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MiRNA-210 induces the apoptosis of neuronal cells of rats with cerebral ischemia through activating HIF-1α-VEGF pathway

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Abstract. – OBJECTIVE: This study aims to explore the expression of micro-ribonucleic acid (miRNA)-210 in the cerebral cortex of rat model with global cerebral ischemia, and determine its function on the regulation of the apoptosis of neuronal cells.

MATERIALS AND METHODS: Rat models of global cerebral ischemia were established *in vitro.* Rats were euthanized at 24 h after reperfusion and the cerebral cortex was collected. The expression of miRNA-210 was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Neuronal cells were transfected by liposomes *in vitro* and cells were divided into neuronal cell group (group i), neuronal cell + miRNA-210 mimic group (group ii) and neuronal cell + miRNA-210 inhibitor group (group iii). The cell apoptosis rate and gene and protein expressions of HIF-1α, VEGF and Caspase-3 were measured.

RESULTS: The level of miRNA-210 in rats with global cerebral ischemia was remarkably higher than that in rats from sham operation group ($p<0.05$). The apoptosis rate of neuronal cells was increased evidently when miRNA-210 was overexpressed, and the expressions of HIF-1α, VEGF and Caspase-3 were elevated markedly at both mRNA and protein levels.

CONCLUSIONS: Our data indicate that miRNA-210 expression is upregulated in the rats with global cerebral ischemia, and the rise of miRNA-210 level increases the apoptosis of neuronal cells through the activation of HIF-1α-VEGF signaling pathway.

Key Words:
Global cerebral ischemia, miRNA-210, HIF-1α, VEGF, Cell apoptosis.

Introduction

Global cerebral ischemia injury often causes disability of the patients, of which, the major factors include delayed neuronal death. The tissue injured by ischemia is not effectively recovered after the blood flow is restored, further aggravating the damage[1]. Currently, the apoptosis of neuronal cells is regarded as the main way of neuronal damage caused by cerebral ischemia-reperfusion injury[2]. Micro-ribonucleic acid (miRNA) is a type of small non-coding RNA molecule with a length of less than 22 nucleotides. It has been reported that miRNA suppresses protein translation or degrade messenger RNA (mRNA) of target genes by specific binding of target genes. It, therefore, exerts regulatory effects on cell proliferation, differentiation and apoptosis[3,4]. The miRNA-210 is expressed in a variety of hypoxic tissues and cells, and it is regulated by hypoxia-inducible factor-1α (HIF-1α) in hypoxic environments[5]. It has been indicated that, through the results of microarray expression profiles, miRNA-210 was proposed to be involved in angiogenesis after the ischemia-reperfusion injury by means of vascular endothelial growth factor (VEGF) signaling pathway[6]. In this research, we focus to evaluate the miRNA-210 expression by establishing cerebral ischemia-reperfusion model and investigate the role of miR-210 in the apoptosis of neuronal cells.

Materials and Methods

Laboratory Animals and Reagents

Healthy Sprague-Dawley (SD) rats weighing 250-280 g were bought from Nanjing Better Biotechnology Co., Ltd. (No. J001, Nanjing, Jiangsu, China). HIF-1α monoclonal antibody was purchased from Shanghai LMAI Biotechnology Co., Ltd. (Shanghai, China). Secondary antibody

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was obtained from Shanghai Yu Bo Biological Technology Co., Ltd. (Shanghai, China). BCA protein assay kit was provided by Shanghai Y-J Biological Technology Co., Ltd. (Shanghai, China). Protein extraction kit was from Bestbio (Beijing, China). TRIzol reagent kit for total RNA extraction was from Shanghai Kang Lang Technology Co., Ltd. (Shanghai, China). SYBR Green quantitative polymerase chain reaction (qPCR) kit was offered by BioTeke Corporation (Beijing, China). Annexin V-fluorescein isothiocyanate (FITC) apoptosis assay kit was acquired from BD (San Jose, CA, USA) and Lipo 2000 liposomal transfection kit was from Invitrogen (Carlsbad, CA, USA). This research was approved by Lanzhou University Second Hospital Ethics Committee.

Grouping of Laboratory Animals

A total of 40 healthy male SD rats were equally randomly divided into sham operation group (group A) and global cerebral ischemia group (group B). The SD rats in both groups were sacrificed by euthanasia at 24 h after ischemia-reperfusion.

