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miR-497-5p promoted neuronal injury in ischemic stroke by inhibiting the BDNF/TrkB/PI3K/Akt pathway

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Abstract. The aim of this study was to investigate the molecular mechanism by which miR-497-5p regulates neuronal injury after ischemic stroke through the BDNF/TrkB/Akt signaling pathway. PC12 cells were used to construct a stroke injury model by oxygen-glucose deprivation/reoxygenation (OGD/R). The expression level of miR-497-5p was measured by RT-qPCR. CCK-8 kit was used to detect cell viability. Cell apoptosis and reactive oxygen species (ROS) were detected by flow cytometry. MDA and SOD detection kits were used to detect MDA content and SOD activity. A double luciferase reporter system was used to verify the targeting relationship between miR-497-5p and BDNF. The expression of BDNF, TrkB, p-TrkB, Akt and p-Akt was detected by Western blot. We have found that miR-497-5p expression was inhibited after treatment with OGD/R. Simultaneously, cell apoptosis, MDA content and ROS were upregulated, while cell viability and SOD were significantly decreased in PC12 cells. The effects of OGD/R on PC12 cells were reversed with the downregulation of miR-497-5p. A double luciferase reporter assay demonstrated that miR-497-5p negatively targets BDNF. BDNF inhibited cell apoptosis and oxidative stress injury in PC12 cells. These findings suggest that miR-497-5p aggravates neuronal injury in experimental model of ischemic stroke by inhibiting the BDNF/TrkB/PI3K/Akt signaling pathway.

Key words: Ischemic stroke — PC12 cells — miR-497-5p — BDNF — TrkB/Akt signaling pathway

Introduction

Ischemic stroke, which is caused by cerebral ischemia, is a disease that causes brain tissue necrosis. Ischemic stroke can affect the health of patients and may even be life-threatening at any time (Zhang et al. 2019; Feske 2021). The pathogenesis of these diseases is very complicated, but existing studies have shown that the stimulation of neuronal cells by inflammatory factors will cause oxidative stress, which will lead to cell apoptosis, eventually leading to the development of diseases (Ye et al. 2019). The molecular mechanism underlying neuronal cell damage is the key to the development of stroke, and in-depth study could provide a new direction for effective treatment of ischemic stroke.

Many endogenous microRNAs (miRNAs) are involved in the regulation of oxidative stress and abnormal cell apoptosis in neurons caused by ischemic stroke (Eyileten et al. 2018). miR-497-5p expression was shown to be upregulated in neuronal cell injury, and downregulation of miR-497-5p could inhibit neuronal apoptosis and relieve the progression of this brain disease (Cai et al. 2019). Brain-derived neurotrophic factor (BDNF) plays an important role in the repair of neuronal cell damage after stroke (Yan et al. 2022). Studies have shown that BDNF can bind to the tropomyosin receptor kinase B (TrkB) receptor and subsequently activate the antiapoptotic protein kinase B (Akt) signaling pathway

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to relieve cell damage (Ahmed et al. 2021; Liu et al. 2021). However, there are no reports in the literature suggesting that miR-497-5p regulates neuronal cell injury after ischemic stroke through regulation of BDNF. Therefore, this study is focused on the relationship between the targeting of miR-497-5p and BDNF and the expression of their related signaling axes involved in the regulation of neuronal damage in experimental model of ischemic stroke to provide a more theoretical basis for the follow-up study of ischemic stroke.

Material and Methods

Distribution of the experimental groups

(1) NC group: negative control; (2) OGD/R: oxygen-glucose deprivation/reoxygenation (disease model); (3) OGD/R+miR-497-5p inhibitor: miR-497-5p inhibitor was transfected after OGD/R; (4) OGD/R+miR-497-5p inhibitor+si-BNDF: miR-497-5p inhibitor and si-BNDF were transfected after OGD/R.

Cell culture and construction of the OGD/R model

PC12 cells were purchased from Sunncell Biotechnology Co., Ltd. (Wuhan, China). PC12 cells were cultured in DMEM (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) at 37°C and 5% CO_2. Cells in logarithmic growth phase were placed in glucose-free medium and cultured at 37°C under hypoxic conditions (85% N_2, 10% CO_2, 5% O_2) for 6 h to induce OGD/R injury. The cells were then reoxygenated in complete medium containing 4.5 g/l glucose for 24 h (5% CO_2, 37°C). Cells cultured under conventional culture conditions were used as the control group (NC).

