Neuroscience Letters 690 (2019) 11–16

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

MicroRNA-21 attenuates oxygen and glucose deprivation induced apoptotic death in human neural stem cells with inhibition of JNK and p38 MAPK signaling

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ARTICLE INFO

Keywords:
Human neural stem cells
Oxygen and glucose deprivation
microRNA-21
Neuroprotection

ABSTRACT

Neural stem cells (NSCs) persist in the mammalian brain throughout life and protect against hypoxia-ischemia injury. NSCs are being increasingly recognized as a novel therapeutic target for various neurological disorders. Previous research indicates that miR-21 attenuates hypoxia-ischemia induced apoptotic death in various cell types. However, whether miR-21 plays a role in this protective effect mediated by NSCs is unknown, particularly in human NSCs (hNSCs). The present study investigated whether miR-21 could prevent hNSC injury induced by oxygen and glucose deprivation (OGD). Upon challenge with OGD treatment, loss of cell viability was observed in cultured hNSCs, as shown by CCK-8 assay. Moreover, quantitative real-time PCR (qRT-PCR) analysis indicated that expression of miR-21 increased in a time-dependent manner. TUNEL staining and Western blotting analysis showed that overexpression of miR-21 inhibited excessive hNSCs death induced by OGD treatment. Accordingly, knock down of miR-21 attenuated the neuroprotective effect observed in response to OGD treatment. Furthermore, JNK and p38 MAPKs inhibition was observed after overexpression of miR-21, and knock down of miR-21 had the opposite effect. We suggest that miR-21 prevents OGD-induced hNSCs death and apoptotic-associated protein activities through inhibiting JNK and p38 pathways in cultured hNSCs. Our findings may help to develop strategies for enhancing resident and transplanted NSCs survival after hypoxia-ischemic brain damage.

1. Introduction

Stroke is the second most frequent cause of death after coronary artery disease, accounting for 6.3 million deaths (11% of the total) [1]. Approximately 87% of strokes are ischemic, the rest being hemorrhagic [2]. Traditional therapies such as surgery and drugs have limited effect on lost functional recovery. As a result, neural stem cells (NSCs) replacement therapy as a novel treatment is now receiving increasing attention. NSCs are self-renewing, multipotent cells that generate neurons, astrocytes, and oligodendrocytes in the nervous system. They persist in the mammalian brain throughout life and are activated in response to brain injury, whereby they may participate in central nervous system (CNS) repair and functional recovery [3]. However, harsh brain microenvironments caused by hypoxic-ischemic brain damage makes survival of resident and transplanted NSCs challenging. Hence, conferring anti-hypoxic ischemic injury properties to NSCs may contribute to strategies in combating existing hypoxic-ischemic brain damage.

MicroRNAs are small noncoding RNA molecules (containing approximately 22 nucleotides) that are found in plants, animals and some viruses, functioning in RNA silencing and posttranscriptional regulation of gene expression, ultimately regulating cell behavior [4]. MicroRNA-

Abbreviations: NPCs, neural stem cells; CNS, central nervous system; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; I/R, ischemia-reperfusion; OGD, oxygen and glucose deprivation; ROS, reactive oxygen species; SVZ, subventricular zone; SGZ, subgranular zone; qRT-PCR, quantitative real-time PCR

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https://doi.org/10.1016/j.neulet.2018.09.060
Received 7 June 2018; Received in revised form 8 September 2018; Accepted 27 September 2018
Available online 03 October 2018
0304-3940/

21 (miR-21) is particularly important in terms of its regulatory role in cell survival. In cultured cortical neurons, overexpression of miR-21 substantially suppressed oxygen and glucose deprivation (OGD) induced apoptotic cell death, whereas anti-miR-21 exacerbated OGD-induced death [5]. MiR-21 has an important role in anti-apoptotic effect of cardiac progenitor cell [6]. MiR-21 was significantly upregulated in oxidative stress-induced exosomes and miR-21 overexpression decreased the expression of cleaved caspase-3 [7]. Inhibition of miR-21 ablated protective effects observed in bone marrow mesenchymal stem cells in response to H_2O_2 treatment [8]. Our previous study showed that hypoxia treatment increases expression of miR-21, and miR-21 overexpression promotes proliferation of rat NSCs [9]. Collectively, these data indicate that miR-21 may play a key role in regulating cell survival induced by OGD. However, whether miR-21 plays a role in the protective effect mediated by NSCs is unknown, particularly in human NSCs (hNSCs).

