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Puerarin inactivates NLRP3-mediated pyroptotic cell death to alleviate cerebral ischemia/reperfusion (I/R) injury through modulating the LncRNA DUXAP8/miR-223-3p axis

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Abstract: NLRP3 inflammasome-mediated cell pyroptosis aggravates the development of cerebral ischemia/reperfusion (I/R) injury, and the aim of this study is to investigate the potential utilization of the Chinese medicine, Puerarin, in treating this disease. Through conducting *in vitro* and *in vivo* experiments, the present study illustrated that Puerarin regulated LncRNA double homeobox A pseudogene 8 (DUXAP8)/miR-223-3p axis to inactivate NLRP3-mediated pyroptotic cell death, resulting in the attenuation of I/R injury. Specifically, the cerebral I/R injury in rat models and hypoxia/reoxygenation (H/R) in primary hippocampus neuron (PHN) cells were inducted, which were subsequently exposed to Puerarin treatment. As expected, we validated that Puerarin suppressed cell pyroptosis and rescued cell viability in I/R rat hippocampus tissues and H/R PHN cells. Next, through bioinformatics analysis, we noticed that miR-223-3p targeted both LncRNA DUXAP8 and NLRP3 mRNA, and both LncRNA DUXAP8 ablation and miR-223-3p overexpression inactivate NLRP3-mediated cell pyroptosis to rescue cell viability in H/R PHN cells. Interestingly, we evidenced that Puerarin restrained LncRNA DUXAP8 expressions, but upregulated miR-223-3p in I/R rat tissues and H/R PHN cells, and the protective effects of Puerarin on H/R PHN cells were abrogated by overexpressing LncRNA DUXAP8 and silencing miR-223-3p. Collectively, we concluded that Puerarin regulated LncRNA DUXAP8/miR-223-3p/NLRP3 signaling cascade to attenuate I/R injury.

Introduction

Ischemic stroke accounts for approximately 87% of all stroke patients worldwide, and about 15 million people suffer from stroke each year (Benjamin *et al.*, 2018). Currently, the main treatment therapies for cerebral ischemia includes pharmacological and mechanical reperfusion strategies (Zhang *et al.*, 2016; Zhao *et al.*, 2017), and researchers and clinicians agreed that timely ischemia-reperfusion strategy is effective to prevent neurological death and dysfunction (Zhang *et al.*, 2016; Zhao *et al.*, 2017), however, the adverse effects caused by this strategy, including intracerebral hemorrhage (Kimiwada *et al.*, 2019; Zhang *et al.*, 2017a), brain edema (King *et al.*, 2018; Nakano *et al.*, 2018), etc., still remains a serious challenge in ischemic stroke treatment. In addition, the pathogenesis mechanisms of cerebral ischemia/reperfusion (I/R) injury are very complicated, which can be attributed to

metabolic energy failure (Galkin, 2019; Vannucci *et al.*, 2004), intracellular Ca2+ elevation (Sun *et al.*, 2018; Zhang *et al.*, 2017b), mitochondrial injury (Gong *et al.*, 2018; Zhang *et al.*, 2013), and cell pyroptosis-mediated inflammation (Guo *et al.*, 2016; Zhao *et al.*, 2019). Among those mechanisms, NLRP3 inflammasome-mediated pyroptotic cell death played an important role to aggravate I/R injury development, and pharmacological inactivation of NLRP3 inflammasome has been proved as effective strategies to attenuate I/R injury in multiple pre-clinical studies (An *et al.*, 2019; He *et al.*, 2019; Ma *et al.*, 2019; Qu *et al.*, 2019; Ye *et al.*, 2019).

As a well-known Chinese medicine, Puerarin (7,4-dihydroxyisoflavone-8β-glucopyranoside) is one of the main bioactive isoflavone extracted from the Puerariae Lobatae Radix (*Pueraria lobata* (Willd.) Ohwi) root, and this drug has been recorded in the Pharmacopoeia of the People's Republic of China (2020 edition, Part I) for the treatments of cardio- and cerebrovascular diseases in China (Zhang *et al.*, 2019d; Zhang *et al.*, 2019e). Of note, Puerarin exerted its neuroprotective effects to alleviate cerebral I/R injury in rat models (Ling *et al.*, 2018; Liu *et al.*, 2013; Wang *et al.*, 2018; Zhou *et al.*, 2014), and the available clinical data

hinted that Puerarin was effective for the treatment of ischemic stroke in China (Liu et al., 2016). For example, Jin-Feng Wang et al. (2018) found that Puerarin protected rat brain against I/R injury by inactivating the AMPK-mTOR-ULK1 pathway mediated autophagy, and Liu et al. (2013) evidenced that Puerarin activated the cholinergic anti-inflammatory pathway to alleviate cerebral I/R injury. However, as the results of the complicated nature of Chinese medicine, the underlying mechanisms are still largely unknown. Interestingly, researchers noticed that Puerarin inactivated NLRP3-meidated cell pyroptosis to regulate I/R injury (Guan et al., 2020; Wang et al., 2020). Specifically, the data provided by Zi-Kuang Wang indicated that Puerarin regulated the SIRT1/NF-kB pathway to suppress NLRP3 inflammasome in myocardial I/R injury (Wang et al., 2020), and Guan et al. (2020) proved that Puerarin ameliorated I/R-induced retinal ganglion cell damage through regulating the TLR4/NLRP3 pathway, which enlightened us that Puerarin might protect neurons from cerebral I/R-induced death through inhibiting NLRP3-mediated pyroptotic cell death.

Targeting the competing endogenous RNA (ceRNA) networks has been reported as a feasible strategy to attenuate cerebral I/R (Wan et al., 2020; Zeng et al., 2019; Zhang et al., 2019b; Zhong et al., 2019). Interestingly, recent data suggested that targeting the LncRNAs/miRNAs axis was capable of suppressing cerebral I/R development (Wan et al., 2020; Zeng et al., 2019; Zhang et al., 2019b; Zhong et al., 2019) and NLRP3-mediated cell pyroptosis (Hu et al., 2019; Yu et al., 2018). According to the previous publications, the NLRP3 mRNA could be targeted and degraded by miR-223-3p, resulting in the suppression of cell pyroptosis (Dong et al., 2019; Wan et al., 2018; Zhang et al., 2019c). In addition, upregulation of miR-223-3p suppressed necroptosis (Qin et al., 2016) and microglial M1 polarization mediated inflammation (Zhao et al., 2020) in I/R rats, hence we selected miR-223-3p for further analysis. Moreover, LncRNA double homeobox A pseudogene 8 (DUXAP8) potentially acted as RNA sponges for miR-223-3p, but no literatures reported the potential regulating effects of LncRNA DUXAP8 in cerebral I/R injury or cell pyroptosis. Furthermore, multiple miRNAs could be regulated by Puerarin treatment (Zhao et al., 2020), but it was still unclear whether Puerarin regulated the LncRNA DUXAP8/miR-223-3p axis in hippocampus neural cells.

