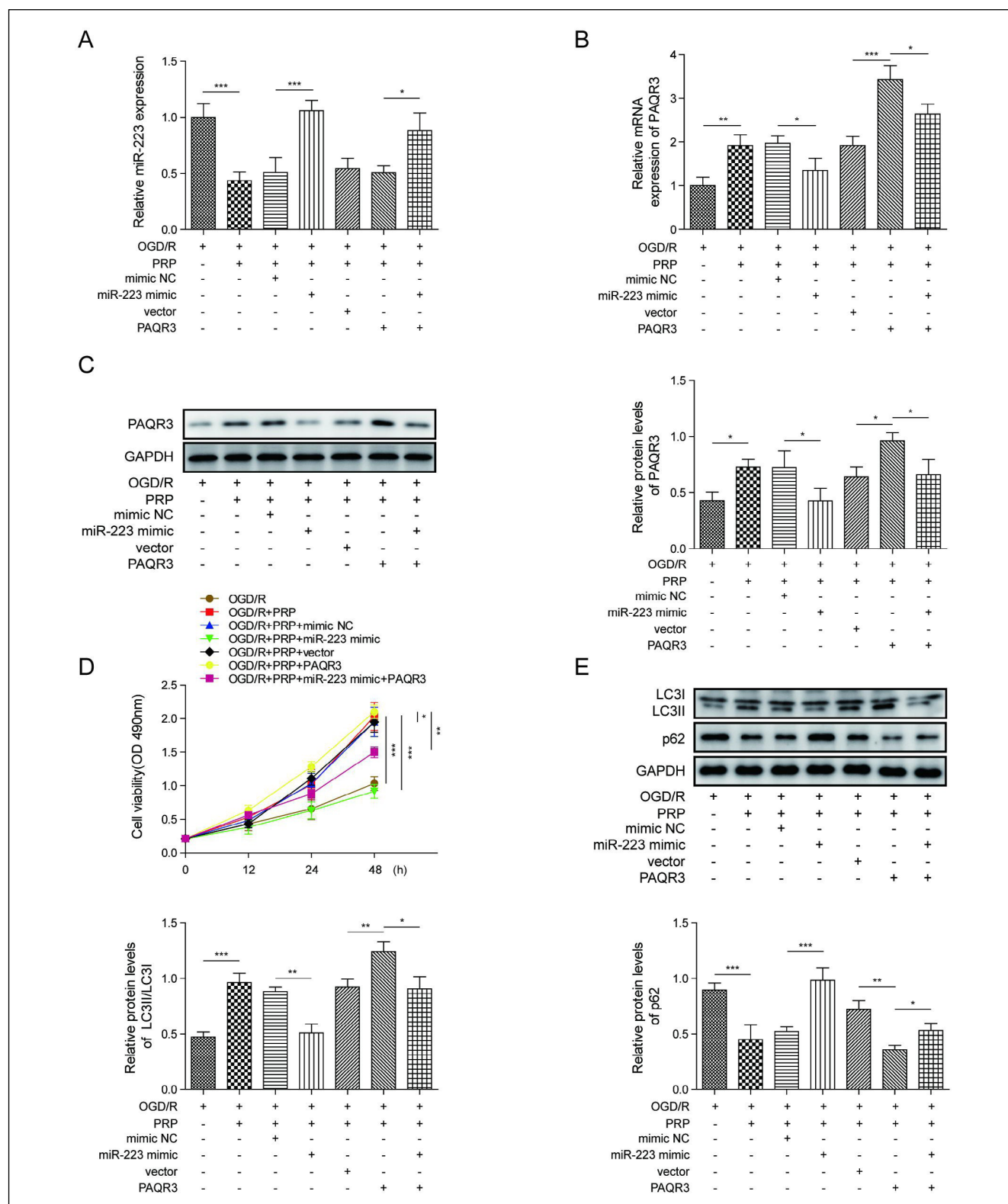
Fig. 5. PRP increased cell autophagy through miR-223/PAQR3 and affected OGD/R cells autophagy and viability. (A and B) qRT-PCR was used to detect the expression levels of miR-223 and PAQR3 in cells that simultaneously overexpressed miR-223 and PAQR3. © Western blot detection of PAQR3 protein level in cells overexpressing both miR-223 and PAQR3. (D) MTT assayed for cell activity after overexpression of miR-223 and PAQR3. (E) Western blots detection of LC3I, LC3II and p62 proteins level in cells after overexpression of miR-223 and PAQR3. *P<0.05, **P<0.01, ***P<0.001. Data were presented as mean ± SD. At least three independent experiments were performed.