In this study, we attempted to elucidate the role of miR-21 on neuroprotection in cultured hNSCs in response to OGD treatment. The results showed that OGD treatment promoted expression of miR-21, and miR-21 overexpression protected hNSCs from OGD-induced cell death. Furthermore, JNK and p38 MAPK signaling pathways might be involved in mediating this phenomenon. Our research indicates that miR-21 could serve as a possible target for preventing OGD-induced cell death and may help to develop strategies for enhancing graft and host NSCs survival after hypoxia injury in the CNS.

2. Materials and methods

2.1. Human NSCs culture

Thirteen-week human fetal cortexes from selectively terminated normal pregnant women were obtained from the Department of Gynecology and Obstetrics, The First Affiliated Hospital of Xi'an Medical University. Informed consent was obtained before specimen collection, and the experimental protocols were approved by the Ethics Committee of Xi'an Medical University and followed the guidelines of the Declaration of Helsinki. Specimen collection was conducted in accordance with the guidelines of the National Institutes of Health. hNSCs were prepared from 13-week human fetal cortex as previously described with minor modifications [10]. For hNSCs culture, after removal of the meninges, the cortex was mechanically dissociated into single-cell suspensions in serum-free DMEM/F12 basal medium. After centrifugation at 4 °C, cells were cultured in serum-free complete medium consisted of DMEM/F12 (1:1), 1% penicillin-streptomycin, 1% N2, 2% B27, 20 ng/ml epidermal growth factor (EGF) and 10 ng/ml basic fibroblast growth factor (bFGF). All materials used in cell culture were purchased from Life Technologies (California, USA). For single-cell adhesive culture, single hNSCs in serum-free complete medium were allowed to attach onto poly-D-lysine-coated coverslips.

2.2. Oxygen-glucose deprivation (OGD) treatments

On day 2 after plating, hNSCs were cultured in a glucose-free DMEM medium (Life Technologies, USA) and placed in an anaerobic workstation (Bugbox; Ruskinn Technology) of 5% CO_2, 0.3% O_2 and 94.7% N_2 at 37 °C for up to 0.5, 1, 2, 4, 6, 8, 12 or 24 h. The control cultures were incubated in standard conditions for the same duration.

2.3. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, California, USA) according to the manufacturer's instructions for the analysis of miRNA expression. Total RNA (1 μg) was used as a template for reverse transcription using the First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). qRT-PCR was performed using a SYBRGreen PCR kit (Fermentas, Vilnius, Lithuania) in IQ5 Optical System real-time PCR machine (Bio-Rad, California, USA). Relative gene expression was calculated using the 2-(ΔΔCt) method, and miR-21 expression was normalized to U6 expression. Data analyses were performed according to methods described in previous studies [9].

2.4. Transfection

Cells were plated in poly-D-lysine-coated 24-well or 6-well plates. Transient transfections were performed using Lipofectamine 2000 (Invitrogen, California, USA) in accordance with the manufacturer's instructions. MiR-NC, miR-21 mimic and miR-21 inhibitor were purchased from GenePharma (Shanghai, China).

2.5. Cell viability assay

Cell viability was evaluated by using the Cell Counting Kit-8 (Roche, Germany) in accordance with the manufacturer's instruction, and then detected using a multi-microplate spectrophotometer (Epoch, BioTek, USA).

2.6. TUNEL staining

To measure apoptotic death in hNSCs, terminal-deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) assay was carried out following the manufacturer's instruction (Roche Diagnostics, IL, USA). After TUNEL staining, cells were counter-stained with DAPI (1 μg/ml) before mounting. Microscopy and imaging were performed in an Olympus BX51 fluorescence microscope. Images were processed using Image-Pro Plus 5.0 software. Ten random fields were counted for each sample using the 20× objective. Data are presented as the percentage of TUNEL positive cells from the total number of cells (DAPI-stained cells).