Preparation of Animal Models

Animal models were prepared according to the methods reported by Xing et al[7]. The rats were fasted for 24 h before operation, whose skin along the cervical posterior midline was opened after intraperitoneal anesthesia, and the fascia and muscle were bluntly dissected layer by layer until the visual field was exposed. A medical high-frequency bipolar electrocoagulation needle was inserted deeply into the vertebral artery until the bone around the pterygoid pore turned white, and local tissues and skin were disinfected after the vertebral artery was closed permanently. 24 h later, the abdominal cavity was anesthetized and disinfected, and then the fascia, common carotid artery and vagus nerve were bluntly dissected. After that, the rats were connected to a multi-channel electrophysiological system, and the common carotid artery was clipped using a noninvasive micro-artery clamp. Next, the artery was released 10 min later, which was regarded as the start of ischemia-reperfusion. In this research, if fluctuations approaching the baseline or straight line were displayed on the rat's electroencephalogram, or if coma, bilateral mydriasis and absent corneal reflex occurred within 1 min after clipping, the models of global cerebral ischemia were established successfully. The rats in group A were only subjected to electric coagulation of the vertebral artery, and the common carotid artery was dissected and exposed but not clipped.

Detection of miRNA-210 Expression Level in Cerebral Cortex via Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

The RNA content at A260/A280 was quantified using quantitative spectrophotometry in strict accordance with the instructions of total RNA extraction kit. The sample total RNA was reversely transcribed according to the instructions of miRNA RT kit, and the primers of miRNA-210 were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd (Shanghai, China).

RT system: 5×RT buffer solution (10 μL), 2.5 mM deoxyribonucleoside triphosphate (dNTP) Mix (4 μL), 40 U/μL RNase inbibitor (1 μL), 200 U/μL Moloney murine leukemia virus (M-MLV) reverse transcriptase (1 μL) and RNase-free pure water adding the system to 50 μL. Reaction conditions: at 42°C for 60 min and 70°C for 10 min. The total RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA), which was stored at -20°C after the reaction.

Real-time quantitative PCR system (15 μL): RT product (4 μL), 1× SYBR Green I Master Mix (25 μL), 0.5 μM miRNA-specific forward primer (2 μL), 0.5 μM universal reverse primer (2 μL), rhodamine-X (ROX) (1 μL) and RNase-free pure water (16 μL), with a total volume of 50 μL. Reaction conditions: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, 40 cycles in total. U6 was taken as the internal reference, and ABI7500 qPCR software was applied to analyze the relative copy number of target gene and internal reference gene: RQ (Relative Quantity) $=2^{-\Delta\Delta Ct}$.

Transfection of Neuronal Cells

Transfection was performed as per the operating instructions in the Lipo 2000 transfection kit. The overexpression and suppressed expression vectors of miRNA-210 were constructed *in vitro*, which were divided into 3 groups, namely, neuronal cell group (group i), neuronal cell + miRNA-210 mimic group (group ii) and neuronal cell + miRNA-210 inhibitor group (group iii).

Detection of Neuronal Cell Apoptosis via Flow Cytometry

After transfection for 24 h, 3 groups of neuronal cells were collected and pipetted to a density of 1×10^6/mL. Next, 100 μL cell solution was taken into a flow cytometry tube using a pipette, and the cell apoptosis was determined according to the operating instructions in the Annexin V-FITC apoptosis assay kit.

Detection of mRNA Expressions of HIF-1α, VEGF and Caspase-3 in Neuronal Cells via RT-qPCR

The RNA content at A260/A280 was quantified by virtue of the quantitative spectrophotometry in strict accordance with the instructions of total RNA extraction kit. The total RNA in the cells was reversely transcribed in the light of the instructions of RT kit.