Hence, by establishing in vitro and in vivo models, the present study managed to investigate whether Puerarin attenuated the development of cerebral I/R injury through modulating the LncRNA DUXAP8/miR-223-3p/NLRP3 signaling cascade, while protein designations should be in regular capital letters.

Materials and Methods

In vivo I/R rat models establishment

We bought the Sprague-Dawley (SD) rats (males, N = 40, aged 10 weeks, weighed 240–290 g) from the SiPeiFu Biotech (Beijing, China), and all the rats were fed under the specific-pathogen-free (SPF) circumstances with 12-hour light–dark cycle, standard humidified atmosphere (60 ± 5%) and were freely accessible to water and food. The Puerarin was bought from North China Pharmaceutical Co., Ltd. (Shijiazhuang, China), and was diluted in the physiological PBS buffer at the final concentration of 25 mg/mL for further utilization at 100 mg/kg each rat according to our preliminary data (data not shown) and previous literature (Wang et al., 2018; Zhou et al., 2014). Then, the middle cerebral artery occlusion (MCAO) method was used to establish I/R rat models with the protocols as follows: the rats were intraperitoneally injected with 10% chloral hydrate at the concentration of 350 mg/kg for anesthetization, and a monofilament nylon suture was inserted into the internal carotid to generate MCAO for 90 min, which were then removed and allowed reperfusion for 48 h. Finally, the rat hippocampus tissues were separated and collected for further analysis. We declared that all the animal experiments were approved by the Ethics Committee of Ningbo Rehabilitation Hospital (No. 2018NBR67362).

2,3,5-Tripthenyltetrazolum Chloride (TTC) staining for the measurement of infarcted volume

TTC staining assay was performed to evaluate the infarcted area volume in rat brain in keeping with the standard experimental procedures (Zhang et al., 2019a). Briefly, the rat brains were collected and prepared as sections with 2 mm thickness, which were further stained with 2% TTC (Sigma, MO, USA) for 30 min at 37°C, and the tissues were subsequently fixed with 4% formalin overnight at room temperature. The infarcted area was observed, and the volume was calculated.

Cell culture and induction of cellular H/R models

Based on the experimental protocols provided by the previous work (Qin et al., 2020), the male newborn rat (N = 5) were used for the isolation and purification of the primary hippocampus neuron (PHN) cells, which were maintained in the Neuro-basal Medium (Invitrogen, USA) containing poly-D-lysine (Invitrogen, USA), 2% B27plus glucose (5 g/L) and 0.25% Glumax, in the incubator with standard culture conditions. At day 4 post-culture, the PHN cells were subjected to hypoxic stimulation to induct H/R cellular models as previously described (Qin et al., 2020), which were further stimulated by Puerarin (80 μM) for 0, 24, and 48 h, respectively. Also, the HEK293T were obtained (ATCC, USA) and cultured in the DMEM medium (Gibco, USA) with 10% FBS (Gibco, USA).

Delivery of vectors into cells

A commercial third-party company GenePharma (Shanghai, China) was employed to design and synthesize the LncRNA DUXAP8 overexpression/downregulation vectors, and the mimic and inhibitor for miR-223-3p were obtained from GenePharma (Shanghai, China), we delivered the above vectors into the PHN cells by using the Lipofectamine 2000 transfection kit (Invitrogen, USA). At 24 h post-transfection, Real-Time qPCR was conducted to examine the transfection efficiency.

Real-Time qPCR analysis

Total RNA was extracted from cells and tissues by using the commercial TRIZOL reagent (Beyotime, Shanghai, China),

which were further reversely transcribed into cDNA and quantified by performing Real-Time qPCR analysis. The detailed experimental procedures and primers could be found from the previous work (Zhao *et al.*, 2020).

Western Blot analysis

The RIPA lysis buffer (Beyotime Biotech, Shanghai, China) was used for protein extraction and BCA kit (Beyotime Biotech, Shanghai, China) was used for protein quantification. The proteins were separated by 10% SDS-PAGE, and were transferred onto PVDF membranes (Millipore, USA). The membranes were blocked and were sequentially incubated with primary/secondary antibodies. Finally, the Western Blot Hyper HRP Substrate (TAKARA, USA) was used for protein bands visualization.

Examination of cell proliferation and viability

The primary hippocampus neuron (PHN) cells were cultured in the 96-well plates and treated with 10 μL of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) solution (5 mg/mL) for 4 h incubation at 37°C, and the supernatants were carefully discarded and the 96-well plates were added with dimethyl sulfoxide (DMSO). Then, the plates were vortexed, and a microplate reader (ThermoFisher, USA) was used to evaluate cell proliferation. Also, the cells were stained with trypan blue staining buffer (Merck, USA) to determine cell viability, and the cells stained with blue were regarded as dead cells.

Cell apoptosis examination

In this study, we respectively used TUNEL apoptosis kit (Beyotime, Shanghai, China) and the Annexin V-FITC/PI staining reagent (Invitrogen, USA) followed by Flow Cytometer (Becon Dickinson FACS Calibur, USA) detection to evaluate cell apoptosis in PHN cells, and the protocols were well-documented in the manufacturer's instructions.

Validation of the binding sites among LncRNA DUXAP8, miR-223-3p and NLRP3 mRNA

The targeting sites of miR-223-3p with LncRNA DUXAP8 and 3' untranslated region (3' UTR) of NLRP3 were predicted, and the targeting sites in LncRNA DUXAP8 and NLRP3 were mutated and named as Mut-LncRNA DUXAP8 and Mut-NLRP3. In addition, their wild-type counterparts were shown as Wt-LncRNA DUXAP8 and Wt-NLRP3. The above sequences were cloned into the p-MIR-luciferase reporter plasmids (GenePharma, Shanghai, China), and were co-transfected with miR-NC, miR-223-3p mimic and inhibitor into the HEK293T cells. At 48 h post-transfection, the relative luciferase activities were measured.

Enzyme linked immunosorbent assay (ELISA)

The supernatants of the PHN cells were collected, and the expression levels of IL-1β and IL-18 were examined by using their corresponding ELISA kit (RAPIDBIO, CA, USA) in keeping with the producers' instruction. The optical density (OD) values were measured by using a microplate reader (ThermoFisher Scientific, USA) with 450 nm wavelength.

