Am J Transl Res 2019;11(5):3007-3017
www.ajtr.org /ISSN:1943-8141/AJTR0082529

Original Article

MiR-21b-3p protects NS2OY cells against oxygen-glucose deprivation/reperfusion-induced injury by down-regulating cyclooxygenase-2

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Received July 13, 2018; Accepted December 23, 2018; Epub May 15, 2019; Published May 30, 2019

Abstract: Recent studies have shown abnormal expression levels of cyclooxygenase-2 (COX-2) and miR-21b-3p in cerebral ischemia-reperfusion (I/R) rat models. Decreased COX-2 expression could reduce brain injury and thus could be a target of miR-21b-3p according to the miRNA databases (miRDB) analysis. However, its functions and underlying mechanisms in I/R injury remain unclear. In our study, we have established an oxygen/glucose deprivation and reperfusion (OGD/R) model by using NS2OY cells. The expression of miR-21b-3p and COX-2 was determined by quantitative real-time PCR or Western blot, and the fluorescence intensities were detected by fluorescence in situ hybridization (FISH) or immunofluorescence. After transfection and OGD/R treatments, the functions of miR-21b-3p and COX-2 on cell viability and apoptosis were detected using cell-counting kit 8, Edu staining, flow cytometry and Hoechst staining, respectively. Finally, dual-luciferase reporter assay was used to explore the relationship between miR-21-b-3p and COX-2. The results have showed that COX-2 mRNA and protein expression were significantly increased; however, the expression of miR-21b-3p was remarkably reduced in NS2OY cells after OGD/R treatment. The changes were most remarkable in OGD 2 h/R24 group. Function analysis has showed that when NS2OY cells were exposed to OGD/R injury, overexpressed miR-21b-3p significantly downregulated COX-2 expression, increased cell viability and decreased apoptosis. In addition, knocking down the expression of COX-2 could also increase cell viability and decrease apoptosis. Dual-luciferase reporter assays showed miR-21b-3p as the target of 3'-UTR of COX-2. Therefore, we concluded that OGD/R-induced injury by down-regulating COX-2.

Keywords: Oxygen/glucose deprivation and reperfusion, cyclooxygenase-2, miR-21b-3p, cell viability, apoptosis

Introduction

Ischemia-reperfusion is a pathological condition, which is characterized by tissue damage [1]. Brain ischemia is associated with a high rate of morbidity and poses threat to human health. However, its therapy is still unavailable. Cerebral ischemia reperfusion injury includes neuronal injury and neurological deficits [2, 3], which are usually caused by ischemia and inflammation [4]. Deprivation of blood supply results in a significant decrease in oxygen and glucose, and the lack of energy and nutrition, in turn, leads to the death of cerebral neurons [5]. The injury is attributed to the post-ischemic inflammatory response, which leads to an exacerbated, cascaded injury [6-9]. Ischemia-induced cell death is one of the most serious effects caused by brain ischemia, and the loss of neurons by apoptosis results in cerebral infarction and brain damage [10, 11]. Therefore, it is necessary to inhibit apoptosis in order to treat cerebral ischemia-reperfusion injury.

COX-2 is an isoform of cyclooxygenases, which catalyzes the transformation of arachidonic acid to prostanoids. Its expression levels are varied between different tissues and could be regulated by biologic events, such as injury, inflammation and proliferation [7, 12, 13]. In acute or chronic ischemia, COX-2 usually acts as a prominent inflammatory mediator to induce

subsequent cascade reactions [14-16]. Many studies have shown that the expression levels of COX-2 are increased in chronic inflammatory diseases and various cancers [17-19]. The protein levels of COX-2 are significantly upregulated after cerebral I/R injury [20-22]. Increased COX-2 expression and activity could lead to higher levels of arachidonic acid, which would result in neuronal apoptosis and death due to its direct excitotoxic effects [23, 24]. COX-2 inhibitors, including Valdecoxib and Rofecoxib, could reduce brain damage to some extent; however, overexpression of COX-2 significantly increases the injury [25-27]. Due to its important, but deleterious roles, downregulation of COX-2 could be a potential treatment for ischemia.

