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MiR-124 affects the apoptosis of brain vascular endothelial cells and ROS production through regulating PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: The apoptosis of vascular endothelial cells (VEC) is related to ischemic stroke. Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB) signaling pathway can upregulate Bcl-2 expression, reduce reactive oxygen species (ROS) production, and induce apoptosis. The level of miR-124 was significantly increased after cerebral ischemia. This study aimed to investigate the role of miR-124 in regulating PI3K expression, brain VEC apoptosis, and ROS production.

MATERIALS AND MATERIALS: The expressions of miR-124, PI3K, p-AKT, and Bcl-2 in brain VEC of rats from the sham group and middle cerebral artery occlusion (MCAO) group were detected. Bioinformatics analysis showed the complementary binding site between miR-124 and PI3K mRNA. ROS content and cell apoptosis were detected by flow cytometry. Rat brain VEC were cultured in vitro and treated by oxygen-glucose deprivation (OGD) for 6 h. VEC were divided into four groups, including miR-NC, miR-124 inhibitor, pIRES2-blank, and pIRES2-PI3K, and were further treated by OGD.

RESULTS: MiR-124 expression, ROS content, and cell apoptosis were markedly increased, whereas the levels of PI3K, p-AKT, and Bcl-2 were markedly reduced in rat VECs from MCAO group compared with those in the sham group. OGD treatment significantly induced VECs apoptosis, upregulated miR-124 expression and ROS content, and down-regulated the levels of PI3K, p-AKT, and Bcl-2. MiR-124 inhibitor or transfection of pIRES2-PI3K plasmid apparently enhanced PI3K, p-AKT, and Bcl-2 expressions, alleviated apoptosis and decreased ROS content in VEC induced by OGD.

CONCLUSIONS: Our data demonstrated that miR-124 induced the apoptosis of brain vascular endothelial cells via the down-regulation of PI3K/AKT signaling pathway and promotion of ROS production.

Key Words:
Ischemic stroke, MiR-124, PI3K/AKT, Brain vascular endothelial cell.

Introduction

Cerebral disease (CVD) is a type of important disease that presents the major cause of disability and death. Ischemic CVD (ICVD) represents common cerebrovascular disease accounting for 70-80% of stroke. It is featured as high morbidity, mortality, and disability rates, resulting in heavy burden to family and society[1,2]. Vascular endothelial cells (VECs) play a crucial role in regulating vascular inflammation, maintaining homeostasis of the vascular system and the balance between coagulation and fibrolytic system[3,4]. Ischemic stroke can induce VEC injury, which aggravates the brain damage. Therefore, it is closely related to the occurrence and development of ischemic stroke[5-7]. Reactive oxygen species (ROS), as an important oxidative stress molecule, participates in promoting the occurrence of ischemic stroke through the mediation of VECs damage[8,9]. Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB) signaling pathway is involved in multiple biological processes, such as cell proliferation, survival, and apoptosis[10]. Several studies[11-13] showed that PI3K/AKT signaling pathway positively affected B-cell lymphoma-2 (Bcl-2) expression, alleviated oxidative damage and cell apoptosis. MiRNA is a type of endogenous single-stranded noncoding RNA at the length of 22-25 nt from the eukaryote. It exerts to the degrading or inhibiting function by binding with the 3'-UTR of targeted RNA[14]. MiRNA is involved in regulating cell proliferation, survival, apoptosis, oxidative stress, and ROS production. Thus, it is related to atherosclerosis[15], coronary heart disease[16], stroke[17], and cancer[18,19]. Accumulative evidence revealed that the level of miR-124 was significantly elevated after ischemic brain injury[20-22]. This study aimed to investigate the mechanism of miR-124 in regulating the proliferation of brain vascular endothelial cells.