Forward primer of HIF-1α: 5'-ACCACAGGATTGATGCCCAAG-3'

Reverse primer: 5'-CGATGACACGGAAACTGAAG-3'

Forward primer of VEGF: 5'-CAGAGGCAGGTAATGGAGACA-3'

Reverse primer: 5'-TTTCTCCGCTCTGAACAAGG-3'

Forward primer of Caspase-3: 5'-ACGGGACTTGGAAAGCATC-3'

Reverse primer: 5'-TAAGGAAGCCTGGAGCACAG-3'

RT system: reverse transcriptase Mix (1 μL), RT primer (1 μL), dNTP Mix (4 μL) and RNase-free pure water (4 μL). Reaction conditions: at 35°C for 15 min and 85°C for 5 s, and cooling to 4°C. The total RNA was reversely transcribed into cDNA, which was stored at -20°C after the reaction. Real-time quantitative PCR system (20 μL): SYBR Green Mix (10 μL), RT product (2 mL), 10 μM forward primer (0.4 μL), 10 μM reverse primer (0.4 μL), ROX (0.4 μL) and RNase-free pure water (6.8 μL). Reaction conditions: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s and annealing at 60°C for 31 s, 40 cycles in total. β-actin was regarded as the internal reference, and ABI7500 qPCR software was applied to analyze the relative copy number of target gene and internal reference gene: RQ (Relative Quantity) $=2^{-\Delta\Delta Ct}$.

Detection of Protein Expressions of HIF-1α, VEGF and Caspase-3 via Western Blotting

The total protein in cells was extracted according to the instructions of total protein extraction kit, the concentration of the extracted protein was measured, and the protein was preserved at -70°C for standby use. Gel in different concentrations was prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting was performed for 35 min after polyvinylidene difluoride (PVDF) membrane transfer at the gel positions of the two types of protein selected according to the marker bands, followed by sealing in 5% skim milk powder at 37°C for 90 min, incubation with primary antibody at 4°C overnight, addition of Tris-buffered saline and Tween 20 (TBST) and shaking and washing for three times. Then, the secondary antibody was added for incubation at 37°C for 1 h, and TBST was added and placed on the shaking table for shaking and cleaning for 15 min for 3 times, followed by color development with electrochemiluminescence (ECL) liquid in a dark room, exposure, development and fixation. Finally, the protein was scanned using ChemiDoc™ MP imaging system, and the grayscale values of the target band and internal reference band were measured and corrected by means of Image J professional image analysis software.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was adopted for data statistics and analysis. Continuous data are presented as means ± standard deviation (SD), and were analyzed by using one-way ANOVA, with the Tukey's post-hoc test. Two-tailed p-values <0.05 were considered statistically significant.

Results

Expression of miRNA-210 in Cerebral Cortex

The expression of miRNA-210 in rats from global cerebral ischemia group was statistically higher than that in sham operation group ($p<0.05$) (Figure 1).

Change in Apoptosis of Neuronal Cells

Compared with that in group i, the number of apoptotic cells after transfection with miR-210 mimic was significantly increased but was statistically reduced by using miR-210 inhibitor ($p<0.05$) (Figure 2, 3).

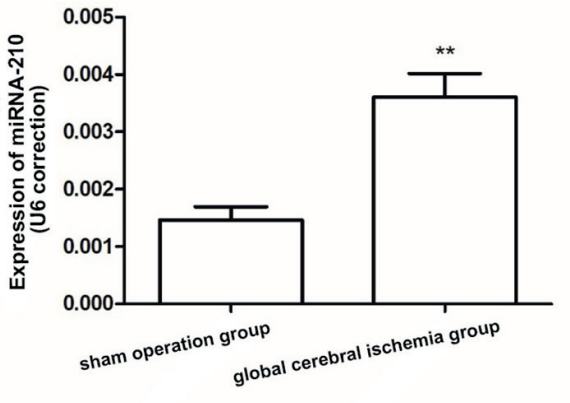

Figure 1. Expression of miRNA-210 in cerebral cortex. $^{**}p<0.01$ *vs*. sham operation group.

Gene Expressions of HIF-1α, VEGF and Caspase-3 in Cells

According to the RT-qPCR results, the over expression of miR-210 significantly increased the expressions of HIF-1α, VEGF and Caspase-3 compared to group i, whereas those levels were statistically diminished after the transfection of miR-210 inhibitor ($p<0.05$) (Figure 4).

Protein Expressions of HIF-1α, VEGF and Caspase-3

The results of Western blotting assay also indicated that expressions of HIF-1α, VEGF and Caspase-3 were remarkably higher than those in group i and group iii (Table I and Figure 5).