Collection and analysis of the data

SPSS 18.0 software was used to analyze the data, which were shown as mean ± SD. Specifically, the one-way ANOVA analysis was used for the comparisons among different groups. Individual experiment was repeated for at least 3 times, and *$P < 0.05$ was considered as statistical significance.

Results

Involvement of NLRP3-mediated cell pyroptosis in I/R rat models and H/R cellular models

Initially, according to the previous publications (An *et al.*, 2019; He *et al.*, 2019; Ma *et al.*, 2019; Qu *et al.*, 2019; Ye *et al.*, 2019), we established the *in vivo* I/R rat models. In Fig. 1A, the results showed that the white unstained infarcted area was lager in I/R rats but not their normal counterparts ($P < 0.01$). Next, the hippocampus tissues were collected from the brain of the rats for further analysis. The hippocampus tissues were stained by TUNEL, and the results in Fig. 1B showed that I/R treatment significantly increased the TUNEL-positive apoptotic cell ratio in rat tissues ($P < 0.01$), and the data in Suppl. Fig. S1A supported that I/R significantly increased neurological deficit score in rats, as determined by using the Longa scoring system. Consistently, the expression levels of cleaved Caspase-3 and Bax were increased ($P < 0.05$, Fig. 1C), while the Cyclin D1 and CDK2 were increased ($P < 0.05$, Fig. 1D) in rat tissues, suggesting that I/R suppressed hippocampus viability *in vivo*. Interestingly, as shown in Fig. 1E, we noticed that I/R also upregulated NLRP3 and ASC to induce cell pyroptosis in rat hippocampus tissues ($P < 0.05$). In addition, the H/R models in the PHN cells were inducted to simulate the real conditions of I/R injury *in vitro*. As expected, cell proliferation (Fig. 1F) and viability (Fig. 1G) were significantly decreased in the PHN cells treated with H/R, in contrast with the normal PHN cells ($P < 0.05$). Also, the levels of NLRP3, ASC, IL-1β and IL-18 in PHN cells ($P < 0.05$, Fig. 1H) and their supernatants ($P < 0.05$, Figs. 1I and 1J) were also increased by H/R.

Puerarin inactivated NLRP3-mediated pyroptotic cell death to attenuate I/R and H/R injury in vivo and in vitro

As previously described (Guan *et al.*, 2020; Wang *et al.*, 2020), Puerarin exerted its neuroprotective effects during I/R injury, which were validated by our following experiments in Figs. 2A–2I and Suppl. Fig. S1A and S1B. Specifically, the rats were divided into four groups, including Sham, I/R group, Puerarin (100 mg/kg) group, and I/R + Puerarin (100 mg/kg) group, and the results in Suppl. Figs. S1A and S1B showed that Puerarin decreased neurological deficit score and infarcted area volume in I/R rats. Further Western Blot analysis results showed that Puerarin downregulated cleaved Caspase-3 and Bax ($P < 0.05$, Fig. 2A), and upregulated Cyclin D1 and CDK2 ($P < 0.05$, Fig. 2B) in rat hippocampus tissues. Besides, NLRP3 and ASC expressions were also downregulated by Puerarin treatment ($P < 0.05$, Fig. 2C). In addition, our *in vivo* results were supported by the following *in vitro* experiments, and the results suggested that Puerarin rescued cell proliferation ($P < 0.05$, Fig. 2D) and viability ($P < 0.05$, Fig. 2E) in PHN cells treated with H/R. Also, we evidenced that Puerarin attenuated cell apoptosis in H/R-treated PHN cells ($P < 0.05$, Fig. 2F). Finally, the aggravating effects of H/R on NLRP3, ASC, IL-1β and IL-18 in the PHN cells (Fig. 2G) and their supernatants (Figs. 2H and 2I) were abrogated by
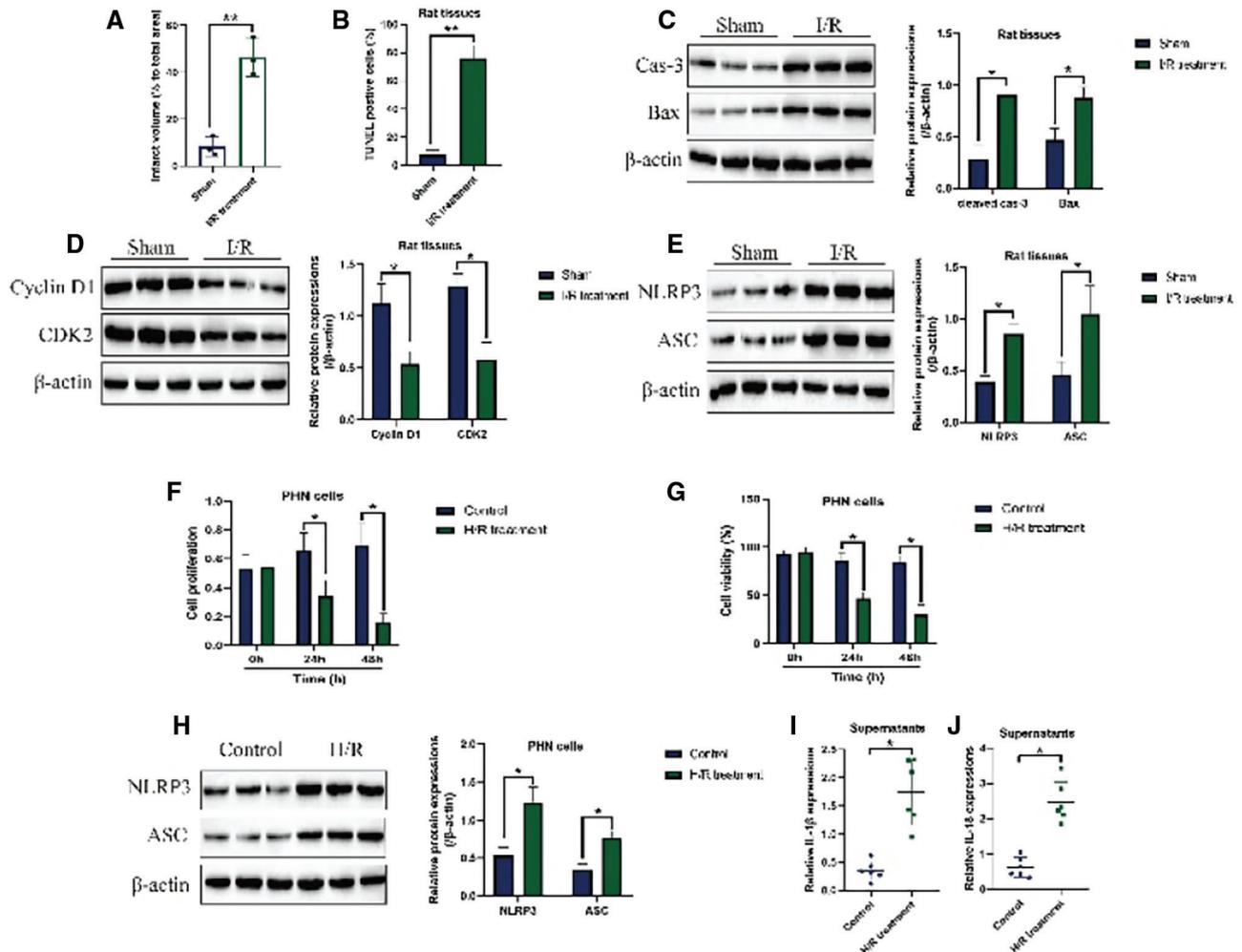
FIGURE 1. NLRP3-mediated cell pyroptosis was closely associated with *in vivo* I/R injury in rat hippocampus tissues and *in vitro* H/R injury in PHN cells. (A) The rat brain was obtained, and the white unstained infarcted area was observed and recorded. (B) Cell apoptosis in rat tissues was determined by TUNEL staining assay. Western Blot was used to examine (C) apoptosis, (D) cell cycle, (E) pyroptosis associated biomarkers. The cellular functions, including (F) cell proliferation and (G) viability were respectively examined. The expression status of pyroptosis markers were examined in the (H) cells and (I–J) supernatants. Each experiment was repeated three times; *$P < 0.05$, **$P < 0.01$.