MicroRNAs (miRNAs) are a subtype of short sequences, conserved, non-coding RNAs that downregulate the expression of their target genes. In the central neurons, miRNAs play a crucial role. MiR-21 is involved in the inflammation response and delays negative-regulatory functions [28, 29]. Many studies have showed that miR-21 participates in acute I/R injury, for example miR-21 is significantly downregulated in kidney, liver and myocardium [30-32]. In addition, overexpressed miR-21 could protect neurons against I/R injury, increase cell viability and decrease apoptosis [32, 33]. These evidences indicate that miR-21 could be used in the treatment of I/R injury. However, the mechanism by whichmiR-21 exerts neuroprotective effects during I/R is not fully elucidated. Analysis by miRDB showed that COX-2 could be a target of miR-21b-3p. OGD/R model is one way to better study I/R injury *in vitro* [34-36].

In this study, we established OGD/R models using NS2OY cells. We found that after OGD/R treatment, the COX-2 expression was significantly enhanced; however, the expression of miR-21b-3p was significantly reduced. To explore the function of COX-2 and miR-21b-3p in OGD/R injury, we established miR-21b-3p overexpression model using miR-21b-3p mimics and COX-2 knock-down model using COX-2 siRNA. Our results showed that overexpressed miR-21b-3p or knock-down COX-2 could protect NS2OY cells against OGD/R-induced injury and miR-21b-3p could downregulate the expression of COX-2 by targeting its 3'-UTR.

Materials and methods

Cell culture and OGD/R treatments

NS2OY cell-line was obtained from ATCC (Manassas, VA, USA). The cells were initially cultured in normal DMEM medium containing with 10% fetal bovine serum (FBS) and maintained in 37°C at 5% CO_2 and 95% O_2 humidified incubator. To establish OGD/R models, NS2OY cells were washed (PBS, 3 × 10 minutes; 37°C), cultured in glucose-free DMEM and grown in hypoxia chamber filled with 2% O_2, 6% CO_2 and 92% N_2 for different hours at 37°C. Finally, NS2OY cells were cultured in DMEM medium under normal conditions for 24 hours (re-oxygenation). After OGD/R treatment, NS2OY cells were continually cultured in normal conditions.

Quantitative real-time PCR assay

Total RNAs of NS2OY cells were extracted by Trizol reagent (#9109, Takara, China). After RNA quantities were determined, 5 μg of RNA was transcribed to cDNA using a cDNA synthesis kit (#6130, Takara). QRT-RCR was conducted using SYBR Premix Ex Taq II (#RR820A, Takara) on Applied Biosystems® 7500 Real-Time PCR System. The primer sequences were: miR-21 5'-ACA CTC CAG CTG GGT AGC TTA TCA GAC TGA-3' (forward); 5'-GTG TCG TGG AGT CGG CAA TTC-3' (reverse). COX-2: 5'-CCA GCA CTT CAC CCA TCA GTT-3' (forward); 5'-ACC CAG GTC CTC GCT TAT GA-3' (reverse). Relative expressions were determined based on $2^{-\Delta\Delta Ct}$ method [37].

Fluorescence in situ hybridization

After OGD/R treatment, the cells were resuspended in Carnoy's solution and centrifuged at 1000 rpm for 6 minutes. 100 μL material was dropped on clean glass slides. The slides were prehybridized with a hybridization solution (#544-01331, WAKO) for 1 h at 37°C. The probes (100 nM) were added to slides. After denaturation at 74°C for 6 minutes, the slides were placed in humid chambers overnight at 35°C. The slides were washed with 0.4 × SSC (4 × 5 minutes; 45°C) and followed by 2 × SSC containing 0.05% Tween20 at room temperature for 1 min. The nuclei were counterstained with DAPI (Life Science) and coverslipped. The sequences of the miR-21 probe were: 5'-TCA ACA TCA GTC TGA TAA GCT A-3'.

Western blot

Total protein of NS20Y cells was extracted using cell lysis buffer containing proteinase inhibitors. The concentration was measured using BCA kit (#23225, Pierce). Equal quantity of proteins was electrophoresed using 10% SDS-PAGE and transferred to PVDF membranes (Millipore). After washing three times with TTBS, the membranes were blocked by 5% milk for 2 h. The membranes were incubated with COX-2 antibody (1:500, ab52237, Abcam) and GAPDH antibody (1:10000, sc420485, SantaCruz) at 4°C overnight. After washing with TBST, the blots were blotted using species-specific HRP-conjugated secondary antibodies for 2 h. Finally, the bands were analyzed with chemiluminescence reagents using Gel Imaging System (#1708370, Life Science).

Immunofluorescence staining

The NS20Y cells were fixed with 4% paraformaldehyde for 1 h and washed by PBS. Then, the cells were incubated with block solution for 2 h at RT. Immunolabelling was performed using anti-rabbit COX-2 (1:1000, ab52237, Abcam) antibody overnight in 4°C. After washing (PBS, 5 × 6 minutes; 37°C), the cells were incubated with secondary antibody (1:200, ab15007 3, Abcam) and DAPI (1:100, Invitrogen, CA, USA) for 2 h at RT protected from light. The cells were washed again in dark and imaged with a confocal laser-scanning microscope.

