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Original Article

miR-148a regulates inflammation in microglia induced by oxygen-glucose deprivation via MAPK signal pathways

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Abstract: Inflammation occupies an important place in the progression of acute ischemic strokes. Microglia, a kind of resident immune cell in the central nervous system (CNS), plays a vital role in neuroinflammatory processes. MicroRNAs have been shown to take part in inflammatory response. miR-148a has been reported to be related to acute ischemic strokes. However, the specific connection between miR-148a and cerebral ischemia remains unclear. The current study aimed to figure out the roles of miR-148a in the inflammatory activation of microglia. It was found that miR-148a was downregulated in activated microglia cells induced by Oxygen-Glucose Deprivation/Reoxygenation (OGD/R). Subsequently, microglial cells were transfected with miR-148a mimics or inhibitors to alter expression of miR-148a. In addition, cell viability was measured using an MTT assay. Microglial cell cycle distribution and apoptosis were evaluated using flow cytometry. Results showed that upregulation of miR-148a in primary microglial cells suppressed the secretion of inflammatory mediators, such as TNF-α, IL-1β, and IL-10. In contrast, miR-148a downregulation promoted expression of the inflammatory mediators, indicating that miR-148a might play a justifiable role in regulating neuroinflammation. In addition, it was discovered that overexpression of miR-148a in microglia observably inhibited the activation of p38MAPK, ERK, and JNK. Furthermore, miR-148a overexpression effectively decreased mRNA and protein expression of the above proteins and aggravated OGD/R-induced cell apoptosis. In contrast, miR-148a downregulation was neuroprotective. The current study demonstrates that miR-148a could prevent OGD/R injury by inhibiting microglial activation, thus attenuating neuroinflammation via MAPK pathways and suppressing apoptotic genes.

Keywords: miR-148a, activated microglia, OGD/R, cerebral ischemia, MAPK pathways

Introduction

Ischemic strokes are a major public health problem, characterized by a high morbidity, mortality, and disability [1]. Due to sudden onset and rapid progression, there are few effective therapies for acute cerebral ischemic injuries. Development of novel treatments for acute ischemic strokes is urgently needed. MicroRNAs (miRNAs) are ribonucleic acids with short (17–25 nucleotides long) non-protein coding [2]. They are important regulators of gene expression and play important roles in the initiation and progression of several diseases [3]. It has been estimated through bioinformatics approaches that 30% to 80% of protein-coding genes may be under the regulation of miRNAs [4], with each miRNA targeting up to several hundred genes [5, 6]. Related reports have shown that miRNAs, such as miRNA-126, -146a, -27b, -17-5p, and -424, play significant roles in inflammatory response and have a profound impact on neuroprotection after cerebral ischemia [7]. Studies have demonstrated an association between miR-148a and ischemic strokes, with decreased miR-148a levels in the blood of patients suffering from acute ischemic strokes, compared to normal people [8]. However, the function of miR-148a in acute cerebral ischemia has not been elucidated.

Studies have revealed that post-stroke neuroinflammation is a key determinant of acute outcomes and long-term prognoses [9, 10]. After

occurrence of ischemic strokes, the ensuing neuronal cell death triggers a cascade of inflammatory responses contributing to secondary brain damage [11]. Microglia, the major source of cytokines and other immune molecules, will be activated and play a vital role in regulating inflammatory reactions by releasing diverse inflammatory cytokines, such as interleukin-1 beta (IL-1β), necrosis factor-α (TNF-α), and interleukin-10 (IL-10) [12, 13]. Suppression of microglial activation following strokes can prevent brain injury, representing an attractive therapeutic strategy for stroke patients [14].

Mitogen-activated protein kinase (MAPK) signaling pathways are involved in directing cellular response to oxidative stresses and cellular apoptosis, as well as inflammation and cytokine stimulation [15, 16]. Previous studies have reported that overexpression of miR-148a significantly inhibits cutaneous squamous cell proliferation and metastasis via downregulation of MAPK pathways [17].

The current study aimed to figure out whether miR-148a is involved in microglia-mediated inflammatory responses under OGD/R injury. This study constructed a microglial cell-based oxygen-glucose deprivation and reperfusion (OGD/R) model to mimic cerebral ischemic injuries. This study then transfected primary microglial cells with miR-148a mimics or inhibitors to investigate the roles of miR-148a in microglia activation during OGD/R injury, further exploring potential downstream mechanisms involved in the process.