Cell transfection

PC12 cells in logarithmic growth phase were seeded in 6-well plates for conventional culture. According to the instructions of the cell transfection kit, si-BDNF, miR-497-5p mimic, miR-497-5p inhibitor, and their negative control, si-NC, miR-NC mimic and miR-NC inhibitor (designed and provided by GenePharma, China) were transfected by Lipofectamine^{TM} 2000. Transfection efficiency was measured by RT-qPCR or Western blot after 48 h.

RT-qPCR

Total RNA was extracted from cells by using a total RNA extractor (Sangon Biotech, China), 1 μl of RNA sample was collected, and RNA integrity was detected by 1% agarose gel electrophoresis. One microliter of the RNA sample was taken after dilution to measure the optical density (OD) value based on the OD_{260}/OD_{280} ratio to identify total RNA purity. A cDNA synthesis kit (Vazyme, Nanjing, China) was used to reverse transcribe 2 μg mRNA into cDNA. Then, cDNA was used as the amplification template in the SYBR Green Master Mix (Vazyme, Nanjing, China) reaction system. ABI QS3 Fluorescence PCR (Thermo Fisher, USA) was performed. The total reaction mixture was 25 μl, incorporating 10 ng of cDNA, 12.5 μl of L SYBR Premix Ex Taq II and 10 μmol/l of each of the forward and downstream primers, and ddH_2O was used to bring the total volume to 25 μl. The reaction routine was as follows: 95°C 30 s; 95°C 5 s, 60°C 30 s 39 cycles; 95°C 10 s, 65°C 6 s, 95°C 5 s. The results were calculated using the $2^{-\Delta\Delta Ct}$ method. U6 served as an internal control.

Detection of cell viability by CCK-8

Cells in each group were collected and seeded in 96-well plates at 5×10^3 cells/well. Three wells were set up for each group, and the cells were placed in an incubator at 37°C and 5% CO_2. After culturing for 48 h, CCK-8 solution was added according to the instructions of the CCK-8 kit (Beyotime, China) and incubated for 2 h. Subsequently, the optical density of each well at 450 nm was detected on a microplate reader (BioTek, USA).

Detection of cell apoptosis and ROS levels

Cell apoptosis detection: Cells in the logarithmic growth phase were stained using an Annexin V-FITC/PI kit (Beyotime, China) according to the manufacturer's instructions, and cell apoptosis was analyzed using flow cytometry (Beckman, USA).

Reactive oxygen species (ROS) detection: PC12 cells were cultured in serum-free medium and loaded with 2',7'-dichlorofluorescein diacetate (DCFH-DA, Solarbio, China) at a final concentration of 10 μM, and then cells were cultured according to the method for subsequent processing in the literature. Finally, the fluorescence intensity was measured by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Detection of MDA content and SOD activity using kits

According to the operating procedures of the MDA and SOD detection kits (Solarbio, China), the absorbance of MDA at 532 nm and SOD at 560 nm were measured by an enzyme marker (BioTek, USA), and the MDA content and SOD activity were calculated according to the manufacturer's manual.

Detection of the targeting relationship between miR-497-5p and BDNF

The pmirGLO-BDNF-WT/MUT vector was constructed, and PC12 cells were transfected with BDNF-WT or BDNF-

MUT with miR-497-5p mimic or miR-NC mimic. Luciferase activity was detected after 48 h.

Western blot

Proteins were extracted using RIPA lysis buffer (Sangon Biotech, Shanghai). Protein concentrations were determined according to the specifications of the bicinchoninic acid assay (BCA, Solarbio, China) kit. Fifty micrograms of protein were loaded *per* lane, and total proteins were separated by SDS-PAGE (5% concentrated gel, 10% separating gel), transferred to PVDF membranes (Millipore, USA) and then blocked with 5% nonfat milk powder for 1.5 h at room temperature. The PVDF membranes were incubated with antibodies against BDNF (ab108319, 1:1000, Abcam), p-AKT (ab38449, 1:1000, Abcam), AKT (ab131168, 1:1000, Abcam), TrkB (ab187041, 1:5000, Abcam), phospho Y705-TrkB (ab229908, 1:1000, Abcam), p-PI3K9 (ab278545, 1:1000, Abcam), PI3K (ab140307, 1:2000, Abcam) and GAPDH (ab9485, 1:2500, Abcam). The membranes were incubated overnight at 4°C, the primary antibody was removed the next day, and the membranes were incubated at 25°C for 2 h. Secondary antibodies (1:4000, ab97051, Abcam) were added and incubated at 25°C for 1 h. Band gray values were analyzed by ImageJ.