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Materials and Methods

Main Reagents and Materials

Healthy male SD rats at 6-8 weeks old and weighted 220-250 g were purchased from Selleck (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) medium, Fetal bovine serum (FBS), and collagen II were bought from Gibco (Waltham, MA, USA). RNA extraction kit was obtained from Roche (Indianapolis, IN, USA). Rabbit anti-rat PI3K antibody was derived from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-rat Bcl-2, AKT, p-AKT, and β-actin antibodies were got from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP) conjugated secondary antibody was provided by Jackson ImmunoResearch (West Grove, PA, USA). Earle's balanced salt solution was prepared by HAICR (Nanjing, China). Glucose was purchased from Sigma (Temecula, CA, USA). The pGRE-luc luciferase reporter plasmid RIPA, cell apoptosis detection kit, and ROS detection kit were bought from Beyotime (Beijing, China). Lipofectamine 2000 was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Q... Tect SYBR Green RT-PCR Kit was got fr... gen (Venlo, Netherlands). Luciferase activi... tection kit was provided by Promega (Fitch... WI, USA). MiR-NC and miR-124 inhibitor w... derived from Ribobio (Guangz... angdon... China). pIRES2 overexpressi... as... was pur... chased from Youbio (Chan..., Huna... China).

Rats were used for a... perim... ll procedures were app... d ... mal ... s Committee of the t... hospita... amen.

Rat Middle C... Artery Occ... on (MCAO) Mo... Esta... ment

The ra... s anesthet... with 10% chloral hydrat... d fixed in supine ... e neck was incised ... er ste... ation to expose right common ... otid ... y, external carotid artery, and inter... d arte... he artery clamp was ... to c... e bif... te of internal carotid and ... al ca... small incision was made usi... ophthal... scissors on the internal carotid ... the intersection. The MCAO wire ... through the incision into the internal carotid for about 18 mm, suggesting the ... caused right MCAO. After the obligate w... was tightened and the redundant thread was cut off, the incision was sutured and the rat was treated with antibiotics. The rat in Sham group was treated by carotid artery exposed without MCAO wire. The rat was sacrificed after three days and brain tissue and blood vessel were collected. The intima was separated and the cells were digested by trypsin.

Neurologic impairment was scored ... Score 0, normal activity without n... logic im... pairment presentation; score 1, ... lly stretch in left forepaw; score 2, turning ... ft when walking; score 4, no sponta... us wa... loss of consciousness. The rat... score 1-3 a... deling were enrolled in t... study. T... e rats i... re 0 or 4, dyspnea, s... chnoi... morrhage, or death after modeli... w... d.

The Isolatio... d Cultiva... Cerebrov... Endothelia... l

The ra... s ki... d soaked in 75% ethanol. The cranial cavity w... ened for the collection of c... ortex tissue. ... washed by PBS for t..., the brain tissue was ... ut into pieces and di... ed by 0.1% co... gen II at 37°C for 1.5 h. After ... ifuged at 2... for 5 min, the cell sediment w... eated by ... glucose solution. Next, the mi... was ... ifuged at 4°C and 4000 rpm for 20 ... en the cell sediment was treated by 0.1% collagen II/dispase at 37°C for 1 h and ... ged at 1000 rpm for 5 min. At last, the cell ... was suspended in DMEM complete medium containing 15% FBS, 1% L-glutamic acid, and 1% penicillin-streptomycin. The cells were passed at 1:4 and were used for an experiment on the 3rd generation.

The Establishment of VEC Oxygen-glucose Deprivation (OGD) Model

VECs from the sham group were seeded in a six-well plate and washed by Earle's balanced salt solution twice. Then the VECs were cultured in glucose-free balanced salt solution at 95% N_2, 5% CO_2, and 37°C for 6 h to construct hypoxia. VECs in logarithmic phase were divided into four groups, including miR-NC, miR-124 inhibitor, pIRES2-blank, and pIRES2-PI3K groups. The cells were treated by abovementioned hypoxia at 72h after transfection.

Flow Cytometry

The cells were digested by trypsin and resuspended in 500 μL Annexin V Binding Buffer. Next, the cells were incubated in 5 μl Annexin V-FITC and 5 μl PI in the dark at room temperature for 15 min. At last, the cells were tested on Beckman Coulter CytoFLEX flow cytometry to evaluate cell apoptosis.

ROS Detection

The cells were digested and incubated in 0.1% DCFH-DA probe diluted by the serum-free medium at 37°C for 20 min. After being washed for three times, the cells were tested on Beckman Coulter CytoFLEX flow cytometry.