2.7. Immunostaining

To identify hNSCs and anoxic cells, NSCs plated on PDL-coated coverslips were fixed in 4% paraformaldehyde for 20 min at room temperature and washed in PBS. Immunostaining was performed as in a previous study [11]. Briefly, cells were stained with suitable first and secondary antibodies (Supplement Table 1), and positive cells were observed using a BX51 fluorescence microscope equipped with a DP70 digital camera (both from Olympus, Japan).

2.8. Western blot analysis

Immunodetection methods were based on a previous study [11]. Briefly, hNSCs were cultured in PDL-coated 6-well plates. After treatment, cells were rinsed three times in PBS followed by incubation in lysis buffer (Pierce, IL, USA) supplemented with Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA) for 15 min on ice, followed by sonication (VCX500, Sonics, CT). Protein concentrations of samples were estimated by BCA assay (Pierce, Rockford, IL, USA). Lysates (20 μg–40 μg for each sample) were subjected to Western blot and probed with suitable first and secondary antibodies (Supplement Table 1). The results were collected using a G: Box gel imaging system (Syngene, UK) and quantified using NIH ImageJ 3.5 software. All Western blot data presented are in samples from at least 3 independent experiments.

2.9. Statistical analysis

All of the data are reported as the mean ± SD of at least three independent in vitro experiments. Multiple comparisons within groups were made to analyze CCK-8, qRT-PCR, immunostaining and Western blot data using Tukey's test after one-way ANOVA. Comparisons between two groups were performed using Student's *t*-test for paired data.

All statistical analyses were performed using SPSS statistical software (version 12.0). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of NSCs from human fetal cortex

After culturing primary hNSCs 3–5 days, diameter 80–120 μm neurospheres were observed in the medium (S1A). hNSCs were then dissociated into single cells, plated onto poly-D-lysine-coated coverslips and cultured in the serum-free complete medium. The purity of hNSCs was determined by immunocytochemical double labeling for the specific markers nestin and SOX2. The results showed that this culture procedure yielded 97.28% ± 4.49% nestin-positive cells, of which 96.41% ± 5.52% expressed SOX2 (S1B–E). To determine differentiating potential, single hNSCs were cultured in differentiation medium supplemented with 1% FBS and lacking bFGF and EGF. The staining results showed that the differentiated cells expressed Tuj1, a neuronal marker for neurons, or GFAP, an astrocytic marker (S1F, G). These findings strongly indicated that these primary cultured cells were hNSCs, and this culture system was used to conduct subsequent experiments.

3.2. OGD treatment stimulates expression of miR-21 in cultured hNSCs

To identify the damaging effects of OGD on hNSCs, CCK-8 assay was used to measure cell viability. The results revealed that cell viability was significantly decreased at 2 h, and over time, the effect became increasingly evident. Of note, most hNSCs exhibited seriously injury at 6 h. Therefore, 4 h of OGD treatment was used in subsequent experiments (Fig. 1A). For further verification, the purity of hNSCs and hypoxia marker expression were assessed by immunocytochemical double labeling for their respective specific markers nestin and HIF-1α, an adaptive expressed protein in response to hypoxia, after OGD preconditioning for 4 h. The results showed that this culture procedure yielded 95.41% ± 7.19% nestin positive cells, with 96.15% ± 6.64% of them expressing HIF-1α (Fig. 1B–E), indicating that OGD treatment for 6 h induces hypoxia in cultured hNSCs. To determine expression of miR-21 in hNSCs after OGD treatment, total RNA from each group was extracted using TRIzol at different time points (from 0.5 to 1 day) for subsequent qRT-PCR analysis. Compared to that of the control condition, expression of miR-21 in response to OGD increased in a time-dependent manner, reaching significance after 0.5 h OGD treatment (Fig. 1F), indicating that OGD treatment stimulates expression of miR-21 in NSPCs.