co-treating cells with Puerarin ($P < 0.05$), indicating that Puerarin attenuated I/R- and H/R-induced hippocampus injury *in vivo* and *in vitro*.

Puerarin negatively regulated NLRP3 through upregulating miR-223-3p
NLRP3 could be negatively modulated by its upstream transcriptional regulators, including miRNAs (Hu et al., 2019; Yu et al., 2018), which were closely associated with I/R injury and could be modulated by Puerarin. Therefore, we speculated that Puerarin might regulate NLRP3-mediated cell pyroptosis through modulating miRNAs. To validate this hypothesis, ten miRNAs that potentially regulated NLRP3 was selected, and we found that only miR-223-3p, but not other types of miRNAs, could be downregulated by H/R, but it was upregulated by Puerarin in PHN cells ($P < 0.05$, Fig. 3A). Consistently, the results in Fig. 3B showed that I/R treatment also decreased miR-223-3p levels in rat hippocampus tissues, which could be significantly increased by Puerarin ($P < 0.05$, Fig. 3B). Next, the targeting sites of miR-223-3p with 3' untranslated regions of NLRP3 mRNA were predicted, and the targeting sites in NLRP3 mRNA were

subsequently mutated (Fig. 3C). As shown in Fig. 3D, the dual-luciferase reporter gene system assay validated that miR-223-3p bound to the 3'UTR of NLRP3 mRNA. Subsequently, miR-223-3p was downregulated and upregulated in the H/R PHN cells (Fig. 3E), and the following Real-Time qPCR and Western Blot analysis results evidenced that miR-223-3p negatively regulated NLRP3 expressions in H/R treated PHN cells at both transcriptional ($P < 0.05$, Fig. 3F) and translational (Fig. 3G) levels. In addition, the inhibiting effects of Puerarin on H/R-induced cell pyroptosis in PHN cells were partly reversed by silencing miR-223-3p ($P < 0.05$, Figs. 3H and 3I).