Dual Luciferase Activity Detection

The PCR products containing the full length of PI3K gene 3'-UTR or mutant segment were cloned to pGRE-luc. Next, it was transformed to DH5α competent cells and sequenced to select the plasmid with the correct sequence. Then pGRE-PI3K-3'-UTR-wt (or pGRE-PI3K-3'-UTR-mut) was co-transfected to HEK293T cells using Lipofectamine 2000 together with miR-124 inhibitor or miR-NC. The luciferase activity was detected using the Passive Lysis Buffer and Stop&Glo solution after being cultured for 48 h.

qRT-PCR

Total RNA was extracted using RNA Isolation Reagent and adopted for PCR reaction by QuantiTect SYBR Green RT-PCR Kit. The reaction system contained 2 μg template RNA, 1.0 μL primers at 0.5 μm/L, 10 μL 2× QuantiTect SYBR Green RT-PCR Master Mix, 0.5 μL QuantiTect RT Mix, and ddH_2O. The PCR reaction was composed of 50°C for 5 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Real-time PCR was performed on Bio-Rad CFX96 to test the relative expression.

Western Blot

Total protein was extracted by RIPA from cells. A total of 40 μg protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. Next, the membrane was blocked by 5% skim milk at room temperature for 60 min and incubated in primary antibodies overnight (PI3K, AKT, p-AKT, Bcl-2, and β-actin, 1:4000, 1:2000, 1:1000, 1:1000, and 1:1000, respectively). Then the membrane was incubated with horseradish peroxidase (HRP)-labeled secondary antibody (1:30000) for 60 min, being washed by PBST for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL).

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation and compared by *t*-test. $p < 0.05$ was considered as statistical significance.

Results

MiR-124 Inhibited PI3K Expre

The *in silico* analysis with MicroRNA online prediction showed the targeted binding site between miR-124 and the 3'-UTR of PI3K mRNA (Figure 1A). Dual luciferase assay revealed that the relative luciferase activity in HEK293T cells was significantly reduced by the transfection of miR-124 mimic, while was recovered after the treatment of miR-124 inhibitor, indicating the regulatory relationship between miR-124 and PI3K mRNA (Figure 1B).

Vascular Intima ROS Production and Cell Apoptosis were Enhanced in Vascular Intima from MetS Rat

Then, detected the ROS content and cell proliferation by flow cytometry assay revealed that ROS content was significantly increased in

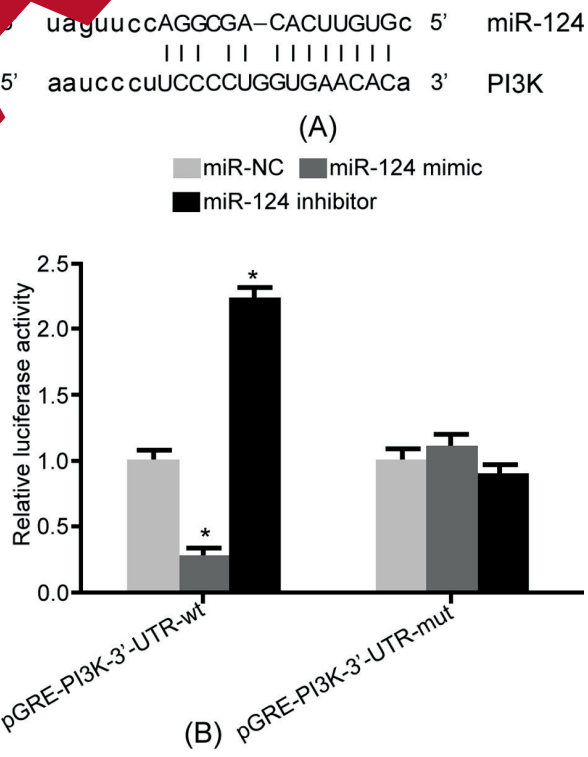

Figure 1. MiR-124 inhibited PI3K expression. ***(A)*** The binding site between miR-124 the 3'-UTR of PI3K mRNA; ***(B)*** Dual luciferase assay. *$p < 0.05$, compared with mimic NC.