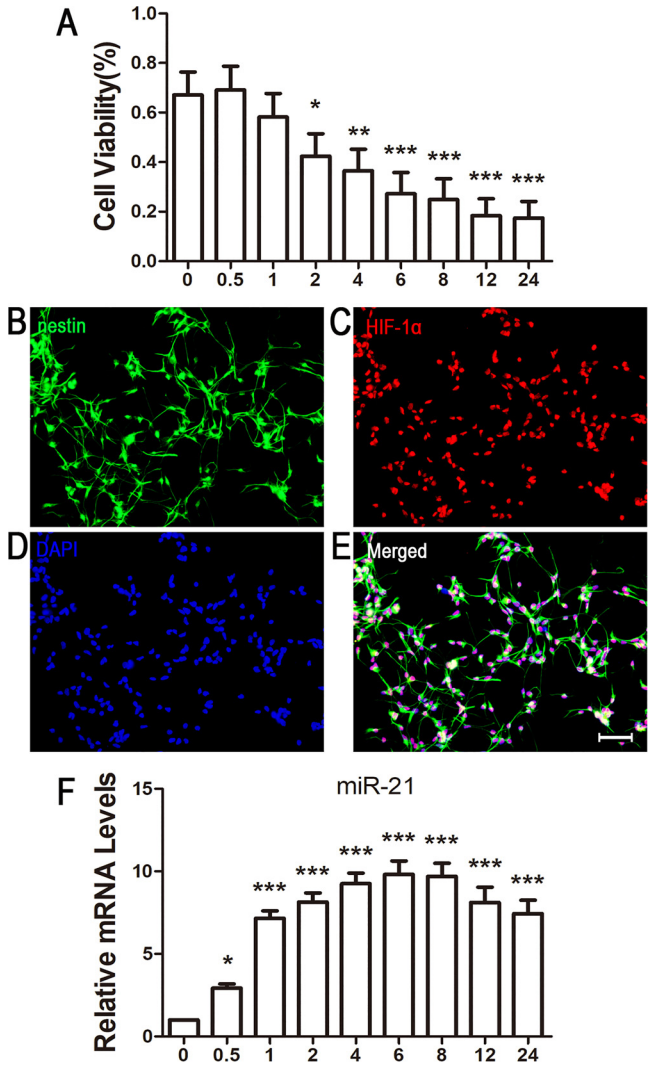

Fig. 1. Expression of miR-21 is enhanced by OGD in cultured hNSCs. Cells were cultured in glucose-free DMEM medium and placed into the hypoxic incubator for 0.5, 1, 2, 4, 6, 8, 12 or 24 h. (A) Cell viability detected by CCK-8 shows decreasing hNSCs viability observed 2 h after hypoxia treatment, and over time, the effect became increasingly evident. (B–E) Immunofluorescence staining shows HIF-1α (red) expressed in cultured NSPCs with nestin$^+$ (green), after 6 h OGD treatment. Nuclei were counterstained with DAPI (blue); scale bars = 40 μm. (F) qRT-PCR analysis of miR-21 expression after hypoxia treatment at different time points (0.5, 1, 2, 4, 6, 8, 12 or 24 h) indicated that expression of miR-21 increased in a time-dependent manner, and significant difference were observed from 0.5 h of hypoxia treatment onward. Each value represents the mean ± SD of three independent experiments (n = 3). *$P < 0.05$, ***$P < 0.001$ versus control (0 h). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. MiR-21 protects hNSCs from OGD-induced cell death

To further examine the neuroprotective effects of miR-21 on OGD-induced hNSC injury, apoptotic cells were distinguished from viable cells by TUNEL staining. As expected, the percentage of apoptotic cells increased markedly 4 h after OGD treatment, as indicated by 31.59% ± 3.23% TUNEL positive cells. More importantly, overexpression of miR-21 significantly decreased TUNEL-positive cells compared to the control group (OGD + miR-NC), while knock down of miR-21 exerted the opposite effect on neuroprotection in hNSCs (Fig. 2A and B). Various apoptotic proteins mediate OGD-induced cell death, in which caspase 3 plays a central role in the execution-phase of apoptosis [12]. To further investigate the neuroprotective effect of miR-21 on hNSCs, we employed Western blotting to detect changes in the expression of pro-caspase 3 and cleaved caspase-3. The results showed that the expression of cleaved caspase-3 was significantly increased after OGD treatment and miR-21 overexpression significantly decreased cleaved caspase-3 expression, while knock down of miR-21 exerted the opposite effect. Meanwhile, changes in pro-caspase-3 expression showed opposing results, indicating that overexpression of miR-21 significantly promote pro-caspase-3 expression (Fig. 2C–E). These results indicate that miR-21 protects hNSCs from OGD-induced cell death.