Materials and methods

Primary microglial cell culture

Primary microglial cells were derived from newborn (less than 24 hours old) C57BL/6 mice (Laboratory Animal Center, Shandong University, Shandong, China) brains. Cerebral cortices were isolated mechanically. Trypsinase was digested and plated into T-75 flasks in DMEM/F12 supplemented with L-glutamine (Invitrogen, Carlsbad, CA), 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA), 100 μg/mL penicillin, and 100 μg/mL streptomycin. After 24 hours, media and the tissues were removed. Fresh media was replaced. On day 14, microglia were isolated from mixed glial cell cultures by shaking the flasks at 300 rpm on a rotary shaker overnight at 37°C. The supernatant was collected. Cells were cultured for 2 days before treatment.

OGD/R model

Primary microglial cells were exposed to OGD/R injury. Briefly, the normal culture medium was removed. The cells were washed three times with PBS. The culture medium was replaced with serum/glucose-free DMEM (Invitrogen-Gibco, Carlsbad, California, USA). Cells were maintained in a hypoxic environment (95% N_2 and 5% CO_2) for 1 hour at 37°C and reperfusion took place for 24 hours under normal culture conditions. The normal control cells received no treatment.

Cell transfection

Primary microglia cells were seeded in 6-well plates at a density of 5×10^5 cells per well and allowed to settle overnight before transfection. To overexpress or suppress miR-148a expression, the cells were transfected with miR-148a mimics, miR-148a inhibitors, or the control vector (RiboBio, Guangzhou, Guangdong, China), using the Ribo FECT™ CP Transfection Kit (RiboBio, Guangzhou, Guangdong, China), according to manufacturer instructions. The cells were then continuously cultured for 48 hours.

Cell viability assay

Cell viability was assessed using MTT assay. Microglia cells were seeded in 96-well plates in DMEM containing 10% FBS, 100 units/mL of penicillin, and 100 mg/mL streptomycin at 37°C in an atmosphere containing 5% CO_2. After 24 hours, unless otherwise specified, the cells were washed with PBS and placed in a culture medium with 200 mM capsaicin for 24 hours. Next, 20 mL of MTT solution (5 mg MTT/mL in PBS) was added to each well and incubated for 4 hours. Formazan crystals were subsequently dissolved in 200 mL of dimethyl sulfoxide and the absorbance was read at 540 nm. The inhibition rate of cell growth was calculated using the following formula: (control group - treated group)/control group × 100.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted in each cell group with Trizol Reagent (Invitrogen, USA), according to manufacturer instructions. Complementary

DNA (cDNA) was synthesized from total RNA using the mRNA Reversed Transcription Kit (Takara 036A, Japan). The cDNA was amplified as a template through the ABI 7900 Sequence Detection System (Applied Biosystem, USA). miR-148a forward: 5'-ACACTCCAGCTGGGTCAGTGCACTACAGAA-3'; reverse: 5'-TGGTGTCGTGGAGTCG-3'. MiR-148a mimic forward: 5'-AAAGUUCUGAGACACUCCGACU-3'; reverse: 5'-UCGGAGUGUCUCAGAACUUUUU-3'. Inhibitors: 5'-AGUCGGAGUGUCUCAGAACUUU-3'. Negative control: 5'-ACG UGA CAC GUU CGG AGA ATT-3'. GAPDH forward: 5'-CATGGCCTTCCGTGTTCCTA-3'; reverse: 5'-CCTGCTTCACCACCTTCTTGAT-3'. Universal temperature cycles of amplification were as follows: 30 seconds at 95°C, 60 seconds at 65°C, and 60 seconds at 72°C, for 40 cycles. The extension was 5 minutes at 72°C. Quantification was performed by the comparative Ct method, using GAPDH as internal control.

ELISA assessment of TNF-α, IL-10, and IL-1β

Quantification of TNF-α, IL-10, and IL-1β was detected using ELISA kits. The cell supernatant was collected for detection analyses. Moreover, 100 μl of diluted standard preparation was added to a 96-well ELISA plate. An equal amount of cell supernatant was also added to the 96-well ELISA plates, followed by incubation for 2 hours at room temperature. Next, the wells were rinsed and replaced with detecting antibody and incubated for 1 hour at room temperature. Subsequently, the plate was rinsed again and added with Avidin-HRP to each well and incubated for another 30 minutes at room temperature. After rinsing several times, 100 μl of TMB hydrogen peroxide urea solution was added into each well and incubated for 15 minutes at room temperature. Finally, the reaction was stopped and the plate was read at 450 nm.

Western blot analysis

Total proteins were extracted via RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA) with protease inhibitors. Protein samples were separated by 10% SDS-PAGE gel electrophoresis and then transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk in 10 mM Tris-HCl containing 150 mM NaCl and 0.1% Tween 20 (TBST) and incubated with primary antibodies overnight at 4°C. Primary antibodies included p38 MAPK (2387, 1:1000), P-p38 MAPK (9211, 1:1000), ERK (4696, 1:1000), P-ERK (4270, 1:1000), JNK (9252, 1:1000), and P-JNK (9251, 1:1000), all from Cell Signaling Technology. After incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), membranes were visualized using the ECL chemiluminescent kit (Amersham Biosciences, Piscataway, NJ, USA).