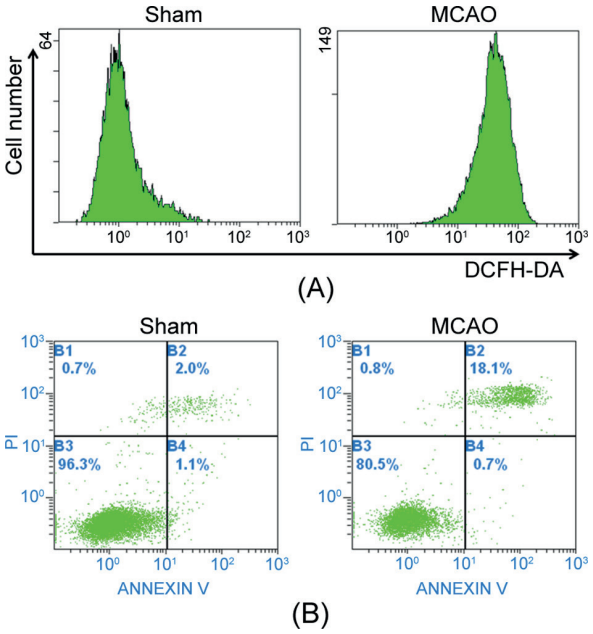

Figure 2. Vascular intima ROS production and cell apoptosis were enhanced in vascular intima from MCAO rat. ***(A)*** Flow cytometry detection on ROS content; ***(B)*** flow cytometry detection on cell apoptosis.

vascular intima from MACO group co with that in sham group (Figure 2A). Mor r, cell apoptosis was aggravated in MCAO g compared with that in sham group (Figure 2B).

MiR-124 level was Upre ted while PI3K Expression was I ited i Vascular Intima from O R

The qRT-PCR de nst t miR 4 expression was ma ly upre ed, whereas the levels of PI3K and Bcl-2 mRNA were apparently reduced in vascular intima from MCAO rat compared with that in sham group (Figure 3A). The result of Western blot exhibit that the expressions of PI3K, p-AKT, and tein were significantly alleviated vascular intima from MCAO rat compar th that in sham group (Figure 3B).

OGD Induced VECs A osis, Pro ROS Production, an evate miR-12 Expression

According to th low cy etry assay, we found t RO tent (F e 4A) and cell apo s (Figure markedly enhanced i from OGD compared with that ormal trol. The qRT-PCR result revealed that miR-12 ression was increased, whe levels of PI d Bcl-2 mRNA were d ed in VECs from OGD group compared that in norm control (Figure 4C). Western further valid d that the levels of PI3K, p-AK and Bcl-2 p in were markedly reduced in VE group compared with that in norma (Figure 4D).

regulation of miR-124 or ement of PI3K Attenuated the Apoptosis of VECs Induced by OGD

After the treatment of miR-124 inhibitor or pIRES2-PI3K transfection, the expressions of PI3K, p-AKT, and Bcl-2 were apparently enhanced (Figure 5A and B), along with the decrease of cell apoptosis (Figure 5C) and ROS content (Figure 5D) in VECs induced by OGD.

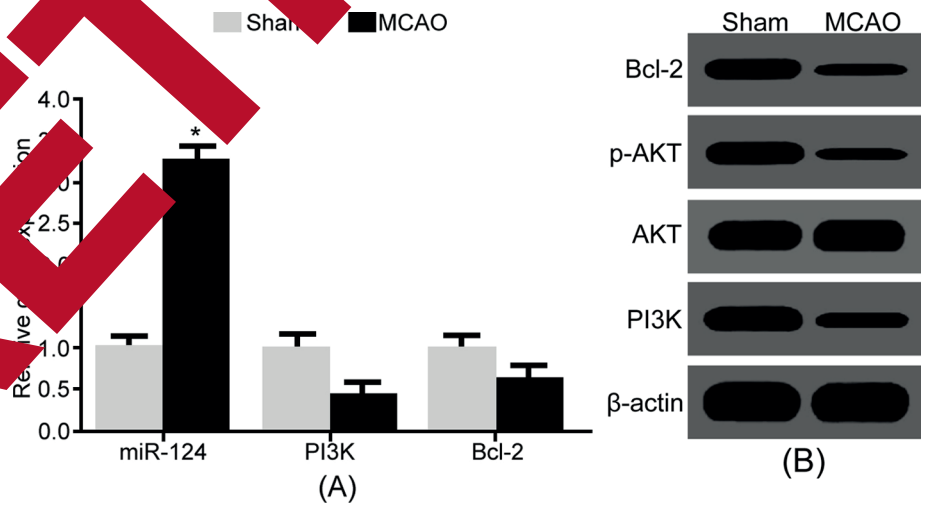

Figure 3. MiR-124 level was upregulated, while PI3K expression was reduced in vascular intima from MCAO rat. ***(A)*** qRT-PCR detection of gene expression; ***(B)*** Western blot detection of protein expression. *$p < 0.05$, compared with sham group.