Statistical analysis

GraphPad Prism 8.0 was used to analyze the experimental data and plot the graphs. The data are from at least 3 replicates of all experiments. The differences between the two groups were compared by independent sample T test. One-way ANOVA was used to analyze the differences among groups. The mean ± standard deviation (SD) is shown in this study. The data were statistically significant if $p < 0.05$.

Results

Upregulation of miR-497-5p expression in PC12 cells after OGD/R

The results of RT-qPCR analysis showed that after PC12 cells were treated with OGD/R, the expression level of miR-497-5p was significantly higher than that in the NC group (Fig. 1). These results showed that the expression of miR-497-5p was upregulated in PC12 cells after OGD/R.

miR-497-5p inhibitor relieves PC12 cell apoptosis and oxidative stress and increases cell viability after OGD/R

The RT-qPCR results showed that compared with that in the NC group or miR-NC inhibitor group, the expression level of miR-497-5p was significantly reduced in the miR-497-5p inhibitor group, which indicated successful transfection of miR-497-5p inhibitor (Fig. 2A). The results of the CCK-8 assay showed that cell viability was significantly inhibited after OGD/R, while cell viability increased after the addition of the miR-497-5p inhibitor (Fig. 2B). Similarly, OGD/R promoted cell apoptosis and ROS levels. The miR-497-5p inhibitor decreased cell apoptosis and ROS levels in comparison to the OGD/R group (Fig. 2B–D). MDA content and SOD activity were measured by detection kits. The results showed that compared with that in the NC group, MDA content was significantly increased and SOD activity was significantly decreased in the OGD/R group, and the above results were reversed after the addition of the miR-497-5p inhibitor (Fig. 2E,F). The results show that miR-497-5p promoted cell apoptosis and oxidative stress in PC12 cells after OGD/R.

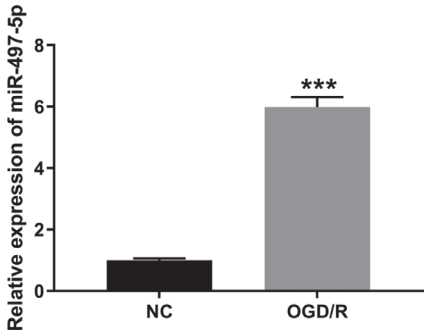

Figure 1. miR-497-5p was upregulated in PC12 cells after OGD/R. NC, negative control; OGR/D, oxygen-glucose deprivation/reoxygenation. *** $p < 0.001$ *vs.* NC.

Targeting relationship between miR-497-5p and BDNF

A binding site for BDNF and miR-497-5p was predicted by the StarBase database (https://starbase.sysu.edu.cn/), and the binding sequence is shown (Fig. 3A). There is an interaction relationship between miR-497-5p and BDNF through double luciferase reporter gene detection (Fig. 3B). RT-qPCR indicated successful transfection of the miR-497-5p mimic (Fig. 3C). In addition, Western blot analysis showed that overexpression of miR-497-5p significantly inhibited the expression of BDNF in comparison to miR-NC mimic (Fig.

Table 1. Primer sequences

Gene	Primer sequences	
miR-497-5p	F:	5'-CGCGATGTTTGGTGTCACAC-3'
	R:	5'- AGTGCAGGGTCCGAGGTATT-3'
U6	F:	5'-CTCGCTTCGGCAGCACA-3'
	R:	5'-AACGCTTCACGAATTTGCGT-3'

F, forward; R, reverse; U6, internal control.