Flow cytometry analysis

Microglial cells (1×10^5 cells/mL) were seeded in 6-well plates and incubated with the cell cycle and apoptotic staining kit [KeyGEN BioTECH; AnnexinV-phycoerythrin (PE)/7-aminoactinomycin D (7-AAD)] at room temperature for 15 minutes, prior to analysis using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Apoptotic cells were obtained and analyzed using Cell Quest Software (version 3.1; BD Biosciences), according to manufacturer protocol. The percentage of cells in the G1, S, and G2 phases was determined using ModiFit software. Results are representative of three independent experiments.

Statistical analysis

Data are presented as mean ± SEM. SPSS 11.7 software was used to carry out statistical calculations. Student's t-test was conducted to compare the two groups. Statistical differences among multiple groups were assessed by one-way analysis of variance followed by Dunnett's post-hoc test. *P*-values < 0.05 indicate statistical significance.

Results

miR-148a downregulated in activated primary microglial cells after OGD/R

Previous studies have shown that miR-148a levels were significantly decreased in the blood of patients with acute ischemic strokes. Inflammation is an important component in the ischemic pathophysiology and microglia are the principal immune cells in the brain. Since a range of miRNAs have been reported to play significant roles in the inflammatory response, it was speculated whether miR-148a had a connection with the activation of microglia. This study exposed primary microglial cells to 1 hour of oxygen-glucose deprivation and 12 hours of

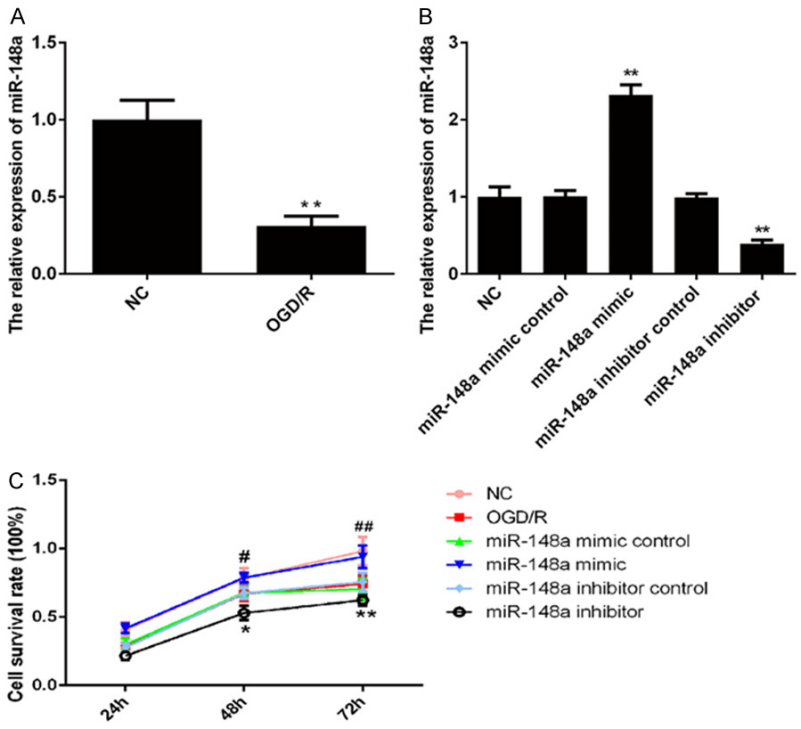

Figure 1. Expression of miR-148a in primary microglial cells under different conditions. A. The level of miR-148a in microglia was suppressed after the cells were exposed to 1 hour of oxygen-glucose deprivation and 12 hours of reperfusion (OGD/R). B. RT-PCR analysis was used to evaluate the efficiency of cell transfection with the miR-148a mimics, inhibitors, or respective controls after 48 hours. miR-148a expression was observably increased after the microglial cells were transfected with the miR-148a mimics, whereas it was decreased following transfection with the miR-148a inhibitors. C. The proliferation rate of microglia cells in the OGD/R group was inhibited, compared with that in the NC group. When cells were treated with miR-148a mimics or inhibitors, miR-148a mimics significantly promoted microglia cell viability and miR-148a inhibitors suppressed cell viability. Values are expressed as the mean ± standard deviation of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. normal control (NC) group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. OGD/R group.

reperfusion to mimic ischemia-reperfusion injury, thus activating microglial cells. Afterward, mRNA levels of miR-148a were detected. As **Figure 1A** shows, expression of miR-148a was significantly decreased after OGD/R, compared with that in normal control microglial cells. Therefore, results suggest that miR-148a might take part in the activation of microglia.