3.4. MiR-21 inhibits OGD-induced phosphorylation of JNK and p38 in hNSCs

In order to explore the intracellular pathways responsible for regulation of hNSCs, neuroprotection by miR-21, we investigated the effects of miR-21 on JNK and p38 MAPKs by Western blot analysis. Single hNSCs were plated onto poly-D-lysinecoated 6-well plates in serum-free complete medium. Twenty-four hours later, synthesized miR-NC, miR-21 mimic or miR-21 inhibitor was transfected into hNSCs and cultured

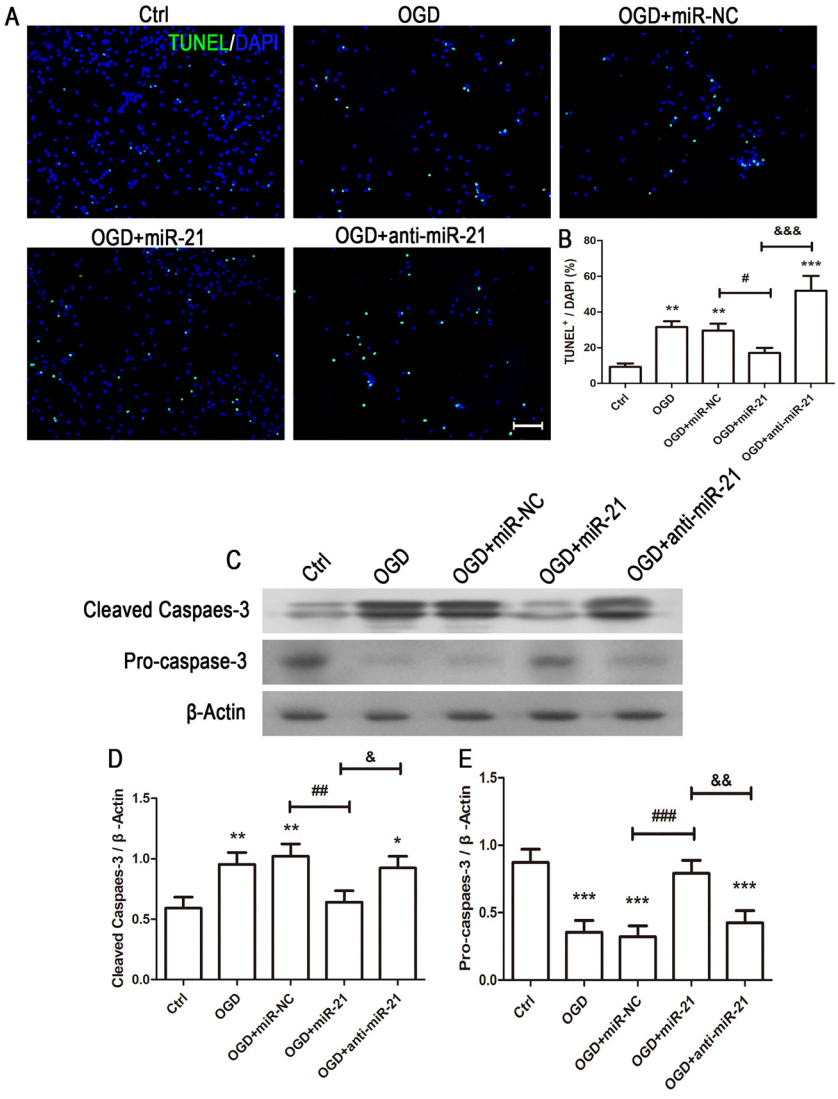

Fig. 2. Overexpression of miR-21 protects hNSCs from OGD-induced cell death.
Single adhesive cultured hNSCs were transfected for 6 h with miR-NC, miR-21 mimic or miR-21 inhibitor using Lipofectamine 2000. One day later, cells were exposed to OGD treatment for 4 h, and apoptotic cells and apoptotic signal proteins were detected by TUNEL staining and Western blotting analysis. Representative images and quantitative analysis for TUNEL staining are shown in (A) and (B), respectively. Data from three independent experiments (n = 3) are presented as the percentage of TUNEL positive cells from total DAPI staining cells. Scale bar 100 μm. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control (Ctrl), $^{\#}P < 0.05$ versus OGD + miR-NC group, $^{\&\&\&}P < 0.001$ versus OGD + miR-21 group. (C) Representative WB images illustrating protein expression of cleaved caspase-3 and pro-caspase-3, with β-Actin used as a reference protein. WB band quantification for the ratio of cleaved caspase-3 (D), pro-caspase-3 (E) to β-Actin are presented, and each value represents the mean ± SD of three independent experiments (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control (Ctrl), $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus OGD + miR-NC group, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ versus OGD + miR-21 group.

for three days. After OGD treatment, the cell lysates were analyzed for the presence of phosphorylated JNK2 and p38 by antibodies to the specific phosphorylation sites of p38 (Thr180/Tyr182) and JNK2 (Thr183/Tyr185). Western blotting bands demonstrated that OGD treatment led to sustained JNK2 and p38 phosphorylation, while miR-21 significantly inhibited OGD-induced phosphorylation of JNK2 and p38. Meanwhile, knock down of miR-21 also activated JNK2 and p38 compared to the controls (Fig. 3A–C). These data indicate that miR-21 attenuates activation of JNK and p38 signaling induced by OGD treatment.