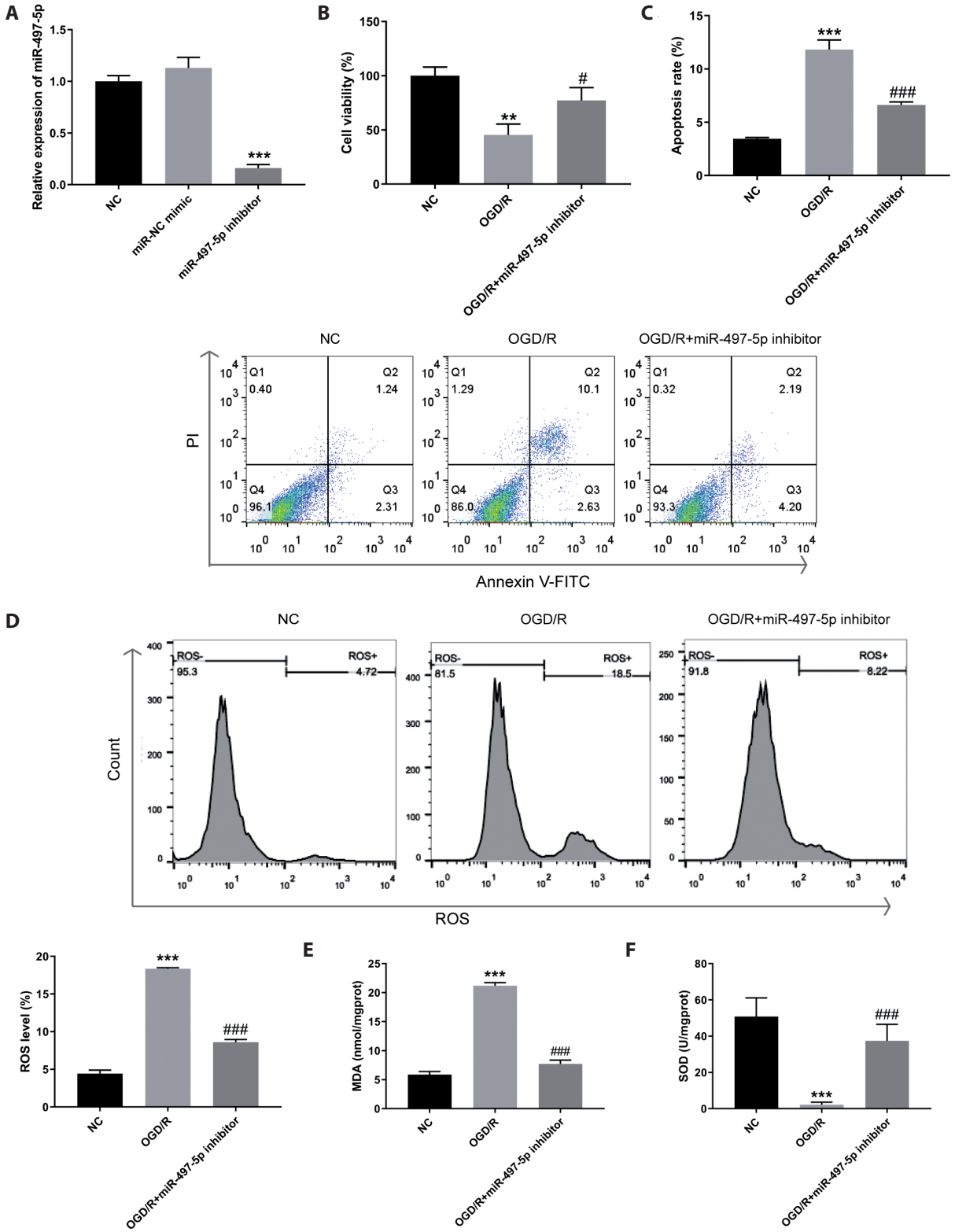

Figure 2. Downregulation of miR-497-5p induced apoptosis and oxidative stress in PC12 cells after OGD/R. **A.** Knockdown efficiency of miR-497-5p was measured by RT-qPCR. **B.** Cell ability was detected by CCK-8 kit. Cell apoptosis (**C**) and ROS (**D**) were detected by flow cytometry. MDA content (**E**) and SOD activity (**F**) were determined by kits. ** $p < 0.01$, *** $p < 0.001$ *vs.* NC or miR-NC mimic; # $p < 0.05$, ### $p < 0.001$ *vs.* OGD/R. NC, negative control; OGR/D, oxygen-glucose deprivation/reoxygenation; PI, propidium iodide.

