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MiR-210 protects cardiomyocytes from OGD/R injury by inhibiting E2F3

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Abstract. – OBJECTIVE: To detect the change in miRNA-210 expression of cardiomyocytes under hypoxia/reoxygenation status. Also, the effect of miR-210 on the apoptosis of cardiomyocytes induced by oxygen-glucose deprivation/reperfusion (OGD/R) and its mechanism through establishing the OGD/R injury model of primary cardiomyocytes in this experiment were investigated.

MATERIALS AND METHODS: The cell model of OGD/R injury was established. The cell apoptosis in each group was detected by methyl thiazolyl tetrazolium (MTT) assay and detection of Caspase-3 activity. The change in miR-210 expression in each group was detected by Real-time fluorescence quantitative polymerase chain reaction (PCR). The high-expression and low-expression miR-210 models were established through the transient transfection of miR-210 mimic and inhibitor to detect the relevant indexes of cell apoptosis. At the same time, changes in mRNA and protein expressions of E2F3 were detected by RT-PCR and Western blotting, respectively. The E2F3 overexpression vector was constructed, and the overexpression vector plasmid and miR-210 mimic were jointly transfected into the cells to detect the relevant indexes of cell apoptosis.

RESULTS: After OGD/R treatment, the activity of Caspase-3 was increased, the survival of cardiomyocytes was significantly inhibited and the expression level of miR-210 was up-regulated in OGD/R injury. Transfection of miR-210 mimic for miR-210 overexpression could alleviate the OGD/R-induced cardiomyocyte injury, while the decrease of miR-210 expression could aggravate the apoptosis of cardiomyocytes. In addition, the high expression of miR-210 could inhibit the protein expression of E2F3, and co-transfection of E2F3 plasmid and miR-210 mimic could reverse the inhibiting effect of miR-210 on the apoptosis of cardiomyocytes.

CONCLUSIONS: We confirmed that miR-210 can inhibit the OGD/R-induced apoptosis of cardiomyocytes, and miR-210, as an upstream factor, plays a protective role in cardiomyocytes through directly inhibiting the protein expression of its target gene E2F3.

Key Words:
miR-210, OGD/R, Cell apoptosis, E2F3.

Introduction

Myocardial ischemia/reperfusion injury refers to a large number of reactive oxygen species (ROS) produced in the process of reperfusion therapy under a certain time conditions after the acute insufficient blood supply due to atherosclerosis, thrombosis or coronary artery spasm, resulting in varying degrees of myocardial damage. Ischemia/reperfusion injury is a complex pathophysiological process that is regulated by a variety of factors. Under normal conditions, low-concentration ROS can regulate the function of vascular cells[1-3], but the excessive oxidative stress leads to the accumulation of ROS, causing cell damage[4] and leading to gene mutation, cell death, protein denaturation, lipid peroxidation and damage to cell membrane structure, etc., thus causing a series of physiological and biochemical disorders[5,6]. In myocardial ischemia-reperfusion injury, the oxidative stress injury will lead to various types of cell damage, including necrosis, apoptosis and autophagy[7-9], among which apoptosis has always been a hotspot in biological research. MiRNA is a kind of micro non-coding RNA sequence with 20-25 nucleotides in length. In the

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past two decades, studies on miRNA have been developed one after another, which is a hot topic in biological research field. The biological effect of miRNA is very extensive and can regulate all the aspects of cell biology, including the cell differentiation, proliferation, angiogenesis and apoptosis, etc.[10-12]; besides, cardiac and vascular physiology and pathology are also regulated by it. MiRNA-210 is considered as the most important hypoxia-associated miRNA in the body. The upregulation of miR-210, as a persistent feature, exists in the hypoxia response in normal cells or tumor cells, and regulates the occurrence and development of cardiovascular diseases from a number of aspects[13,14]. Recent reportss have shown that miRNA-210 is involved in the regulation of apoptosis through a variety of pathways and the ischemia/reperfusion injury process is often accompanied with varying degrees of oxidative stress injury[15]. In this experiment, therefore, it is hypothesized that miRNA-210 may be involved in the development and progression of apoptosis during the oxidative stress injury of cells. In this experiment, the change in miRNA-210 expression of cardiomyocytes under oxygen-glucose deprivation/reperfusion (OGD/R) was detected. Moreover, the effect of miR-210 on the OGD/R-induced apoptosis of cardiomyocytes and its mechanism were investigated through establishing the OGD/R injury model of primary cardiomyocytes.