Table I. Protein expressions of HIF-1α, VEGF and Caspase-3 detected via Western blotting assay.

	Group i	Group ii	Group iii
HIF-1α	0.40±0.03	0.95±0.10*	0.68±0.07*
VEGF	0.31±0.03	1.06±0.11*	0.53±0.08*
Caspase-3	0.27±0.02	0.91±0.09*	0.75±0.07*

Note: *$p<0.05$ *vs*. group i

Discussion

Ischemia and hypoxia of the brain tissues appear in the process of cerebral ischemia-reperfusion injury, further resulting in a variety of complex pathological cascades, which are dynamically controlled by the expression changes of many biomolecules in the body[8]. As essential regulatory factors of gene expression in eukaryotes, miRNAs participate in multiple physiological and pathological processes in cells[9]. Some studies[10] have manifested that miRNA can serve as a biomarker for the diagnosis and treatment of neurodegenerative diseases. MiRNA-210 is ubiquitously expressed in ischemic and hypoxic tissues and cells. Nechemia-Arbely et al[11] revealed that miRNA-210 can bind to the 3'-untranslated region that regulates gene transcripts, thereby playing an efficacious regulatory role in the expression of target genes. Lee et al[12] discovered in an *in vitro* experiment on endothelial cells that miRNA-210 binds to the untranslated region in

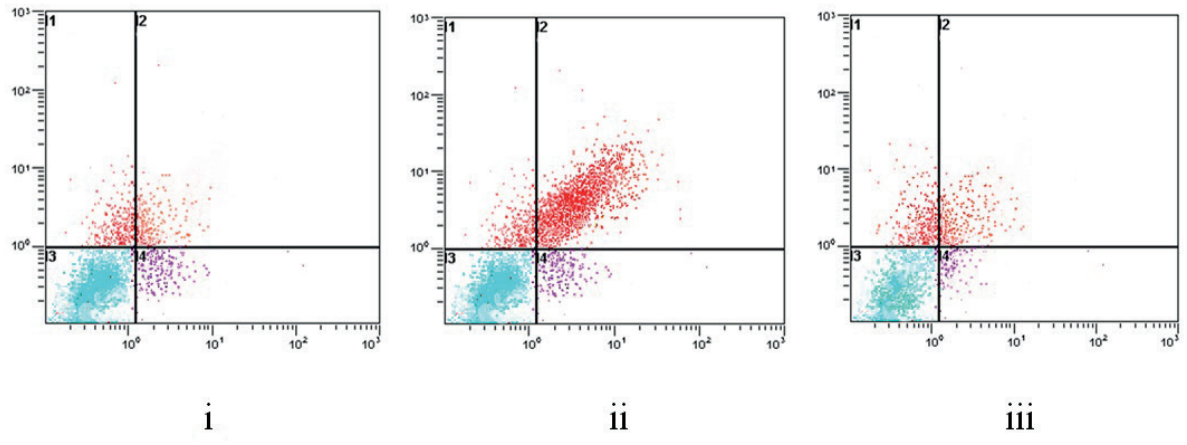

Figure 2. Profile of flow cytometry.

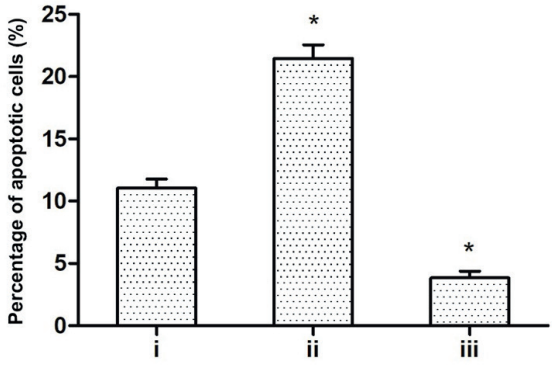

Figure 3. Percentage of apoptotic cells. $^{*}p<0.05$ *vs*. group i.

the mRNA of ephrin A3 (EFNA3) gene, so the expression product of EFNA3 gene is down-regulated markedly. The sensitivity of endothelial cells to VEGF chemotaxis is strengthened, and the formation of new blood vessels is induced. It is presumed that the overexpression of miRNA is possibly associated with the acceleration of angiogenesis in ischemia-reperfusion-related diseases.