The regulating mechanisms of LncRNA DUXAP8-miR-223-3p-NLRP3 axis in vitro
Given that there existed LncRNAs-miRNAs-mRNAs ceRNA networks, and LncRNAs had also been reported to participate in the regulation of I/R injury (Wan et al., 2020; Zeng et al., 2019; Zhang et al., 2019b; Zhong et al., 2019), we next screened out that the miR-223-3p/NLRP3 pathway could be modulated by LncRNA DUXAP8 in PHN cells in Figs. 4A–4I. Specifically, seven LncRNAs containing the
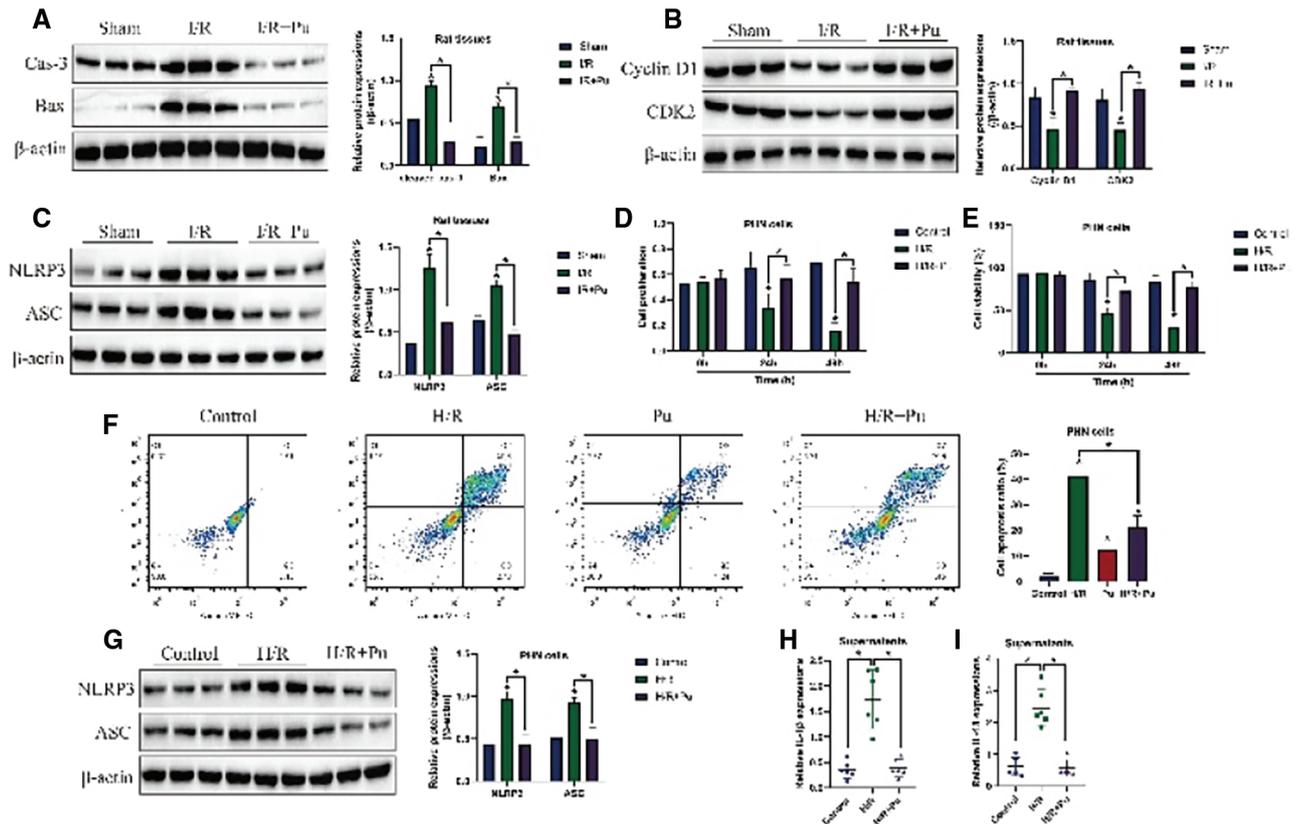
FIGURE 2. Puerarin restrained I/R and H/R injury for potential protection. (A) Cell apoptosis, (B) cell cycle and (C) pyroptosis associated proteins were examined. The cellular biological functions, such as (D) cell proliferation and (E) viability were determined. (F) Cell apoptosis ratio was measured by flow cytometer. (G–I) The pyroptosis associated biomarkers were measured ("Pu" indicated Puerarin treatment). Each experiment was repeated three times; *P < 0.05.
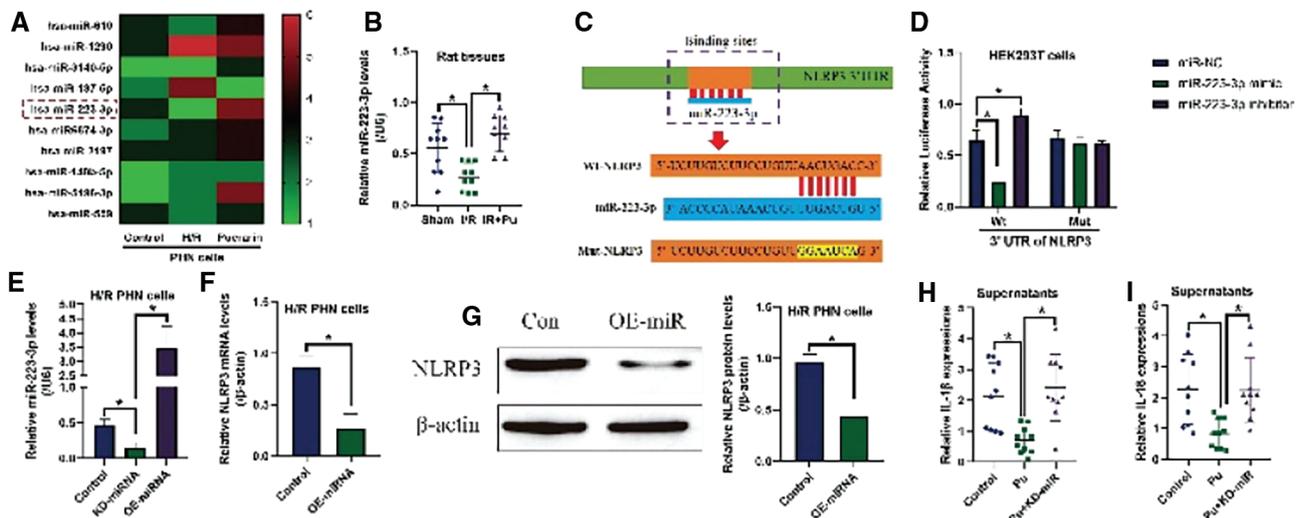
FIGURE 3. The regulatory mechanisms of miR-223-3p and NLRP3. The aberrant expressions of miRNAs were screened by Real-Time qPCR in the (A) PHN cells and (B) rat hippocampus tissues. The targeting sites of miR-223-3p and NLRP3 mRNA were (C) predicted and (D) validated. (E–G) NLRP3 could be negatively regulated by miR-223-3p. (H–I) The pro-pyroptosis cytokines in the supernatants were measured by ELISA ("OE-miR" represented miR-223-3p overexpression). Each experiment was repeated three times; *P < 0.05.

binding sites with miR-223-3p were selected, and further Real-Time qPCR results screened out that LncRNA DUXAP8 was upregulated by H/R treatment in PHN cells, which could be significantly downregulated by Puerarin (P < 0.05, Fig. 4A). Consistently, we evidenced that LncRNA DUXAP8 was also upregulated by I/R and downregulated by Puerarin in rat hippocampus tissues (P < 0.05, Fig. 4B). Hence, the LncRNA DUXAP8 was chosen for further analysis. The targeting sites of miR-223-3p with LncRNA DUXAP8 (Fig. 4C) were predicted, which were validated by the following experiments (Fig. 4D). Next, the overexpression and silencing vectors for LncRNA DUXAP8 were delivered
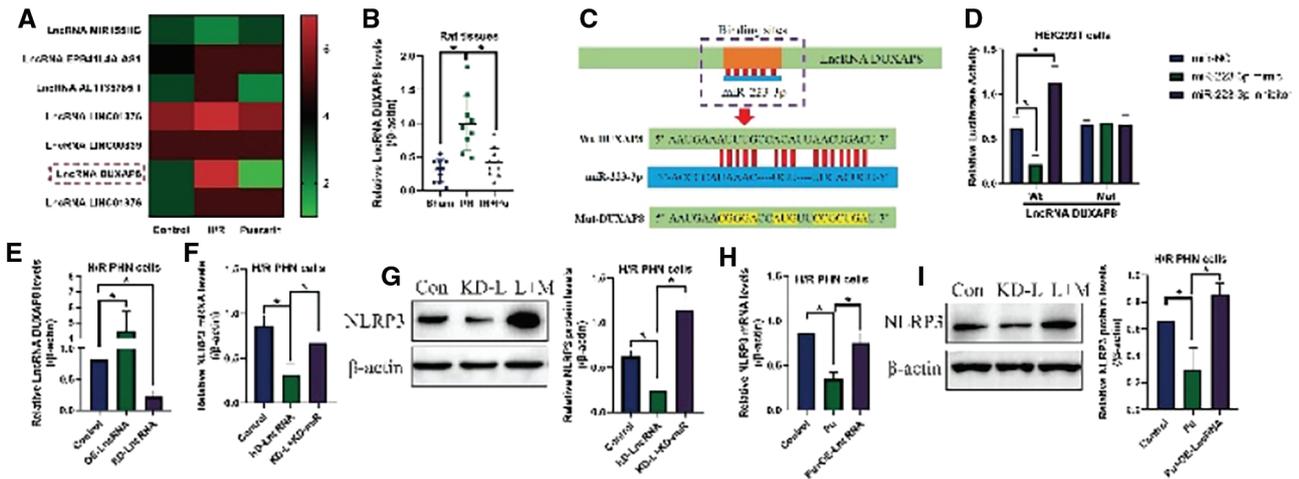
FIGURE 4. LncRNA DUXAP8 positively regulated NLRP3 in the PHN cells by sponging miR-223-3p. (A) Multiple LncRNAs were screened, and (B) LncRNA DUXAP8 was significantly influenced by I/R treatment. (C) Prediction and (D) validation of miR-223-3p-LncRNA DUXAP8 binding sites. (E) LncRNA DUXAP8 was manipulated, and (F, G) the regulating effects of LncRNA DUXAP8 on NLRP3 expressions were examined. (H–I) Puerarin suppressed NLRP3 in PHN cells by regulating the LncRNA DUXAP8/miR-223-3p axis ("Con" indicated Control, "KD-L" suggested knockdown of LncRNA DUXAP8, and "L+M" represented knockdown of LncRNA DUXAP8 plus miR-223-3p ablation). Each experiment was repeated three times; $^*P < 0.05$.