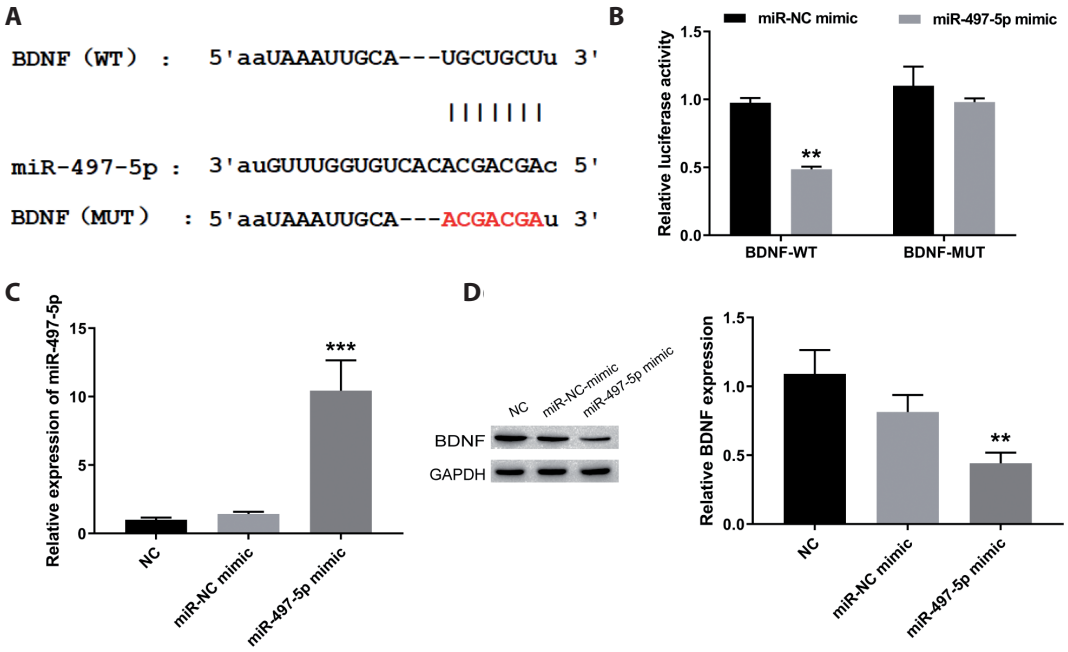

Figure 3. The targeting relationship between miR-497-5p and BDNF. **A.** The targeted site of miR-497-5p and BDNF was predicted by the StarBase database. **B.** The interaction between miR-497-5p and BDNF was verified by a double luciferase reporter. **C.** The transfection efficiency of the miR-497-5p mimic was measured by RT-qPCR. **D.** The expression of BDNF was detected by Western blot. ** $p < 0.01$, *** $p < 0.001$ *vs.* miR-NC mimic. NC, negative control; MUT, mutant; WT, wild type.

3D). The above experimental results indicated that BDNF has a targeted negative regulatory effect on BDNF.

miR-497-5p regulated OGD/R injury in PC12 cells through BDNF

Encouraged by the above results, we further explored the role of miR-497-5p in OGD/R injury by BDNF knockdown. The Western blot results showed that compared with that in the NC group, the expression level of BDNF was reduced in the si-NC group, which indicated successful transfection of si-BDNF (Fig. 4A). The results of the CCK-8 assay showed that cell viability was significantly increased in comparison to OGD/R after addition of miR-497-5p inhibitor, while it was inhibited after transfection of si-BNDF (Fig. 4B). Compared with those in the OGD/R group, the miR-497-5p inhibitor reduced cell apoptosis and ROS levels. Knockdown of BNDF promoted cell apoptosis and ROS levels in comparison to the OGD/R+miR-497-5p inhibitor group (Fig. 4B–D). MDA content and SOD activity were measured by detection kits. The results showed that compared with that in the OGD/R group, MDA content was significantly decreased and SOD activity was significantly increased in the OGD/R+miR-497-5p inhibitor group, and the above results were reversed after knocking down BDNF (Fig. 4E,F). miR-497-5p regulates OGD/R-induced cell apoptosis and oxidative stress levels in PC12 cells through the negative regulation of BDNF.