4. Discussion

Stroke most commonly results from occlusion of a major artery in the brain, giving rise to oxygen and glucose deprivation, excessive production of reactive oxygen species (ROS), and typically leading to the death of all cells within the affected tissue [13]. Studies in mice and rats show that NSCs persists in the brain throughout life, and in the adult mammalian brain, most NSCs are spatially restricted to two specific niches: the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zoon (SVZ) of the lateral ventricles [14]. Moreover, cultured NSCs can be obtained from fetal or adult neurogenesis areas, or induced from embryonic stem cells. These endogenous and exogenous NSCs go through proliferation, migration and maturation, finally integrating into existing neural networks and substituting for neurons lost during CNS disorders [15]. In this study, we observed that miR-21 expression significantly increased after OGD treatment in hNSCs, and miR-21 protected cultured hNSCs from OGD-induced cell death. Furthermore, the neuroprotective effects of miR-21 might be mediated through inhibition JNK and p38 signaling pathways. These results suggest that miR-21 is a novel therapeutic target for various neurological disorders, including stroke. However, this research is purely an in vitro study, and the neuroprotective effects of miR-21 need to be further demonstrated in vivo.

MiR-21, also known as has-mir-21, is a mammalian microRNA encoded by the miR21 gene. Previous research demonstrated that miR-21 plays a key role in protecting various cell types from hypoxia-ischemia injury. In a mouse heart ischemia and reperfusion model, isoflurane protects cells though overexpression of miR-21 [16]. Over-expression of miR-21 protects human umbilical vein endothelial cells from apoptosis during ischemia injury by targeting PTEN [17]. In the CNS, miR-21 protects microglia against OGD-induced cell death though repression of the Fas ligand [18]. Conversely, long-term high expression of miR-21 may be detrimental to the organ by promoting development of renal interstitial fibrosis following OGD injury [19]. Therefore, the long-term effects of miR-21 should be evaluated in hNSCs after OGD injury.

In our study, we observed that miR-21 attenuates JNK/p38 phosphorylation induced by OGD treatment in cultured hNSCs. JNK and p38 MPAKs are key intracellular signaling molecules involved in the regulation of cellular responses to various stresses, such as ionizing