Altered miR-148a expression affected microglia cell viability

MTT results demonstrated that microglia cell viability in the OGD/R group decreased significantly, compared to the NC group. In addition, optical absorption in the miR-148a inhibitor group was significantly decreased, compared with NC and miR-148a inhibitor control groups. In contrast, transfection with miR-148a mimic significantly increased cell viability, compared with NC and miR-148a mimics control groups (**Figure 1C**).

Upregulation of miR-148a in microglia suppressed expression of inflammatory mediators in activated microglia

According to the above results, it was speculated that miR-148a might play a potential role in the activation of microglia. Therefore, this study cultured primary microglial cells to investigate the effects of miR-148a on microglia-mediated neuroinflammation. This study transfected miR-148a mimics or inhibitors into primary microglial cells for 48 hours before subjecting to OGD/R injury. As shown in **Figure 1B**, compared with the normal control group, miR-148a expression was significantly increased after transfection with miR-148a mimics and decreased after transfection with miR-148a inhibitors.

Subsequently, expression of TNF-α, IL-1β, and IL-10 was detected in primary microglial cells after transfection with miR-148a mimics or inhibitors. As shown in **Figure 2**, the microglia were activated after OGD characterized by secretion of TNF-α, IL-1β, and IL-10, as detected by ELISA. In the microglia transfected with miR-148a mimics, expression of TNF-α, IL-1β, and IL-10 were significantly decreased, compared with that in microglial cells exposed to OGD/R injury alone. In contrast, microglia treated with miR-148a inhibitors effectively increased levels of TNF-α, IL-1β, and IL-10. Treating the microglial cells with miR-148a mimic negative controls and miR-148a inhibitor negative controls showed no differences in expression of TNF-α, IL-1β, and IL-10, compared with normal microg-

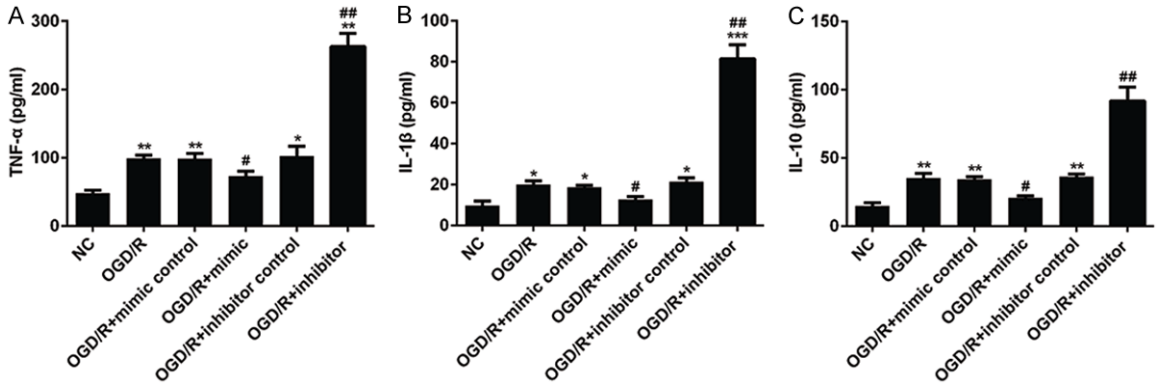

Figure 2. miR-148a regulated the secretion of TNF-α, IL-1β, and IL-10 in activated microglia induced by OGD/R. Quantitative analysis of TNF-α (A), IL-1β (B), and IL-10 (C) expression was measured by ELISA. Expression of TNF-α, IL-1β, and IL-10 in primary microglial cells subjected to OGD/R injury were significantly decreased after transfection with the miR-148a mimics. In contrast, they were increased after transfection with the miR-148a inhibitors. Data are presented as the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. normal control (NC) group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. OGD/R group.

lial cells after OGD. Present results demonstrate that miR-148a participates in modulating the secretion of inflammatory mediators and upregulation of miR-148a in microglial cells suppresses the activation of microglia by decreasing the secretion of inflammatory mediators, such as TNF-α, IL-1β, and IL-10, after OGD/R injury.

miR-148a inhibited activation of p38MAPK, ERK, and JNK in activated microglia

To further investigate the anti-inflammatory molecular mechanisms of miR-148a on microglia, expression of p38 MAPK, ERK, and JNK pathways were detected by Western blot analysis. As shown in **Figure 3**, OGD/R injury induced an apparent upregulation of phosphorylated p38 MAPK, ERK, and JNK in primary microglial cells, compared with normal control cells. After transfection with miR-148a mimic, a significant decrease of phosphorylated p38 MAPK, ERK, and JNK was observed in microglia suffering from OGD/R, compared with the OGD/R group. In contrast, transfecting the microglia with miR-148a inhibitors significantly promoted the activation of MAPK pathways. There was no difference in the expression of MAPK pathways between normal microglia and microglia transfected with miR-148a mimic controls or miR-148a inhibitor controls after exposure to OGD/R. Present results indicate that miR-148a might suppress the activation of microglia through inhibiting p38MAPK, ERK, and JNK pathways.