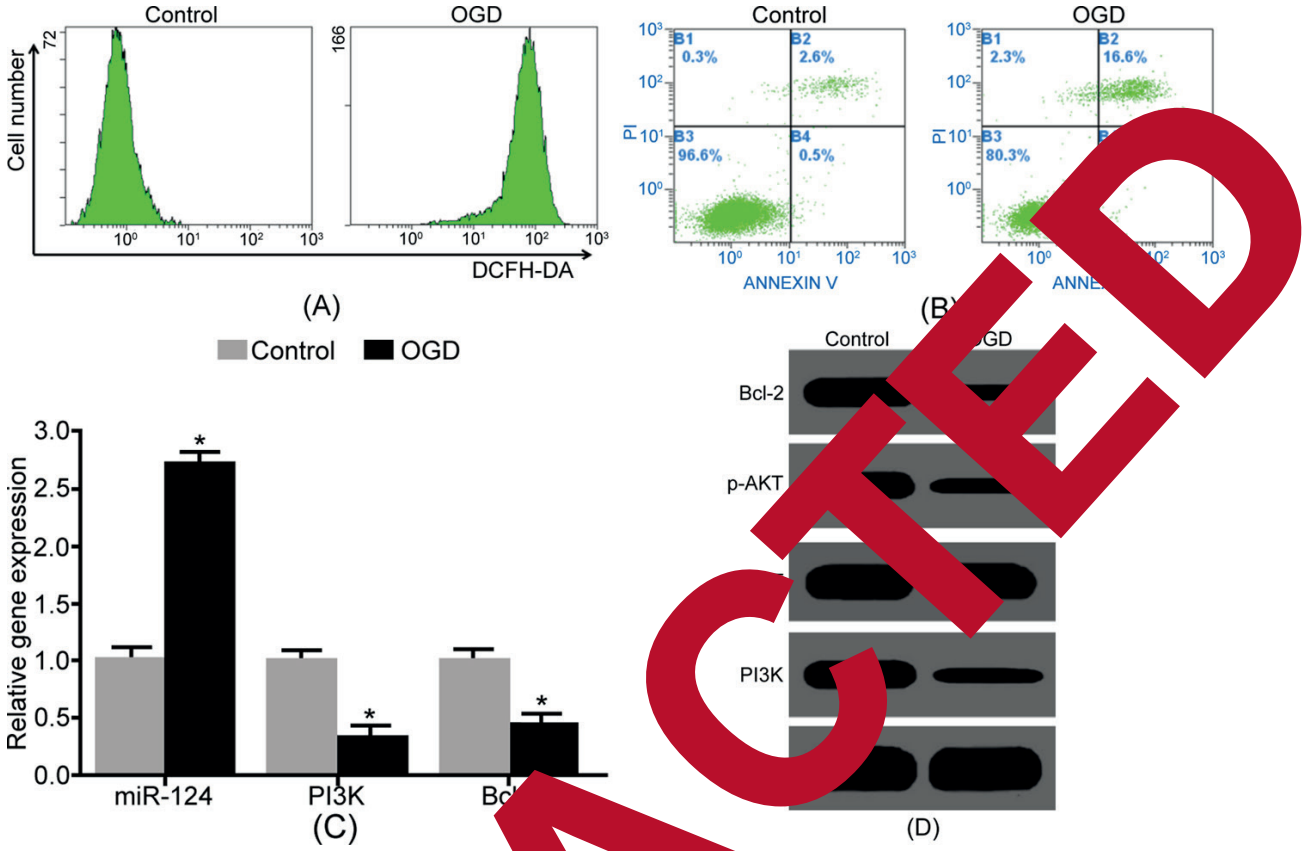

Figure 4. OGD induced VECs apoptosis, promoted ... prod... evated miR-124 expression. ***(A)*** Flow cytometry detection on ROS content; ***(B)*** flow cytometry detection ... optosis; ***(C)*** qRT-PCR detection; ***(D)*** Western blot detection. *$p < 0.05$, compared with control.