Materials and Methods

Extraction, Culture and Transfection of Primary Cardiomyocytes

Primary cardiomyocytes were extracted according to the methods in literature, and cultured with complete medium containing 10% fetal bovine serum (FBS) and 5% horse serum in an incubator containing 5% carbon dioxide at 37°C; the culture medium was replaced every other day. When 70% cells adhered to the wall, cell transfection was performed. For transfection, cells were transfected with miRNAs or siRNAs mixed with Lipofectamine 2000 reagent according to the manufacturer's protocols.

Reagents

MiR-210 and U6 primers were purchased from Applied Biosystems (Foster City, CA, USA); miR-210 mimic and inhibitor and its control (negative control miRNA) were purchased from GenePharma (Shanghai, China); E2F3 small-interfering RNA and its control were also purchased from GenePharma (Shanghai, China); methyl thiazolyl tetrazolium (MTT) assay reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA); Caspase-3 activity assay kit was purchased from Beyotime Biotechnology Research Institute (Shanghai, China); Lipofectamine 2000 transfection reagents were purchased from Invitrogen (Carlsbad, CA, USA).

Establishment of OGD/R Injury Model

First, the hypoxia solution was balanced with mixed gas (95% N_2 and 5% CO_2) for saturated hypoxia solution; then the normal medium of cardiomyocytes was discarded and quickly replaced with the saturated hypoxia solution. The cardiomyocytes were placed in the hypoxic incubator (95% N_2 and 5% CO_2) at 37°C for incubation for 8 h, i.e. the hypoxia (ischemia) model. After hypoxia of cardiomyocytes for 8 h, the hypoxia solution was sucked with a straw and replaced with the medium containing sugar and 10% fetal bovine serum (FBS) for normal culture for 12 h, i.e. the reoxygenation (reperfusion) model.

Detection of Cell Survival Rate Via MTT Assay

The primary cardiomyocytes were inoculated onto the 96-well plate. After adherent growth for 24 h, followed by hypoxia for 8 h and reoxygenation for 12 h according to the experimental design, 10 μL MTT (5 mg/mL) were added into each well for incubation for 4 h. Next, the liquid was removed from each well. Dimethyl sulfoxide (DMSO) was added into each well (150 μL/well) and shaken for 5-10 min to fully dissolve the formazan particles. The absorbance values at 490 nm and 570 nm were measured using the microplate reader. The change in cell survival rate was analyzed by the built-in analysis software.

Total RNA Extraction and RT-PCR

Total RNA was extracted from the cells using the TRIzol kit (Gibco, Grand Island, NY, USA), and reversely transcribed into cDNA via reverse transcriptase with mRNA as the template. PCR amplification was performed using cDNA as the template to obtain the target gene or detect the gene expression. The total RNA extracted was reversely transcribed using the Taqman miRNA reverse transcription kit. U6 was used as an internal normalized reference for miR-93 expressions,

respectively. Expressions were normalized to endogenous controls and fold change gene expression was calculated as $^{2-\Delta\Delta}$Ct.

Western Blotting

The total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) protein lysate, and the protein concentration was quantified using the bicinchoninic acid (BCA) kit, followed by separation via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), polyvinylidene difluoride (PVDF) membrane transfer, sealing at room temperature using 5% skim milk for 1 h. E2F3 protein was identified by E2F3 rabbit anti-human monoclonal antibody (1:1000) and goat anti-rabbit secondary antibody (1:2000) with GAPDH as the control. After incubation with primary antibody for 4 h, the membrane was washed with tris-buffered saline-tween (TBST) for 6 min × 5 times, and after incubation with secondary antibody for 1 h, the membrane was washed again with TBST for 6 min × 5 times. Next, the water was absorbed using filter paper, followed by color development with enhanced chemiluminescence (ECL) kit.

Statistical Analysis

The statistical analysis of experimental data was performed using Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA). Measurement data were presented as mean ± standard deviation; *t*-test was used for the comparison between the two groups, while analysis of variance was used for the comparison among groups. $p < 0.05$ suggested that the difference had statistical significance, and $p < 0.01$ suggested that the difference had remarkably statistical significance.