Altered miR-148a expression regulated cell apoptosis and cycle induced by OGD/R in microglia

miR-148a expression was found to be decreased in microglia during OGD/R injury, suggesting that miR-148a downregulation may play an important role in cerebral ischemia injuries. The present study observed that OGD/R promoted apoptosis in microglia cells, compared with the levels of apoptosis observed in normal control (NC) cells (**Figure 4**). When the cells were treated with miR-148a mimics, the number of apoptotic cells induced by OGD/R injury was effectively increased, compared with the number of apoptotic cells observed among cells subjected to OGD/R injury alone and those treated with miR-148a mimic controls. In contrast, treating the cells with miR-148a inhibitors decreased the number of apoptotic cells. In addition, cells in the G1 stage were significantly increased in the OGD/R, compared with normal control cells. When cells were treated with miR-148a mimics and miR-148a inhibitors, the miR-148a mimics decreased the cells and miR-148a inhibitors increased cells in the G1 stage (**Figure 5**). These experiments suggest that suppression of miR-148a can reduce cell apoptosis and protect microglia cells from OGD/R injury.

Discussion

Strokes are the second most common cause of mortality and are a leading cause of long-term

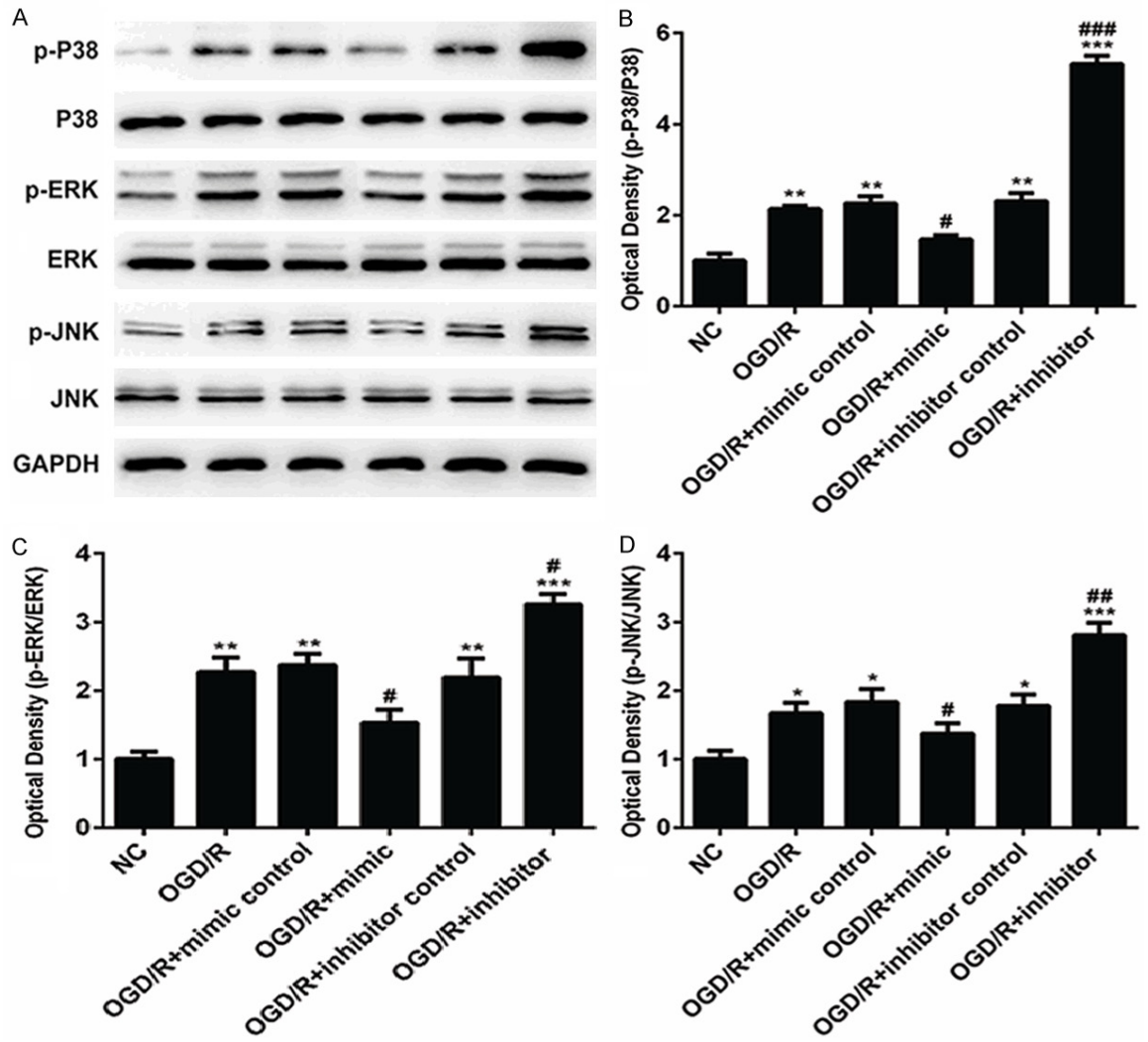