miR-497-5p is involved in PC12 cell injury after OGD/R through the BDNF/TrkB/PI3K/Akt pathway

The Western blot results showed that BDNF and the phosphorylation levels of TrkB, PI3K and Akt in the OGD/R group were significantly lower than those in the NC group, while their levels were significantly enhanced after the addition of the miR-497-5p inhibitor. Compared with those in the OGD/R+miR-497-5p inhibitor group, the expression of BDNF and the phosphorylation levels of TrkB, PI3K and Akt were inhibited in the OGD/R+miR-497-5p inhibitor+si-BNDF group (Fig. 5). These results indicated that miR-497-5p regulated PC12 cell injury after OGD/R through inhibiting the BDNF/TrkB/PI3K/Akt signaling pathway.

Discussion

In China, the majority of deaths caused by brain complications are caused by ischemic stroke. At the same time, approximately 30% of these patients permanently lose the ability to work, which has a negative impact on the life of patients (Ni et al. 2019; Wang et al. 2019). Many studies have

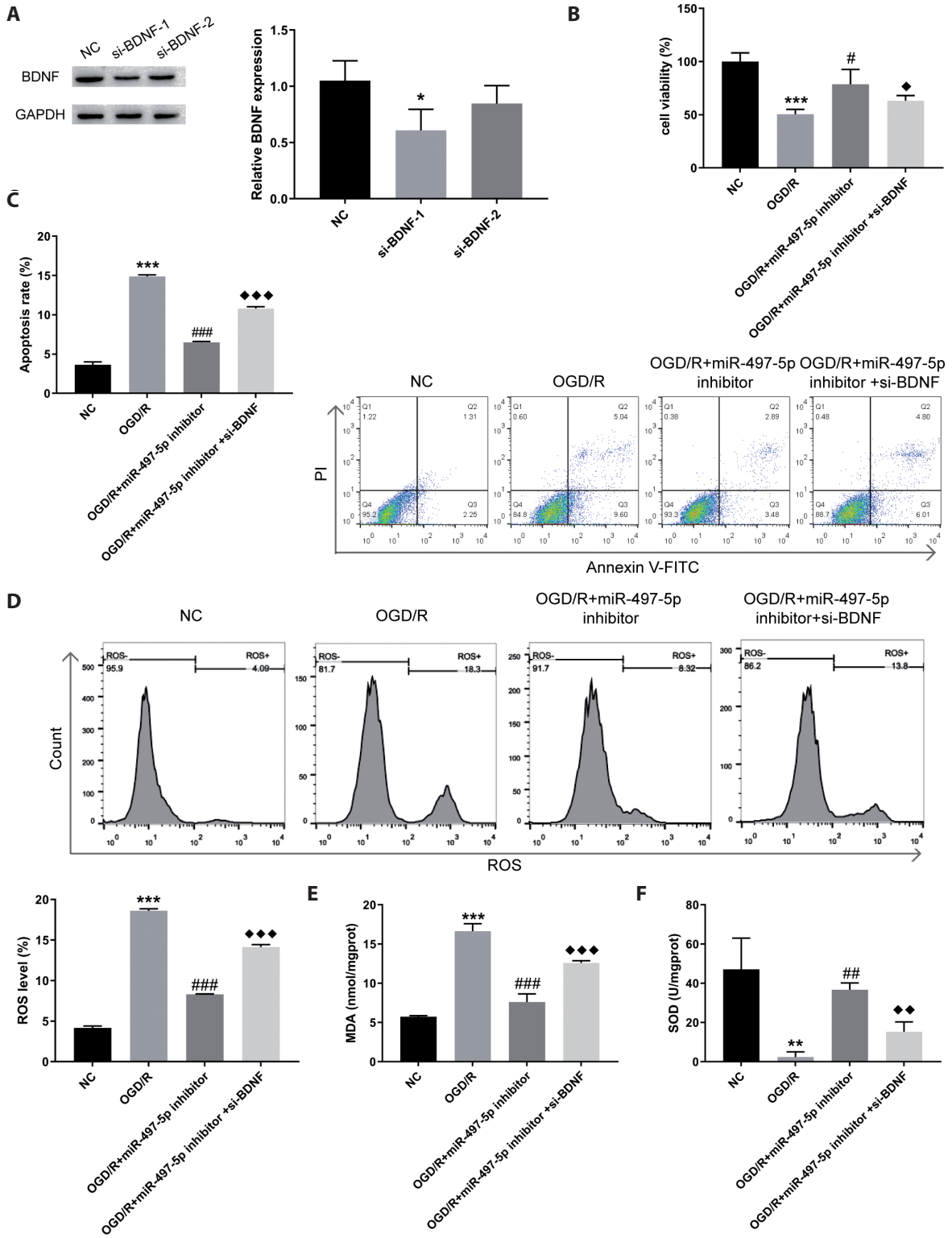

Figure 4. miR-497-5p regulated OGD/R injury in PC12 cells through BDNF. **A.** The knockdown efficiency of BDNF was measured by Western blot. **B.** Cell ability was detected by CCK-8 kit. Cell apoptosis (**C**) and ROS (**D**) were detected by flow cytometry. MDA content (**E**) and SOD (**F**) activity were determined by kits. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* NC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* OGD/R; $^{\blacklozenge}p < 0.05$, $^{\blacklozenge\blacklozenge}p < 0.01$, $^{\blacklozenge\blacklozenge\blacklozenge}p < 0.001$ *vs.* OGD/R+miR-497-5p inhibitor. NC, negative control; OGR/D, oxygen-glucose deprivation/reoxygenation; PI, propidium iodide.

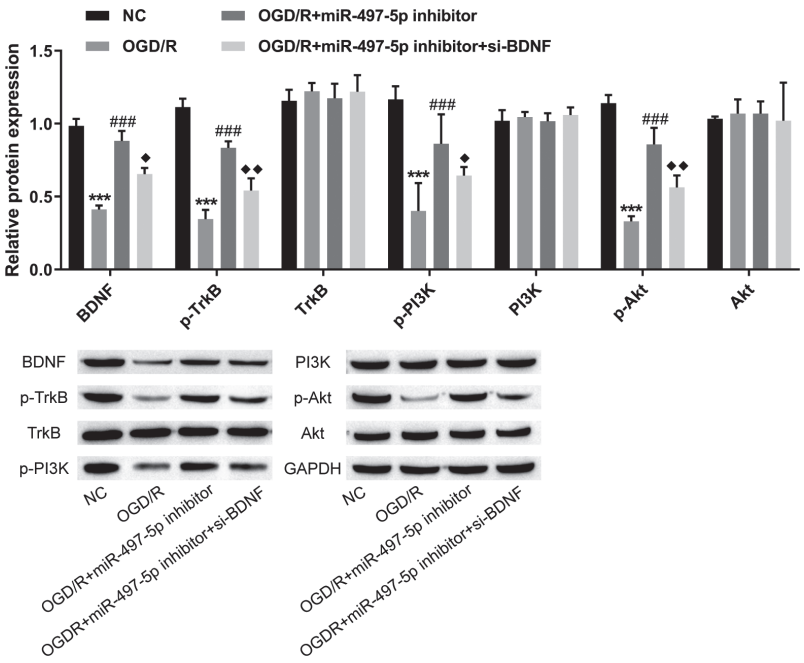

Figure 5. miR-497-5p is involved in PC12 cell injury after OGD/R through BDNF/TrkB/PI3K/Akt. *** $p < 0.001$ *vs.* NC; ### $p < 0.001$ *vs.* OGD/R; ♦ $p < 0.05$, ♦♦ $p < 0.01$ *vs.* OGD/R+miR-497-5p inhibitor. NC, negative control; OGR/D, oxygen-glucose deprivation/reoxygenation.

found that the expression of miRNAs changes after ischemic stroke and is involved in the regulation of the progression of ischemic stroke (Jiang et al. 2019; Li Z et al. 2021; Liu et al. 2022). Downregulation of miR-19a inhibited neuronal apoptosis and relieved the progression of ischemic stroke (Ge et al. 2019). High expression of miR-195 could inhibit the secretion of inflammatory-related factors in neurons and protect against brain injury caused by ischemic stroke (Yang et al. 2018). Some studies have found that miR-497-5p expression is upregulated in neuronal injury and promotes ischemic neuronal apoptosis by regulating apoptotic proteins (Chen Q et al. 2020; Zhu et al. 2021; Sun and Yuan 2022). In this study, PC12 cells were used to establish an OGD/R injury model to simulate neuronal cell injury after ischemic stroke. The expression of miR-497-5p was significantly upregulated in PC12 cells after OGD/R.