Results

Effect of OGD/R on Cardiomyocyte Injury

In order to evaluate the expression level of miR-210 in cardiomyocytes with OGD/R injury, the RNA was extracted after the OGD of primary cardiomyocytes for 8 h and reoxygenation for 12 h; miRNA levels were measured by Real-time quantitative PCR. The results showed that OGD/R could significantly increase the expression level of miR-210 (Figure 1A). In addition, MTT assay and Caspase-3 activity assay showed that the cell apoptosis in OGD/R group was significant (Figure 1B-C).

Role of miR-210 in Hypoxia-Reoxygenation-Induced Cell Injury

In order to further study the roles of up-regulated and down-regulated miRNA-210 in the hypoxia-reoxygenation-induced cell injury, the miRNA-210 mimic and inhibitor were transfected to up-regulate and down-regulate the expression level of miRNA-210, so as to detect the severity of cell damage and the expression of its downstream genes. After the transient transfection of miRNA-210 mimic, miRNA-210 inhibitor, mimic negative control and inhibitor negative control, the changes in miRNA-210 expression were detected via qRT-PCR. The results showed that the expression of miRNA-210 in mimic group was

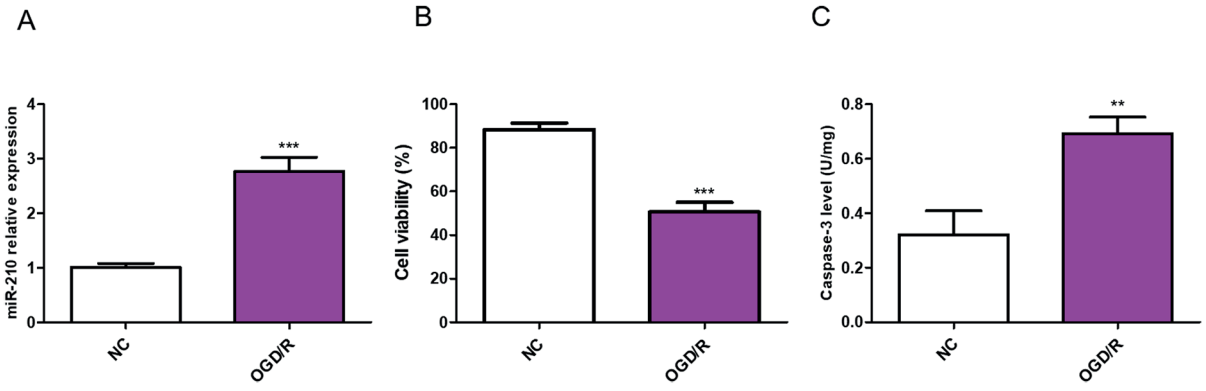

Figure 1. Up-regulation of miR-210 in OGD/R model. ***A,*** qRT-PCR shows that the expression of miR-210 is significantly up-regulated in OGD/R group (n=6). ***B,*** MTT assay shows that the cell survival rate in OGD/R group (n=6) is significantly decreased. ***C,*** Detention of Caspase-3 activity reveals that it is significantly higher in OGD/R group (n=6) than that in control group; ***$p < 0.001$, **$p < 0.01$.

significantly increased compared with that in control group ($p < 0.001$), while the expression level of miRNA-210 in inhibitor group was significantly decreased compared with that in control group; differences were statistically significant ($p < 0.001$), so the model was established successfully (Figure 2A). Furthermore, the effect of OGD/R model on survival rate of cardiomyocytes was detected via MTT assay, and the results showed that the cell survival rate in miRNA-210 mimic-transfected group was significantly increased compared with that in control group ($p < 0.01$), and the cell survival rate in miRNA-210 inhibitor-transfected group was decreased compared with that in control group ($p < 0.01$) (Figure 2B). The activity of Caspase-3 was detected by spectrophotometry, and the results revealed that the activity of Caspase-3 in miRNA-210 mimic-transfected group was decreased compared with that in control group ($p < 0.01$), and the activity in miRNA-210 inhibitor-transfected group was significantly increased compared with that in control group ($p < 0.01$) (Figure 2C).