Discu... n

VECs provide ... echanic... d functional barrier between ... d and peri... l tissue[23]. Besides, VEC... critical fun... ns in the regulation of b... od ves... agulation, and fibrinolysis, th... gh the secre... f various kinds of endoth... cell active substa... . VECs can produce ... S, and ... could be damaged by ROS. Th... liatio... ROS o... VECs injury thus plays a key ... e occu... ce and development of ...emia ... e, hy... ension, and atherosclero-... More... 2 is an important anti-apoptot... ctor th... nhibits Cyt C release, influence ... ionic transmembrane transport, and ... e activation of apoptotic protease ...tivating factor-1 (Apaf-1)[24]. Of note, Bcl-2 also ...ributed to a crucial role in restraining ROS p... tion[25]. Several studies[11-13] demonstrated that Bcl-2 is one of the important target genes of PI3K/AKT signaling pathway. PI3K/AKT signaling pathway plays its role in antioxidation, antagonizing cell apoptosis, and promoting cell survival through upregulating Bcl-2 expression. This study showed that PI3K, p-AKT, and Bcl-2 levels were markedly reduced in rat VECs from MCAO group compared with sham group, which was consistent with the previous study.

Some studies[20-22] showed that miR-124 expression was significantly increased after ischemia stroke. Leung et al[26] revealed that miR-124 expression was markedly upregulated in peripheral blood from ischemia stroke patients compared with healthy control, which was correlated with the increase of NIHSS scoring. Liu et al[27] demonstrated that miR-124 level was abnormally increased in ischemia brain tissue from MCAO mouse. MiR-124 exhibited good diagnostic value to ischemia stroke with the areas under the curve (AUC) at 0.6976[20]. Consistently, we also found that miR-124 expression, ROS content, and cell apoptosis were markedly increased in rat VECs from MCAO group compared with sham group. To further investigate the potential mechanism of miR-124 on VECs, bioinformatics analysis