DISCUSSION

PRP had proven to play an important role in the promoting wound healing in animal models. However, there is no report about PRP in the study of cerebral ischemia. And our study found that PRP could improve cells damage caused by OGD/R damage in N2a cells. The PRP inhibited the expression of miR-223 to increase the expression of PAQR3 which induced cell autophagy after OGD/R treatment. Our results indicated that PRP might play an important role in the future treatment of brain injury. And our study was the first to report the effect of PRP on brain injury and demonstrate the possible regulatory pathways.

PRP was a preparation from plasma enriched with autologous platelet concentrates and contain a lot of growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β1 and vascular endothelial growth factor (VEGF) (Zhou et al., 2017). In fact, PRP therapies were used increasingly for treating musculoskeletal soft tissue injuries, including tendinopathies, tendon tears and cartilage injuries (Moraes et al., 2013). It had been reported that PRP increased the proliferation of chondrocytes and autophagy in osteoarthritic chondrocytes (Moussa et al., 2017). In the meantime, it also was reported that the EPG, which was extracted from PRP, affected the expression of intracellular miRNA-21 in HaCaT cells (Moraes et al., 2013). In our study, we also found PRP can increase autophagy and regulate the expression of intracellular miR-223. More importantly, it was the first time to study the effects of PRP on neural injury and provided a theoretical basis for the possible used of PRP as a treatment for nerve cell injury.

The role of autophagy was an important in progression of certain diseases such as cancer (Velasco, 2018), infectious diseases (Rubinsztein et al., 2015) and neurodegenerative diseases (Tan et al., 2014). Autophagy was also reported in the cerebral ischemia process (Sun et al., 2018; Lu et al., 2019), but the role of autophagy was still controversial in that process. In our study, the results showed the activation of autophagy enhanced the cell viability in the OGD/R injury model. Our results are different from those of others (Sarkar et al., 2020), which might be relative to the selected cell line and treatment methods. Meanwhile it was reported that PAQR3 could enhance autophagy upon glucose starvation experiment in HeLa cells (Xu et al., 2016). We also detected that the high expression of PAQR3 increase autophagy the in OGD/R model in N2a cell in our study. Overall, our data for the first time demonstrated that PRP regulated autophagy of nerve cells through increasing the expression of PAQR3 to protect nerve cells from I/R damage *in vitro*. We suggest that it might be

a new treatment for cerebral ischemia. ERK is found to be involved in PAQR3-mediated cell injury induced by OGD/R. In addition, PAQR3 could induce the cell autophagy. Previous studies indicated that ERK could promote the autophagy (Cagnol et al., 2010; Martinez-Lopez et al., 2013), and PAQR3 could activate ERK signaling. Therefore, we speculate that PAQR3 can induce autophagy by activating ERK signal in brain injury.

Meanwhile, a previous study found that PAQR3 silencing could protect against OGD/R-induced neuronal apoptosis (Qiao et al., 2021), while our research suggested that PAQR3 could protect against OGD/R-induced N2a cell injury. This discrepancy might be due to the different models, different cells or different tissues. In the future, we will further confirm the function of PAQR3 in hypoxia-ischemia-induced brain injury *in vivo*.

CONCLUSION

In summary, our studies provided the important evidence that the PRP improved cell damage caused by OGD/R injury in N2a cell. PRP induced cells autophagy by inhibiting the miR-223/PAQR3 axis, thereby reducing cells damage after OGD/R treatment. Here we reported that the PRP had the neuroprotective activity for the first time and provided a new direction for the therapy of cerebral ischemia.