Effect of miR-210 on E2F3 Protein Expression in Cardiomyocytes

Previous investigation showed that E2F3 is one of target genes of miR-210. In order to further study the effect of miRNA-210 on the expression of E2F3 in OGD/R-induced cardiomyocyte injury, the miRNA-210 mimic, miRNA-210 inhibitor, mimic negative control and inhibitor negative control were transiently transfected. The cells in each group were treated with OGD/R, and changes in mRNA and protein expressions of E2F3 were detected by RT-PCR and Western blotting, respectively. The results revealed that, compared with those in control group, miRNA and protein expressions of E2F3 in miRNA-210 mimic group were significantly inhibited ($p < 0.01$), but those in miRNA-210 inhibitor group were increased; differences were statistically significant ($p < 0.05$) (Figure 3).

Overexpression of E2F3 Could Reverse the Inhibitory Effect of miR-210 on Apoptosis of Cardiomyocytes

The E2F3 overexpression vector was constructed, and the overexpression vector plasmid and miR-210 mimic were jointly transfected into the cells. The total RNA was extracted for RT-PCR, and the total protein was extracted for Western blotting. The results are shown in Figure 4A-B. The expression of E2F3 in E2F3 overexpression vector and miR-210 mimic-transfected group was recovered, proving that E2F3 overexpression vector can resist the inhibiting effect of miR-210 on E2R3 expression. After that, the cell apoptosis in each group was detected via MTT assay and Caspase-3 activity detection. The results revealed that the survival rate in co-transfection group was decreased and the activity of

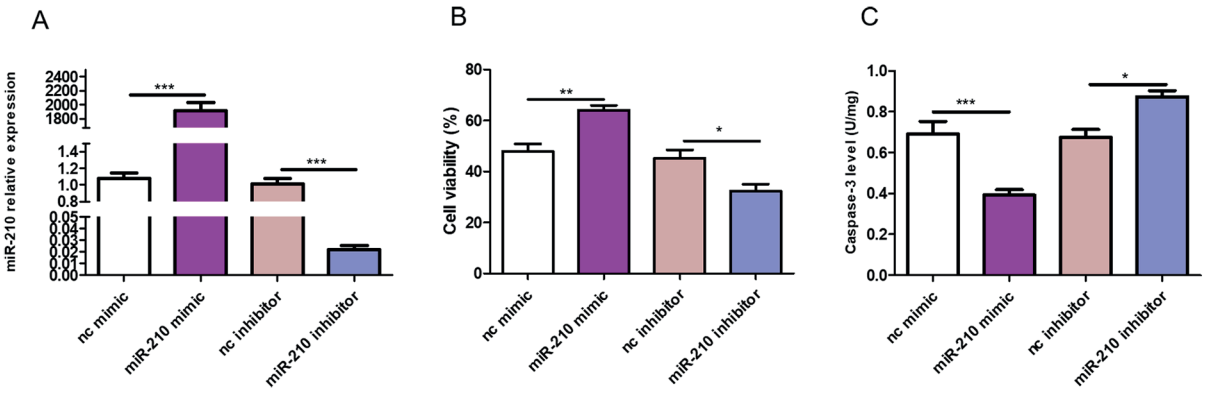

Figure 2. Effect of miR-210 in hypoxia-reoxygenation-induced cell damage. ***A,*** After the transient transfection of miRNA-210 mimic, miRNA-210 inhibitor, mimic negative control and inhibitor negative control for 48 h, the transfection efficiency is verified via qRT-PCR. Compared with that in control group, the expression of miRNA-210 in mimic group is significantly increased, while the expression of miRNA-210 in inhibitor group is significantly decreased (n=6). ***B,*** MTT assay is used to detect the effect of OGD/R model on cardiomyocyte survival rate. Compared with that in control group, the cell survival rate in miRNA-210 mimic-transfected group is significantly increased, but that in miRNA-210 inhibitor-transfected group is significantly decreased (n=6). ***C,*** The activity of Caspase-3 in each group is detected via spectrophotometry. The results show that the activity of Caspase-3 in miRNA-210 mimic-transfected group is significantly decreased compared with that in control group, but that in miRNA-210 inhibitor-transfected group is increased significantly (n=6); ***p <0 .001, **$p < 0.01$, *$p < 0.05$.