into the H/R PHN cells (Fig. 4E), by performing Real-Time qPCR and Western Blot, we found that knock-down of LncRNA DUXAP8 decreased the mRNA and protein levels of NLRP3 in H/R-treated PHN cells, which were reversed by

silencing miR-223-3p ($P < 0.05$, Figs. 4F and 4G). In addition, we found that Puerarin decreased the expression levels of NLRP3 in H/R-treated PHN cells, which were increased by upregulating LncRNA DUXAP8 ($P < 0.05$, Figs. 4H and 4I).
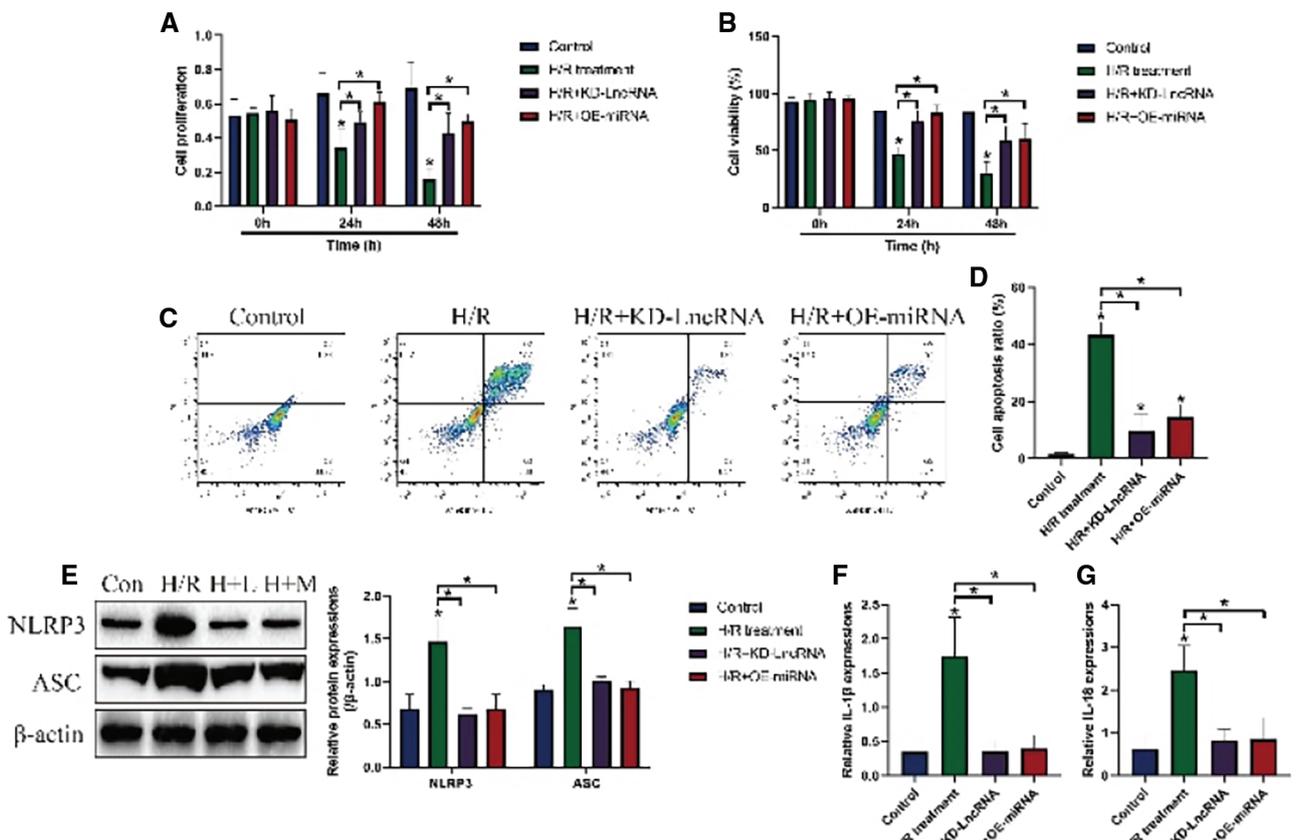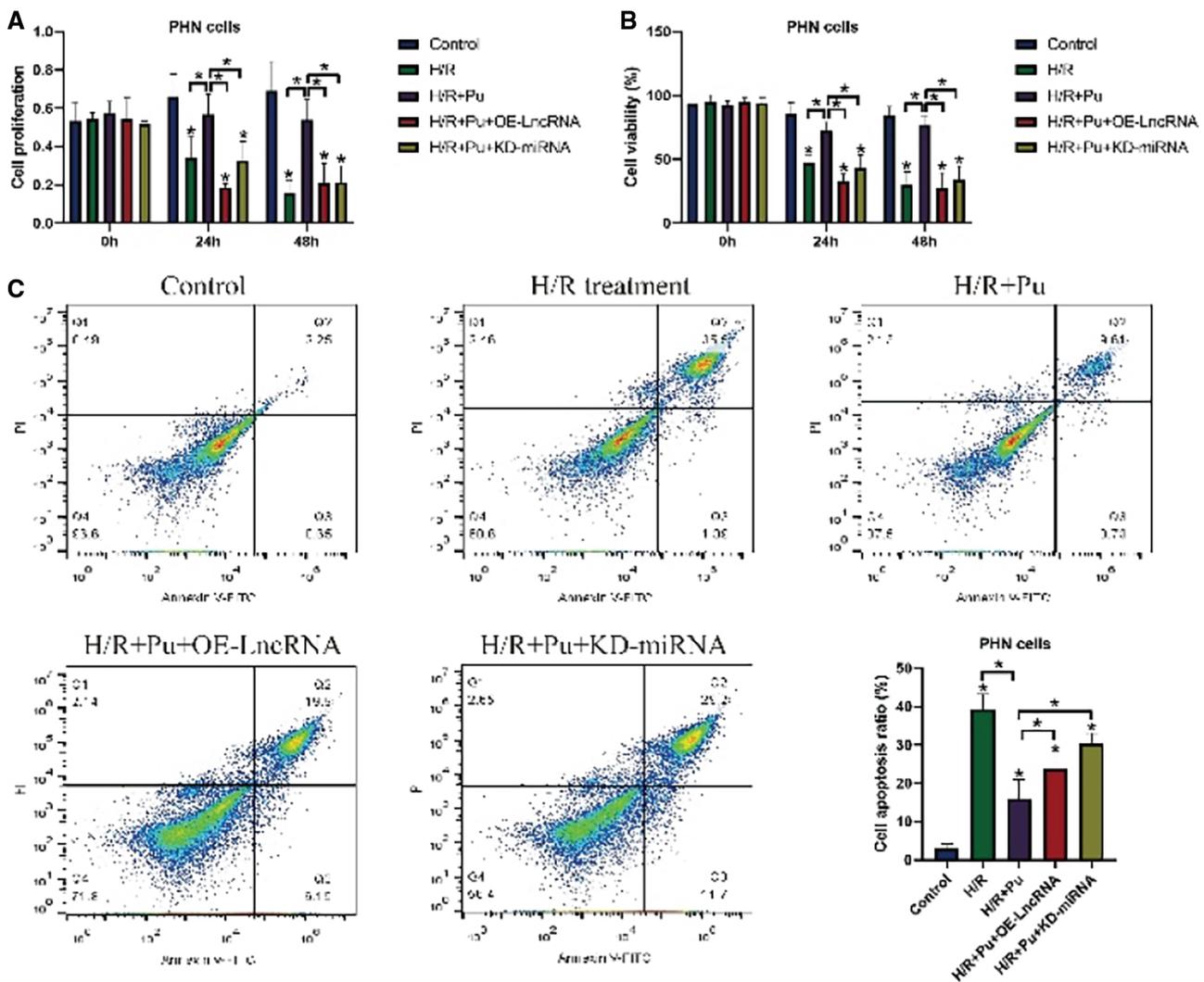