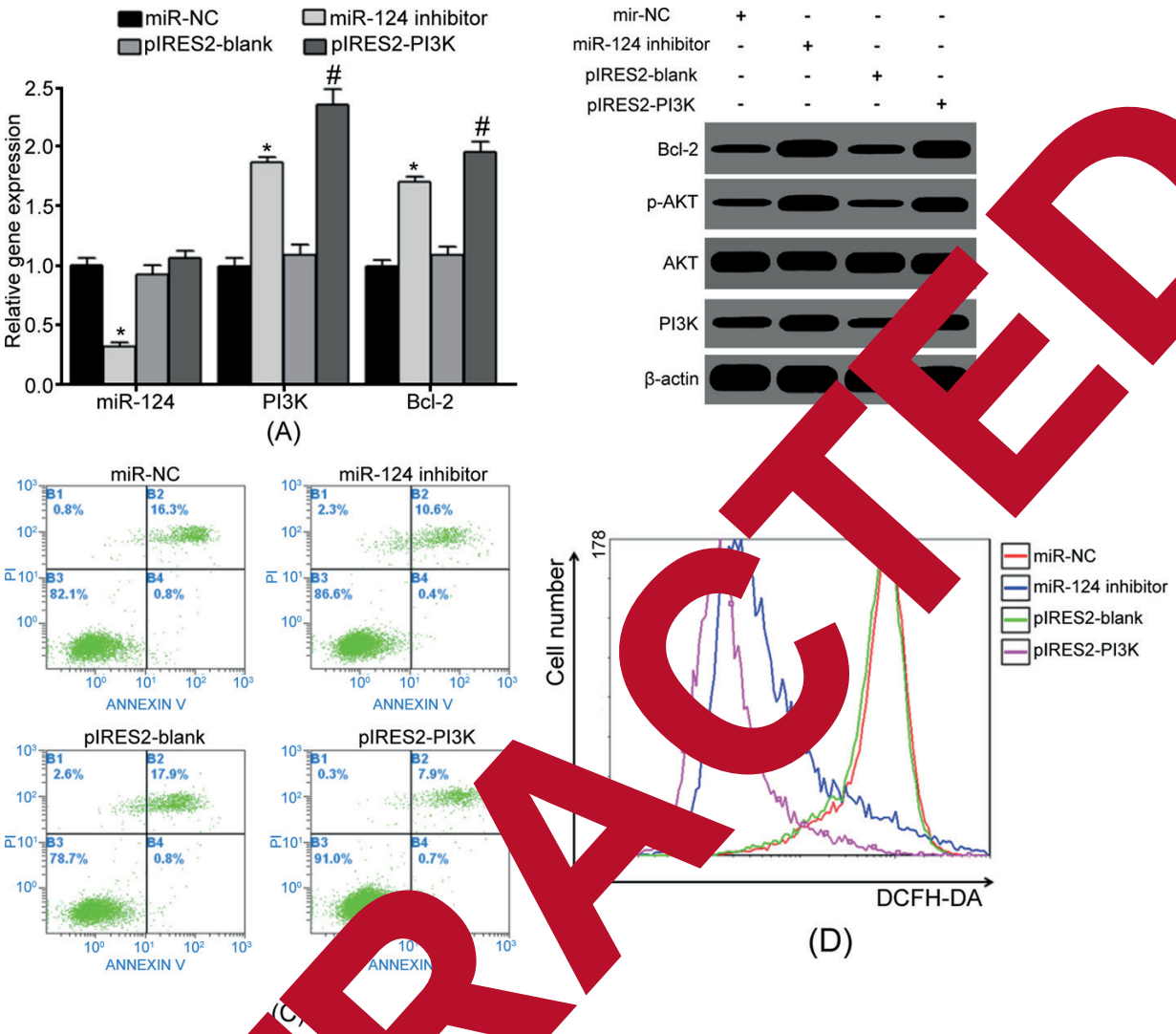

Figure 5. Downregu[illegible] of miR-12[illegible]hancement of PI3K attenuated VECs apoptosis induced by OGD. ***(A)*** qRT-PCR detection; (B) West[illegible]detection; ***(C)*** [illegible]ytometry detection on cell apoptosis; ***(D)*** Flow cytometry detection on ROS content. [a]$p < 0.05$ [illegible]p[illegible]th miR-NC; [b]p [illegible].05, compared with pIRES2-blank.

was [illegible]ormed[illegible] showed the complementary bi[illegible]site l[illegible]een miR-124 and PI3K mRNA. Dual [illegible] assay[illegible]well revealed the regu-[illegible]y re[illegible]ship[illegible]ween miR-124 and PI3K [illegible], sug[illegible]hat miR-124 might mediate the [illegible]liferati[illegible] VECs via the regulation of PI[illegible]KT signaling pathway.

[illegible]exhibited that OGD treatment mar-[illegible]dly increased miR-124 level and apoptosis of [illegible]e cell, suggesting that the elevation of miR-12[illegible]der hypoxia is related to cell apoptosis, which was in accordance with our results. Zhang et al[28] revealed that ischemia treatment apparently inhibited PI3K and AKT functions in myocardium. Sayed et al[29] found that ischemia weakened PI3K and AKT functional activity in myocardial cells. Similar to previous data, our study also observed that the activity reduction of PI3K/AKT signaling pathway was related to ischemia hypoxia-mediated injury. Our further investigation showed that the treatment of miR-124 inhibitor or pIRES2-PI3K transfection apparently enhanced PI3K, p-AKT, and Bcl-2 expressions, along with the decrease of cell apoptosis and ROS content in VECs induced by OGD. Similarly, Ke et al[30] found that the upregulation of PI3K/AKT signaling pathway markedly decreased ROS production and cell apoptosis under ischemia-hypoxia

condition. Xu et al[31] observed that the activation of PI3K/AKT signaling pathway enhanced Bcl-2 expression and weakened cell apoptosis induced by ischemia-hypoxia. All of these studies suggested that reducing miR-124 or upregulating PI3K/AKT signaling pathway play a role in antagonizing cell apoptosis induced by hypoxia, which also supported our results. However, in our study, the regulatory mechanism of miR-124 in model rat has not been fully elucidated, which needs further investigation.

Conclusions

MiR-124 induces the apoptosis of VECs via attenuating PI3K/AKT signaling pathway and promoting ROS production, which provides academic basis for the therapy of hypoxia in the future.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of intere[illegible]

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