Figure 3. Upregulation of miR-148a in microglia suppressed the activation of p38MAPK, ERK, and JNK after OGD/R injury. (A) Protein expression of P-p38MAPK, p38MAPK, P-ERK, ERK, P-JNK, and JNK were detected by Western blotting. The ratio of P-p38MAPK/p38MAPK (B), P-ERK/ERK (C), and P-JNK/JNK (D) markedly decreased in microglial cells that were transfected with miR-148a mimics after OGD/R injury. Data are presented as the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. normal control (NC) group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. OGD/R group.

severe disability in adults. They bring a heavy social burden, worldwide [18]. Acute cerebral ischemia accounts for over 80 percent of all strokes, due to a brain arterial occlusion. Pathophysiological processes following ischemic strokes are complex, represented by a complex series of interlinked molecular and cellular mechanisms that contribute to ischemic cell death.

Recently studies have suggested that miRNAs have a profound impact on the progression of ischemic strokes and are potential targets of novel treatments. Inflammatory response plays an important role in the pathophysiologic progression of ischemic strokes. The relationship between miRNAs and inflammation in ischemic strokes has already been noticed. It has been reported that miR-424 is significantly decreased in circulating lymphocytes of patients with ischemic strokes [19]. miRNA-126 has been reported to inhibit VCAM-1 expression, thus reducing leukocyte adhesion, as well as suppressing inflammatory monocyte recruitment by downregulating chemokine (C-C motif) ligand 2 (Ccl2) [20, 21]. Moreover, miR-146a is considered to be an intrinsic regulator of NF-κB, through which it downregulates inflammatory response in the ischemic brain [22]. More and more miRNAs have been discovered to be involved in the