Cell transfections

MiR-21 mimic, negative control mimic, COX-2 siRNA and negative control siRNA were designed and chemically synthesized as follows: miR-21 mimic, 5'-UAG CUU AUC AGA CUG AUG UUG A-3' (sense), 5'-AAC AUC AGU CUG AUA AGC UAU U-3' (antisense); NC mimic, 5'-UUC UCC GAA CGU GUC ACG UTT-3' (sense), 5'-ACG UGA CAC GUU CGG AGA ATT-3' (antisense); COX-2 siRNA-1, 5'-GGA UUU GAC CAG UAU AAG UTT-3' (sense), 5'-ACU UAU ACU GGU CAA AUC CTG-3' (antisense); NC siRNA, 5'-GAC AAC GGC CAC AAG UTC-3' (sense), 5'-ACU GGC CGU UUA CGU CGC-3' (antisense).

For transfections, the NS20Y cells were seeded onto 24-well plates with antibiotic-free DMEM to achieve an 80% confluence before transfection. Then, the cells were transfected with 1 μm of the constructs using Lipofectamine 2000 (Invitrogen) and cultured in serum-free conditions for 24 hours.

CCK-8 assay

After culturing in 96-well plates for 16 h, the NS20Y cells were transfected with different oligonucleotides for 24 h followed by OGD/R treatment. Cell proliferation was determined at indicated time points (24, 48, 72 h) after OGD/R treatment by CCK-8 (CK04, Dojindo, Japan): 10 μl of CCK8 was added to each well and incubated for 1 h. The absorbance in each well was detected at 450 nm using a microplate reader (S28629, Fisher Scientific).

EdU assay

The NS20Y cells were cultured onto 24-well plates for 16 h and then transfected with different oligonucleotides for 2 h followed by OGD/R treatment. 20 μM of EdU (C10338, Invitrogen) was added during the last 2 h. After OGD/R treatment, the EdU-DNA was measured by Click-iTEdU Imaging Kit (C10638, Thermo Fisher). The cells were washed with PBST and subsequently stained for DAPI. The nuclei of cells were imaged with a confocal laser-scanning microscope.

Flow cytometry assay

Cultured NS20Y cells were seeded onto 6-well plates for 16 h. After transfection and OGD/R treatment, the cells were obtained and washed (by ice cold PBS; 4 × 10 minutes). Cell apoptosis was measure using Annexin V-FITC and PI double staining kit (V13242I, Invitrogen). The cells were stained in 1 X binding buffer with 6 μL Annexin V-FITC and 6 μL PI for 20 min at RT protecting from light. Apoptosis was detected using FACS can flow cytometer (#204316, BD) by Cell Quest Research Software (BD).

Hoechst staining

The NS20Y cells were seeded onto 24-well plates. After transfection and OGD/R treatment, the cells were incubated with Hoechst 33342 (#6432344001, Sigma-Aldrich) for 1 h at RT. After washing with PBS, the samples were imaged. Apoptotic cells showed the overexposed and condensed nuclei.