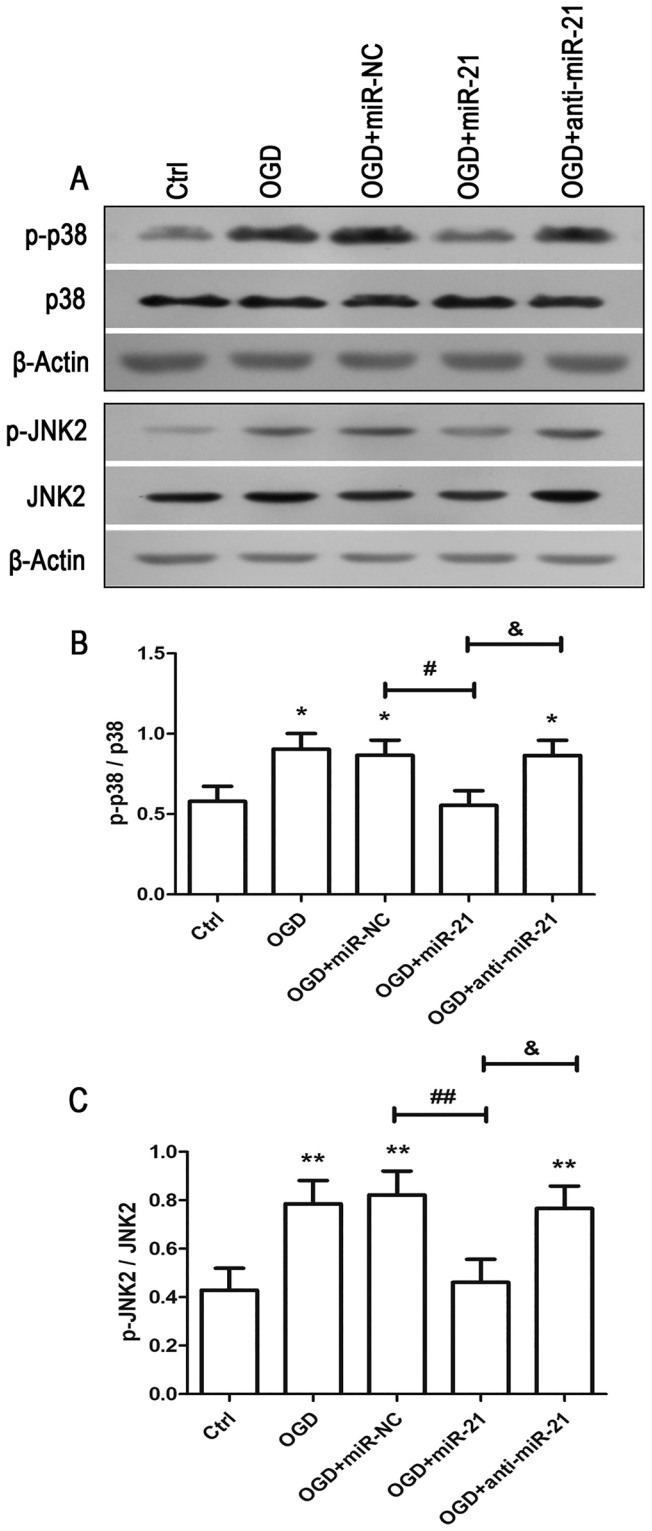

Fig. 3. Overexpression of miR-21 inhibits phosphorylation of JNK and p38 after OGD treatment.

Single NSPCs were plated onto poly-D-lysine-coated 6-well plates and cultured for one day. Then, cells were transfected with miR-NC, miR-21 mimic or miR-21 inhibitor and exposed to OGD treatment for 4 h. Phosphorylation levels of JNK and p38 were detected by Western blotting analysis. (A) Representative Western blot (WB) images illustrate the differential expression of p-p38/p-38 and p-JNK2/JNK2 after treatments. WB band quantification for the ratio of p-p38/p-38 (B) and P-JNK2/JNK2 (C) are presented. Each value represents the mean ± SD of three independent experiments (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (Ctrl), $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ versus OGD + miR-NC group, $^{\&}P < 0.05$ versus OGD + miR-21 group.

radiation, inflammatory cytokines and ischemia/hypoxia [20]. During experimental oxidative stress-induced cell apoptosis, JNK and p38 were activated by H_2O_2 stimulation, resulting in neural stem cell death, and the inhibition of JNK and p38 phosphorylation may play a role in protecting cells from oxidative injuries [21,22]. These phenomena suggest that the miR-21-mediated neuroprotective effects of hNSCs might be due to the inhibition of JNK/p38 phosphorylation.

Acknowledgments

This work was supported by Science and Technology Plan Projects of the Shaanxi Provincial Department of Education (NO. 16JK1643).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neulet.2018.09.060.

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