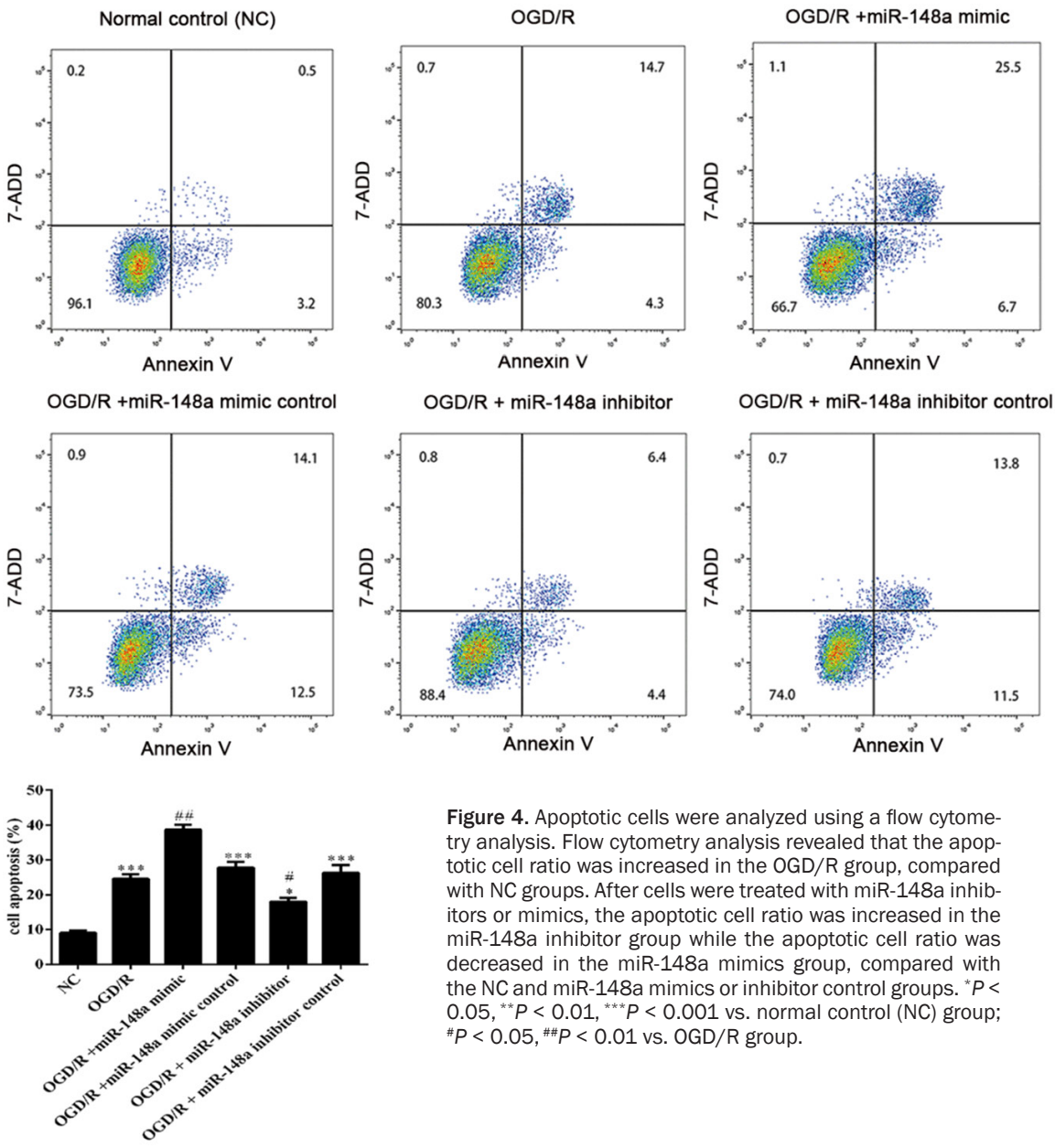

Figure 4. Apoptotic cells were analyzed using a flow cytometry analysis. Flow cytometry analysis revealed that the apoptotic cell ratio was increased in the OGD/R group, compared with NC groups. After cells were treated with miR-148a inhibitors or mimics, the apoptotic cell ratio was increased in the miR-148a inhibitor group while the apoptotic cell ratio was decreased in the miR-148a mimics group, compared with the NC and miR-148a mimics or inhibitor control groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. normal control (NC) group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. OGD/R group.

inflammation of ischemic strokes. It has been discovered that miR-148a was upregulated in the blood of patients with cerebral ischemia. The specific function and molecular mechanisms of miR-148a in the post-ischemic brains, however, are not well understood. The current study aimed to figure out whether miR-148a is involved in inflammation of the brain after focal cerebral ischemia.

Available evidence has shown that microglia are important immune cells in the brain. They can modify their morphology from a resting to an active state. Once ischemic strokes occur, microglia can respond rapidly. This microglial activation gives rise to the overproduction of pro-inflammatory cytokines, which exacerbate the progression of neuroinflammation, thus increasing neurological deficits [12].

In view of this, this study used primary microglia cells exposed to OGD/R to simulate the inflammation of brain after the cerebral ischemia-reperfusion injuries. This study detected expression of miR-148a in microglia cells after normal condition or OGD/R stimulation. It was shown that miR-148a expression was decreased after suffering from OGD/R injury.