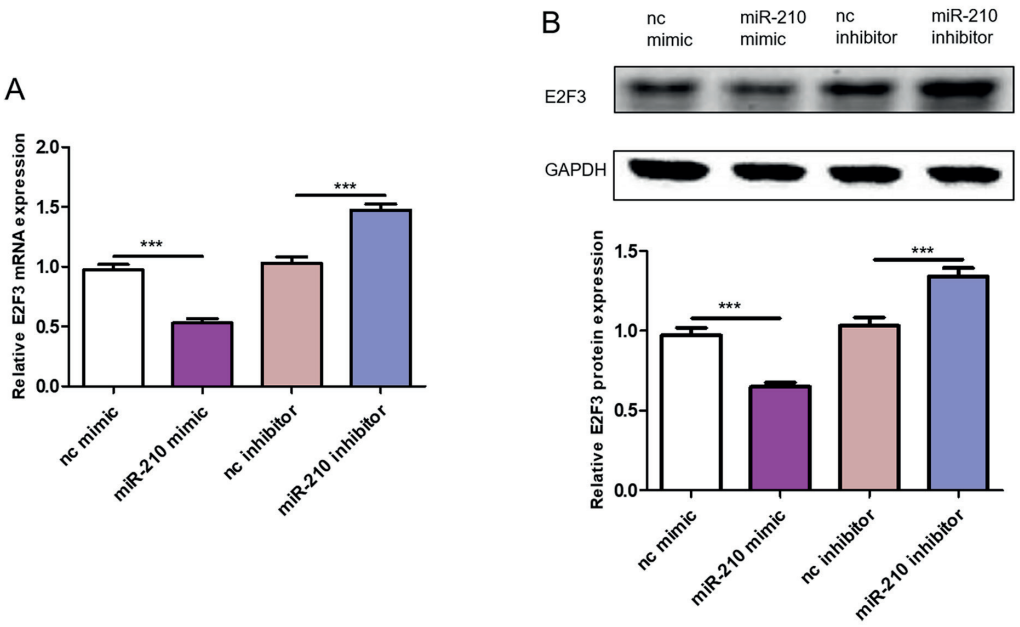

Figure 3. Effect of miR-210 on E2F3 protein expression in cardiomyocytes. ***A,*** After the transient transfection of miRNA-210 mimic, miRNA-210 inhibitor, mimic negative control and inhibitor negative control, the cells in each group are treated with OGD/R and the mRNA expression of E2F3 is detected by RT-PCR (n=6). ***B,*** The change in protein expression of E2F3 is detected by Western blotting (n=6); ***$p < 0.001$.

caspase-3 was increased, compared with those in control group, indicating that the overexpression of E2F3 can reverse the inhibiting effect of miR-210 on cell apoptosis (Figure 4C-D).

Discussion

As we all know, the acute insufficiency of myocardial blood supply due to atherosclerosis,