In this research, the rat models of global cerebral ischemia-reperfusion injury were established successfully. The expression level of miRNA-210 in the tissues at 24 h after reperfusion was evidently higher than that in sham operation group, which is consistent with the findings reported by Yang et al[13]. Barteczek et al[14] also demonstrated that miRNA-210 expression is changed prominently in the rat models of cerebral ischemia and in the serum of the patients. In the *in vitro* cell research, it was revealed that the apoptosis of neuronal cells was increased markedly, and the gene and protein expression of the apoptosis-associated factor Caspase-3 was also elevated when miRNA-210 was overexpressed. Moreover, studies[15,16] have indicated that miRNA-210 is capable of regulating the protein expressions of apoptotic signaling pathway in the brain tissues of animal models of hypoxic encephalopathy and it exerts prominent inhibitory effects on the expressions of apoptosis-associated proteins such as Bax, Caspase-3 and Caspase-9. Meanwhile, it can up-regulate the expression level of anti-apoptotic protein Bcl-2, ultimately contributing to a fairly significant anti-apoptotic effect[17]. The role of growing level of miRNA-210 in ischemic brain tissues still remains unclear at present, but both HIF-1α and miRNA-210, as hypoxia-inducible factors, show close correlation at the expression level in cells under hypoxic environments according to previous *in vitro* studies[18]. The overexpression of miRNA-210 in the brain tissues was shown associated with the generation of local blood vessels and nerve cells[19]. It is also discovered in animal models of cerebral ischemia that the overexpression of miRNA-210 is related to neovascularization after cerebral ischemia[20]. According to this research, the gene and protein expression levels of HIF-1α and VEGF were elevated notably when miRNA-210 expression was upregulated, and a concordance between the expression trends was also observed. On the other hand, the down-regulation of miRNA-210 expression suppressed the gene and protein expression levels of HIF-1α and VEGF obviously. As recent finding

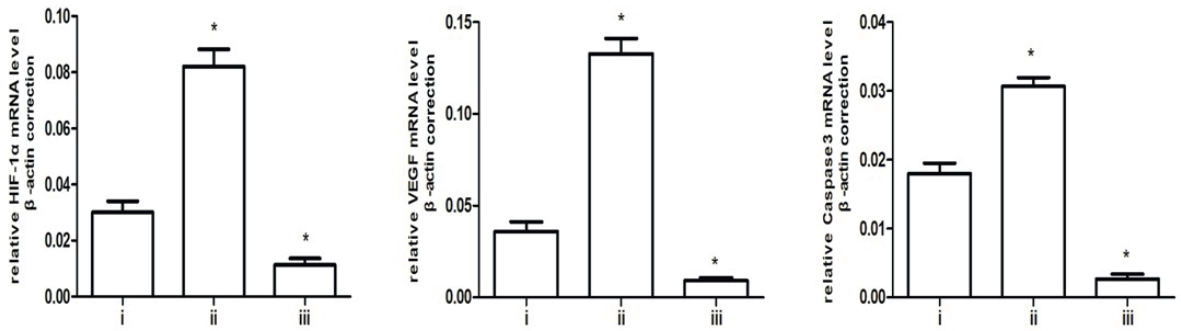

Figure 4. Gene expressions of HIF-1α, VEGF and Caspase-3 in cells. $^{*}p<0.05$ *vs*. group i.

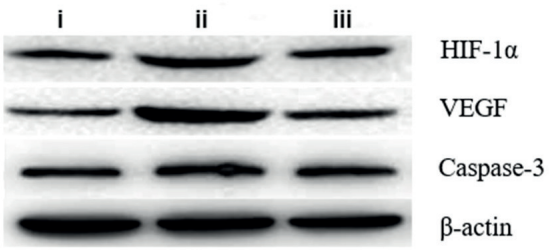

Figure 5. Western blotting images of protein expressions of HIF-1α, VEGF and Caspase-3.

indicated that 1 miR-210 played a protecting role on cardiomyocytes from OGD/R injury[21], our result provides promising leads of miR-210 in the future therapy against ischemia and hypoxia.

Conclusions

Our data demonstrated the increase of miRNA-210 expression in ischemic tissues which induces the apoptosis of neuronal cells by up regulating the HIF-1α-VEGF signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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