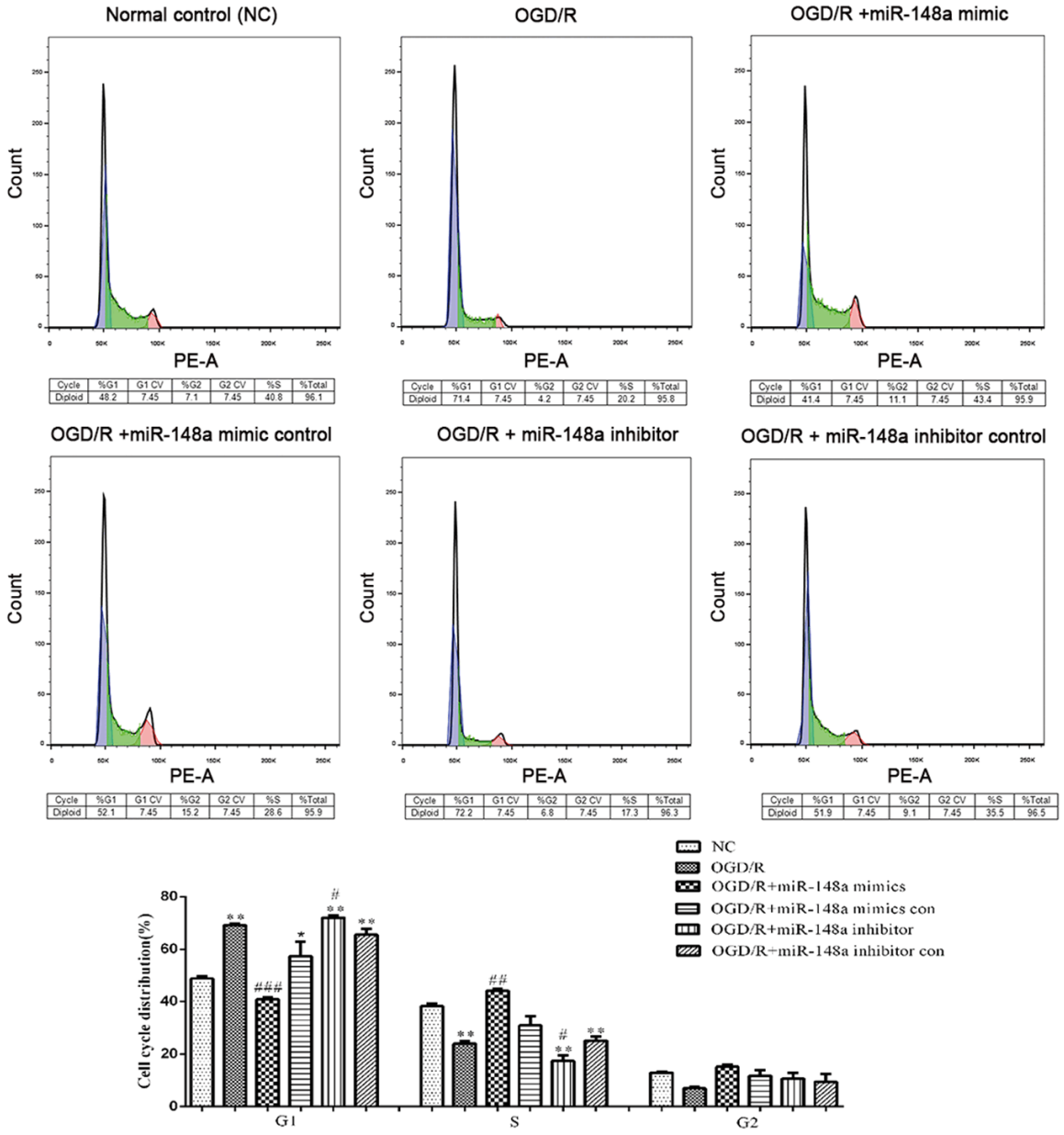

Figure 5. Cell cycle distribution was measured by flow cytometry analysis. Compared with the NC group, the OGD/R groups had a greater number of G1-phase cells and fewer S-phase cells. The miR-148a mimics group had a fewer number of G1-phase cells and higher S-phase cells. In contrast, the miR-148a inhibitor group had a greater number of G1-phase cells and fewer S-phase cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. normal control (NC) group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. OGD/R group.

Present results suggest that miR-148a might take part in the activation of microglia. This study then further transfected the primary microglia with miR-148a mimics or inhibitors, aiming to evaluate the roles of miR-148a in microglia activation. The following important indicators, like TNF-α, IL-1β, and IL-10, were detected. Results showed that overexpression of miR-148a in microglia could suppress inflammatory activation, inhibiting the secretion of TNF-α, IL-1β, and IL-10. When miR-148a expression was downregulated, the secretion of TNF-α, IL-1β, and IL-10 in microglia was significantly increased after subjection to OGD/R injury. This suggests that miR-148a might play an important role in the activation of microglia. More importantly, knocking down miR-148a by transfecting cells with miR-148a inhibitors acceler-

ated OGD/R-induced cell apoptosis and decreased cell viability, while overexpressing miR-148a with miR-148a inhibitors had the opposite effect. Taken together, present results reveal an important phenomenon. miR-148a downregulation can protect cells from apoptosis in OGD/R injury and can upregulate cell proliferation. MAPKs are a family of serine/threonine kinases which play a critical role in cellular responses to various extracellular stimuli [23]. Studies have proven that MAPK pathways are involved in the activation of microglia induced by albumin and LPS [24]. Evidence has demonstrated that MAPK pathways play an important role in the process of microglia activation. Thus, this study explored phosphorylated levels of p38, ERK, and JNK. It was found that p38 MAPK, ERK, and JNK pathways showed significant activation upon the primary microglia exposed to OGD/R. Overexpression of miR-148a in microglial cells dramatically inhibited the activation of MAPK pathways, compared to the normal primary microglia after OGD/R injury. Results suggest that miR-148a might suppress the activation of microglia after OGD/R through inhibiting MAPK pathways.

The current study tentatively explored the roles of miR-148a in microglia activation induced by OGD/R injury, as well as the superficial mechanisms *in vitro*. This study lacked data regarding ischemic brains. Further experiments should be conducted to verify that miR-148a is involved in the neuroinflammation of cerebral ischemia *in vivo*. In addition, the downstream molecular mechanisms involved in MAPK pathways require further exploration.

Acknowledgements

None.

Disclosure of conflict of interest

None.

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