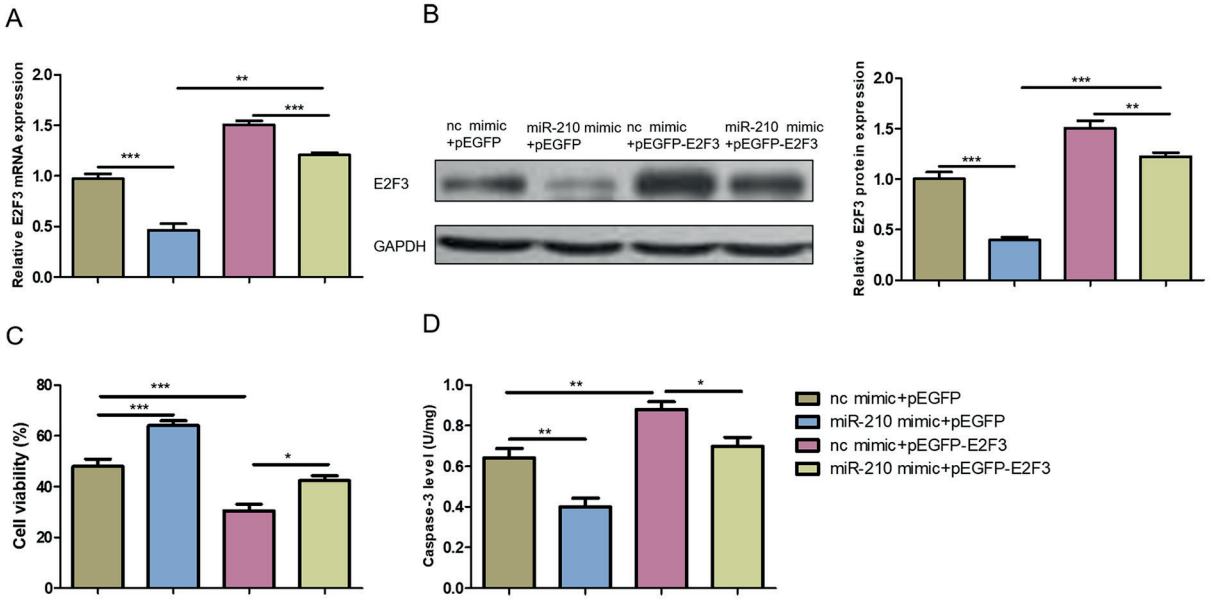

Figure 4. Overexpression of E2F3 can reverse the inhibitory effect of miR-210 on the apoptosis of cardiomyocytes. ***A,*** The overexpression vector plasmid and miR-210 mimic are jointly transfected into cells to extract total RNA for RT-PCR (n=6). ***B,*** The total protein is extracted for Western blotting (n=3). ***C,*** MTT assay is used to detect the cell survival rate (n=6). ***D,*** The activity of Caspase-3 in each group is detected by spectrophotometry (n=6); ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

thrombosis or coronary artery spasm will lead to myocardial ischemia, which is one of the leading causes of cardiomyocyte injury. Although it is very necessary to recover the blood supply in ischemic myocardium as soon as possible, the reperfusion treatment over a period will lead to different degrees of myocardial damage, causing ischemia-reperfusion injury. After the reperfusion is recovered for ischemic myocardium, the ultrastructure, function, metabolic and electrophysiological structures of cells are not improved as expected, but further deteriorate. Such a clinical phenomenon has become one of the decisive factors affecting the prognosis and survival of patients. MiRNA-210 is considered as the most important hypoxia-associated miRNA in the body; the upregulation of miR-210, as a persistent feature, exists in the hypoxia response in normal cells or tumor cells, and regulates the occurrence and development of cardiovascular diseases from a number of aspects. Previous studies[14,16] showed that the overexpression of miR-210 in cardiomyocytes can reduce the production of ROS, thereby inhibiting the apoptosis under oxidative stress and playing a protective role from multiple aspects. In addition, increasing the miR-210 level in mesenchymal stem cells can accelerate the functional recovery after ischemic heart transplantation[17].

MiR-210 regulates the expression of target genes through inhibiting the protein translation or promoting the degradation of specific target gene mRNAs. So far, a number of studies have focused on the relationship between miR-210 and its target genes. The common target genes include E2F3, MNT, Casp8ap2, ephrin-A3, ISCU, COX10, GPD1L and BNIP3[13,18-23], among which the E2F family with was originally found in the study on adenovirus E2 gene, and there are a total of eight members with different functions. E2F3 belongs to the activated subgroup, which can quickly promote the transition of resting cells from G1 phase to S phase, playing an important role in the cell proliferation and apoptosis[24]. Previous researches have confirmed that E2F3, as a target for multiple miRNAs, can play a role in different tumor cells, but its function in the heart is seldom studied. In this work, the OGD/R model was established using the primary cardiomyocytes of rats, and it was confirmed that miR-210 was up-regulated in OGD/R injury model of cardiomyocytes. In addition, the increased activity of pro-apoptotic Caspase-3 and results of MTT assay revealed that OGD/R injury triggered the activation of apoptosis. After that, miR-210 mimic was transfected and the expression level of miR-210 was up-regulated to detect the severity of cell injury. It was found that the high expression of miR-210 increased the survival rate of cardiomyocytes, decreased the severity of cardiomyocyte injury and reduced the apoptosis level after OGD/R. The above experiments indicated that OGD/R injury can up-regulate miR-210 expression level, and miR-210 protects cells in this process. In order to further explore the target of miR-210 in OGD/R injury of cardiomyocytes, the expression of E2F3 was detected. It was found that the mRNA and protein expressions of miR-210 were decreased after the up-regulation of miR-210. Also, it was found that the inhibition of apoptosis was partially offset after the up-regulation of E2F3.

Conclusions

We observed that miR-210 can inhibit the OGD/R-induced apoptosis of cardiomyocytes, and miR-210, as an upstream factor, plays a protective role in cardiomyocytes through directly inhibiting the protein expression of its target gene E2F3. This investigation provided new experimental evidence for the OGD/R injury of cardiomyocytes, a theoretical basis for the protective effect of miR-210 in cardiovascular diseases, and new ideas for further clinical diagnosis and treatment.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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