Oxidative stress is known to activate proapoptotic signaling pathways such as cytochrome c, which induces DNA damage, changes in protein structure and function, and lipid peroxidation during ischemia and reperfusion. Currently, intravenous tissue plasminogen activator (t-PA) and intravascular therapy can be used to treat acute ischemic stroke. However, the disadvantage of these treatments is that reperfusion leads to the production of highly deleterious ROS, generating oxidative stress, which is responsible for most ischemia-reperfusion injuries, thus leading to brain tissue damage (Chen H et al. 2020; Orellana-Urzúa et al. 2020). In this study, the levels of ROS and MDA increased and SOD activity decreased in PC12 cells after OGD/R. When miR-497-5p was knocked down, OGD/R-induced cell apoptosis, ROS and MDA decreased, but SOD increased. The above results show that the miR-497-5p inhibitor protected PC12 cells from oxidative stress damage.

BDNF is a neuroprotective secreted protein that is associated with the survival and growth of neuronal cells (Moraes et al. 2019; Chen Q et al. 2020; Zhang et al. 2020; Wang et al. 2021). It can bind to TrkB extracellularly to activate the PI3K/Akt signaling pathway to prevent neuronal cell damage and death (Ko et al. 2018). In this study, BDNF was found to be the target gene of miR-497-5p through bioinformatics prediction. Further experiments found that miR-497-5p could negatively regulate BDNF. This study elucidated the role of BDNF in OGD/R-induced injury by simultaneously knocking down BDNF and miR-497-5p. The results showed that the miR-497-5p inhibitor promoted cell viability and SOD but inhibited the levels of ROS and MDA. The knockdown of BDNF reversed the above results. The above results show that BDNF protects PC12 cells from oxidative stress damage, which is consistent with previous studies (Wu et al. 2020; Zhao et al. 2021).

In recent years, studies have found that the TrkB/PI3K/Akt signaling pathway can regulate neuronal cell injury. For example, 3,3'-diindolylmethane, beet ethanol extract and mulberry fruit extract relieve oxidative stress-mediated neuronal cell injury by activating the TrkB/PI3K/Akt pathway (Lee et al. 2019; Jeong et al. 2021; Shin et al. 2021). Manganese-dependent upregulation of α-synuclein (α-Syn) expression inhibits the TrkB/Akt signaling pathway to promote neuronal cell injury (Ma et al. 2020). In this study, BDNF and the phosphorylation levels of TrkB, PI3K and Akt

in the OGD/R group were significantly lower than those in the NC group, while BDNF and the phosphorylation levels of TrkB, PI3K and Akt were significantly enhanced after the addition of the miR-497-5p inhibitor. Compared with those in the OGD/R+miR-497-5p inhibitor group, the expression of BDNF and the phosphorylation levels of TrkB, PI3K and Akt were inhibited after knocking down BDNF. Li C et al. (2021) reported that baicalin attenuates OGD/R-induced injury by modulating the BDNF-TrkB/PI3K/Akt and MAPK/Erk1/2 signaling axes in neuron-astrocyte cocultures. In addition, some antioxidant signaling pathways also attenuate hippocampal neuron injury. For example, HO-1 attenuates hippocampal neuron injury *via* the activation of the BDNF-TrkB-PI3K/Akt signaling pathway in stroke (Qi et al. 2014). In this study, miR-497-5p promoted neuronal injury in experimental model of ischemic stroke by inhibiting the BDNF/TrkB/PI3K/Akt pathway.

Conclusion

miR-497-5p expression is significantly upregulated in neuronal cell injury. miR-497-5p targets the negative regulation of BDNF and regulates neuronal cell injury in experimental model of ischemic stroke through the BDNF/TrkB/PI3K/Akt signaling pathway, which provides a more theoretical basis for the study of ischemic stroke.

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Data availability statement. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest. The authors have no conflicts of interest to declare.

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