FIGURE 5. Targeting the LncRNA DUXAP8/miR-223-3p axis rescued cell functions in PHN cells. (A) Cell proliferation and (B) viability were respectively determined. (C–D) Flow cytometer was used to detect the Annexin V-FITC and PI-positive apoptotic cells. The pyroptosis indicators were examined in both (E) PHN cells and (F–G) their supernatants ("Con" represented Control, "H/R" indicated H/R treatment, "H+L" indicated H/R treatment plus LncRNA DUXAP8 knockdown, and "H+M" represented H/R treatment plus miR-223-3p overexpression). Each experiment was repeated three times; $^*P < 0.05$.

Targeting the LncRNA DUXAP8/miR-223-3p axis rescued cell viability in H/R PHN cells

The above data encouraged us to assure whether the LncRNA DUXAP8/miR-223-3p axis involved in regulating the development of I/R-induced injury *in vitro*. To achieve this, LncRNA DUXAP8 was silenced ($P < 0.05$, Fig. 4E), while miR-223-3p was overexpressed ($P < 0.05$, Fig. 3E) in H/R PHN cells, and the results showed that both LncRNA DUXAP8 ablation and miR-223-3p overexpression restored cell proliferation (Fig. 5A) and viability (Fig. 5B) in H/R-treated PHN cells ($P < 0.05$). In addition, the cell apoptosis examination assay results indicated that H/R treatment significantly promoted cell apoptosis in PHN cells, which were reversed by ablating LncRNA DUXAP8 and overexpressing miR-223-3p ($P < 0.05$, Figs. 5C and 5D). Further results validated that silencing of LncRNA DUXAP8 and overexpression of miR-223-3p also downregulated NLRP3, ASC, IL-1β and IL-18 to inactivate NLRP3-mediated cell pyroptosis in H/R PHN cells ($P < 0.05$, Fig. 5E) and the supernatants ($P < 0.05$, Figs. 5F and 5G).

Puerarin promoted cell survival in H/R PHN cells via the LncRNA DUXAP8/miR-223-3p axis

Finally, we validated that Puerarin regulated the LncRNA DUXAP8/miR-223-3p axis to exert its neuroprotective effects on H/R PHN cells. Specifically, the H/R PHN cells were pre-transfected with LncRNA DUXAP8 overexpression vectors ($P < 0.05$, Fig. 4E) and miR-223-3p ablation vectors ($P < 0.05$, Fig. 3E), and were divided into four groups, including Control, Puerarin group, Puerarin + OE- LncRNA DUXAP8 group, and Puerarin + KD-miR-223-3p group. The results showed that Puerarin rescued cell proliferation in H/R PHN cells, which were abrogated by overexpressing LncRNA DUXAP8 and downregulating miR-223-3p ($P < 0.05$, Fig. 6A). Consistently, further data validated that both LncRNA DUXAP8 overexpression and miR-223-3p ablation abrogated the promoting effects of Puerarin on cell viability in H/R PHN cells ($P < 0.05$, Fig. 6B). Furthermore, we evidenced that Puerarin also suppress cell apoptosis in H/R PHN cells by targeting the LncRNA DUXAP8/miR-223-3p axis ($P < 0.05$, Fig. 6C). The above results suggested that

FIGURE 6. Puerarin regulated the LncRNA DUXAP8/miR-223-3p axis to attenuate H/R injury in the PHN cells *in vitro*. PHN cell (A) proliferation and (B) viability were evaluated. (C) Examination of cell apoptosis by flow cytometer ("Pu" indicated Puerarin, OE-LncRNA represented LncRNA DUXAP8 overexpression, and "KD-miRNA" suggested miR-223-3p ablation). Each experiment was repeated three times; *$P < 0.05$.