ACKNOWLEDGMENT

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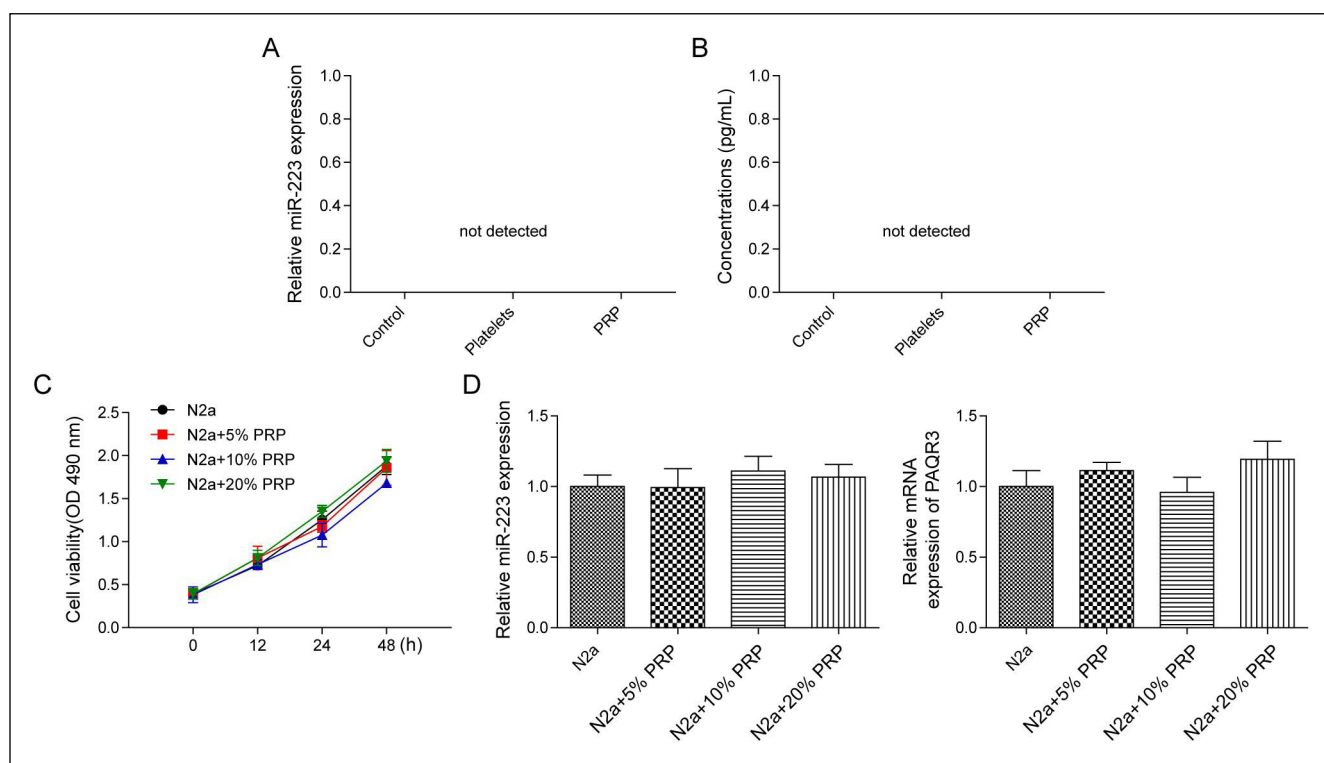
SUPPLEMENTAL MATERIALS

Fig. 1S. The level of miR-223 and PAQR3 in platelets or PRP were not affected, compared with that in saline and PRP does not affect the proliferation and expression of miR-223 and PAQR3 in N2a cells. (A) The level of miR-223 in saline, platelets or PRP was tested by qRT-PCR. (B) The concentration of PAQR3 in saline, platelets or PRP was assessed by ELISA. (C) N2a cells were treated with 5% PRP, 10% PRP or 20% PRP. The viability of N2a cells was assessed by MTT assay. (D) The level of miR-223 and PAQR3 in N2a cells were tested by qRT-PCR. Data were presented as mean ± SD. At least three independent experiments were performed.