Puerarin regulated the LncRNA DUXAP8/miR-223-3p axis to attenuate cell death in H/R PHN cells *in vitro*.

Discussion

Ischemic stroke seriously degrades the life quality of human beings worldwide (Benjamin *et al.*, 2018), and timely pharmacological and mechanical reperfusion are proved as effective strategies for its treatment (Zhang *et al.*, 2016; Zhao *et al.*, 2017). However, recent data indicated that cerebral ischemia/reperfusion (I/R) injury caused serious side effects, such as hemorrhage (Kimiwada *et al.*, 2019; Zhang *et al.*, 2017a), brain edema (King *et al.*, 2018; Nakano *et al.*, 2018), etc., were still a great obstacle for this treatment therapy in clinic, and uncovering the molecular mechanisms of cerebral I/R injury pathogenesis and development of novel therapeutic drugs to attenuate I/R injury might help to solve these problems. This study found that Puerarin regulated a novel LncRNA DUXAP8/miR-223-3p/NLRP3 inflammasome-mediated cell pyroptosis to alleviate cerebral I/R injury. Also, previous publications indicated that NLRP3-meidated cell pyrotosis contributed to the development cerebral I/R injury (An *et al.*, 2019; He *et al.*, 2019; Ma *et al.*, 2019; Qu *et al.*, 2019; Ye *et al.*, 2019), which were validated by our present work. Specifically, the cerebral I/R rat models and H/R PHN cell models were established, and we found that the expression levels of pyroptosis associated signatures were upregulated in both I/R rats and H/R PHN cells, in contrast with their normal counterparts, suggesting that cell pyroptosis occurred and contributed to cerebral I/R injury.

Puerarin a well-known Chinese medicine extracted from the Kudzu (*Pueraria montana* var. lobata) root and has been used as therapeutic drug for multiple cardio- and cerebrovascular diseases in China (Zhang *et al.*, 2019d; Zhang *et al.*, 2019e). Of note, recent publications evidenced that Puerarin also exerted its neuroprotective effects to alleviate cerebral I/R injury in rat models (Ling *et al.*, 2018; Liu *et al.*, 2013; Wang *et al.*, 2018; Zhou *et al.*, 2014), and our experiments supported that Puerarin treatment increased cell survival and suppressed cell apoptosis in both I/R rat hippocampus tissues and H/R PHN cells, implying that Puerarin could be utilized as a therapeutic agent for cerebral I/R injury. Interestingly, previous data suggested that Puerarin inactivated NLRP3-meidated cell pyroptosis to regulate I/R injury (Guan *et al.*, 2020; Wang *et al.*, 2020), and our data showed that Puerarin restrained and inactivated cell pyroptosis in I/R rat models and H/R PHN cell models.

Targeting the LncRNAs-miRNAs-mRNAs ceRNA networks is effective to treat cerebral I/R injury (Wan *et al.*, 2020; Zeng *et al.*, 2019; Zhang *et al.*, 2019b; Zhong *et al.*, 2019) and NLRP3-mediated pyroptotic cell death (Hu *et al.*, 2019; Yu *et al.*, 2018), and we identified a novel LncRNA DUXAP8/miR-223-3p axis that regulated NLRP3-mediated cell pyroptosis to regulate H/R-induced PHN cell injury *in vitro*. Mechanistically, miR-223-3p targeted both LncRNA DUXAP8 and NLRP3 mRNA, and LncRNA DUXAP8 positively regulated NLRP3 expressions by acting as RNA sponge for miR-223-3p, which were partly supported by the previous studies (Dong *et al.*, 2019; Wan *et al.*, 2018; Zhang *et al.*, 2019c) and validated

that LncRNA DUXAP8-miR-223-3p-NLRP3 ceRNA networks existed in the H/R PHN cell models. In addition, previous data suggested that miR-223-3p attenuated cerebral I/R injury (Qin *et al.*, 2016; Zhao *et al.*, 2020), and we evidenced that both LncRNA DUXAP8 ablation and miR-223-3p overexpression promoted cell survival in H/R PHN cells. Furthermore, multiple miRNAs could be regulated by Puerarin (Zhao *et al.*, 2020), and we first identified that Puerarin downregulated LncRNA DUXAP8 and upregulated miR-223-3p in PHN cells. Also, our data showed that the protective effects of Puerarin on H/R PHN cells were abrogated by upregulating LncRNA DUXAP8 and silencing miR-223-3p, implying that Puerarin regulated the LncRNA DUXAP8/miR-223-3p axis to attenuate H/R-induced cell death in PHN cells.

Conclusions

Taken together, the present study evidenced that Puerarin regulated the LncRNA DUXAP8/miR-223-3p axis to inactivate NLRP3 inflammasome-mediated pyroptotic cell death, resulting in the alleviation of cerebral I/R injury.

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Supplementary Figure
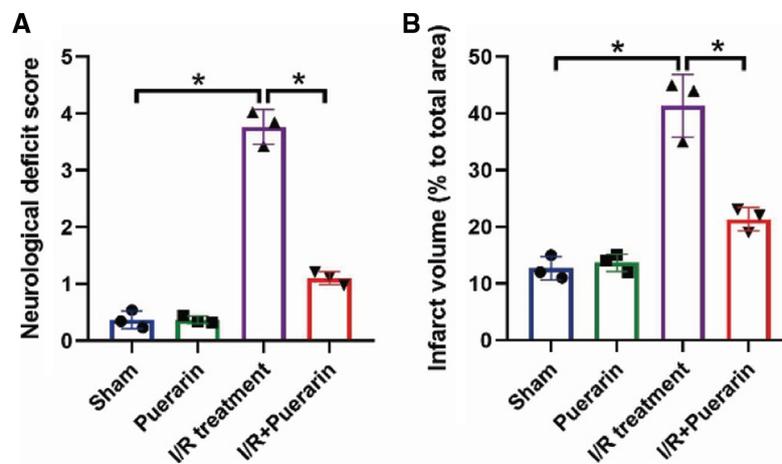
SUPPLEMENTARY FIGURE S1. The effects of Puerarin treatment on (A) the neurological deficit scores and (B) the volume of infarcted area in rat brain respectively by using the Longa scoring system and TTC staining assay. Each experiment was repeated three times; *$P < 0.05$.