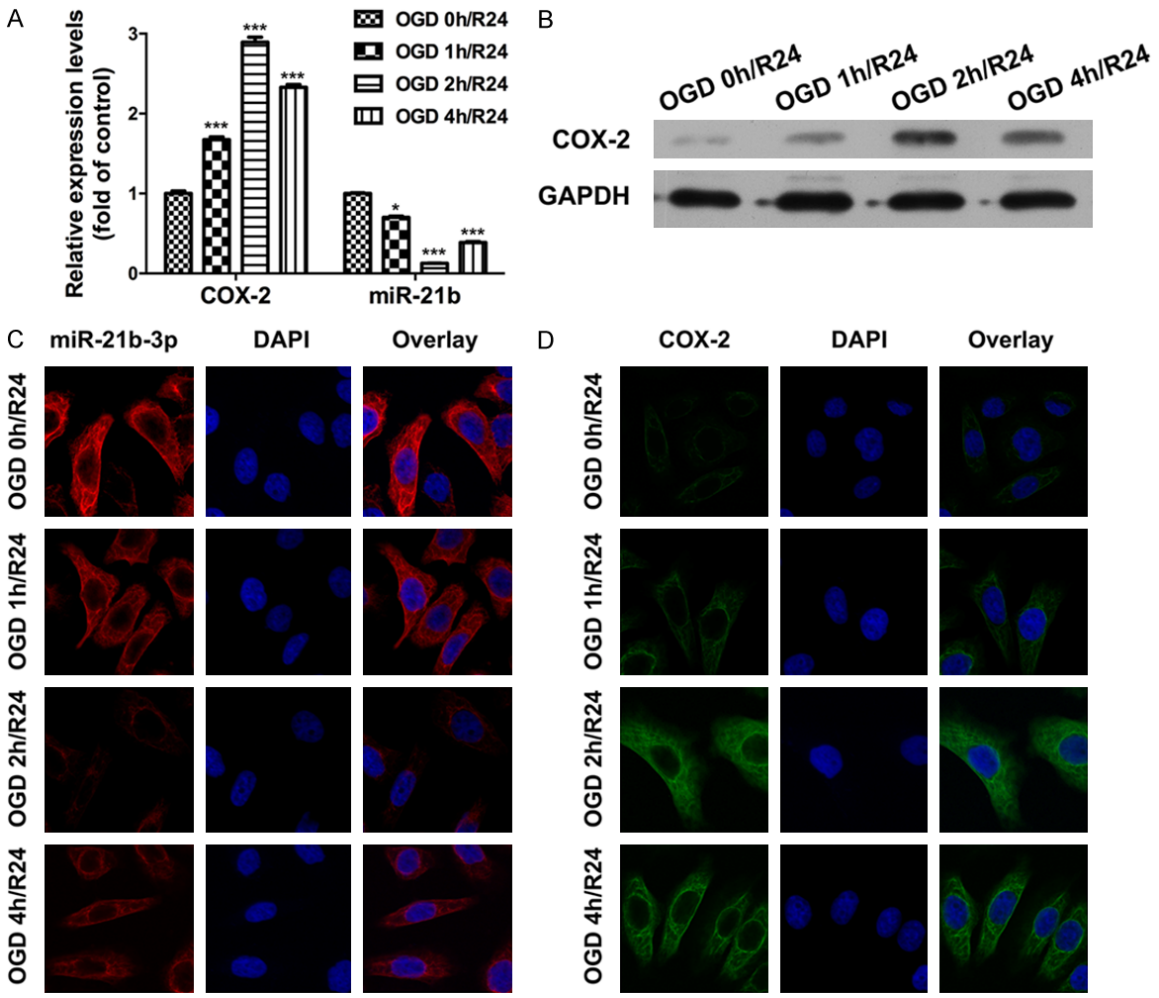

Figure 1. COX-2 expression was increased and miR-21b-3p expression was decreased in NS2OY cells after OGD/R. NS2OY cells were exposed to oxygen-glucose deprivation (OGD) for 0, 1, 2 or 4 h followed by 24 h of re-oxygenation. A. qRT-PCR was performed to analyze the expression levels of COX-2 and miR-21b in NS2OY cells after different OGD/R treatments (**P* < 0.05, ****P* < 0.001 vs. OGD 0 h/R24 group). B. The protein expression level of COX-2 was assessed in NS2OY cells after OGD/R treatments. C. Detection of miR-21b-3p was carried out by miRNA fluorescence *in situ* hybridization (FISH), red represents biotin-labeled probe against miR-21b-3p, blue represents DAPI. Magnification 400 ×, Scale bar = 20 μm. D. Immunohistochemistry assay was used to evaluate the expression of COX-2 in NS2OY cells after OGD/R treatments. Green represents COX-2, blue represents DAPI. DAPI (blue) was used to label nuclei. Magnification 400 ×, Scale bar = 20 μm.

Plasmid construction and dual luciferase activity assay

The fragments of the 3'-UTR of COX-2 containing the miR-21 binding site or mutant miR-140-5p binding sites were amplified using PCR. The sequences of fragments are shown in **Figure 6A**. The fragments were cloned into a luciferase vector psi-CHECK (Promega, Madison, USA) and named COX-2-WT or COX-2-Mut, respectively. TheNS2OY cells were cultured in 24-well plates for 24 h and then co-transfected with COX-2-WT or COX-2-Mut and miR-21b mimics or NC mimics. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega) 48 h after transfection.

Statistical analysis

The data is represented as mean ± SEM and analyzed using Graghpad (Ver. Prism 7, GraphPad Prism Software, La Jolla, CA, USA). Student *t* test was used to evaluate the statistical significance of differences between the groups. A *P* value of < 0.05 was considered to be statistically significant.

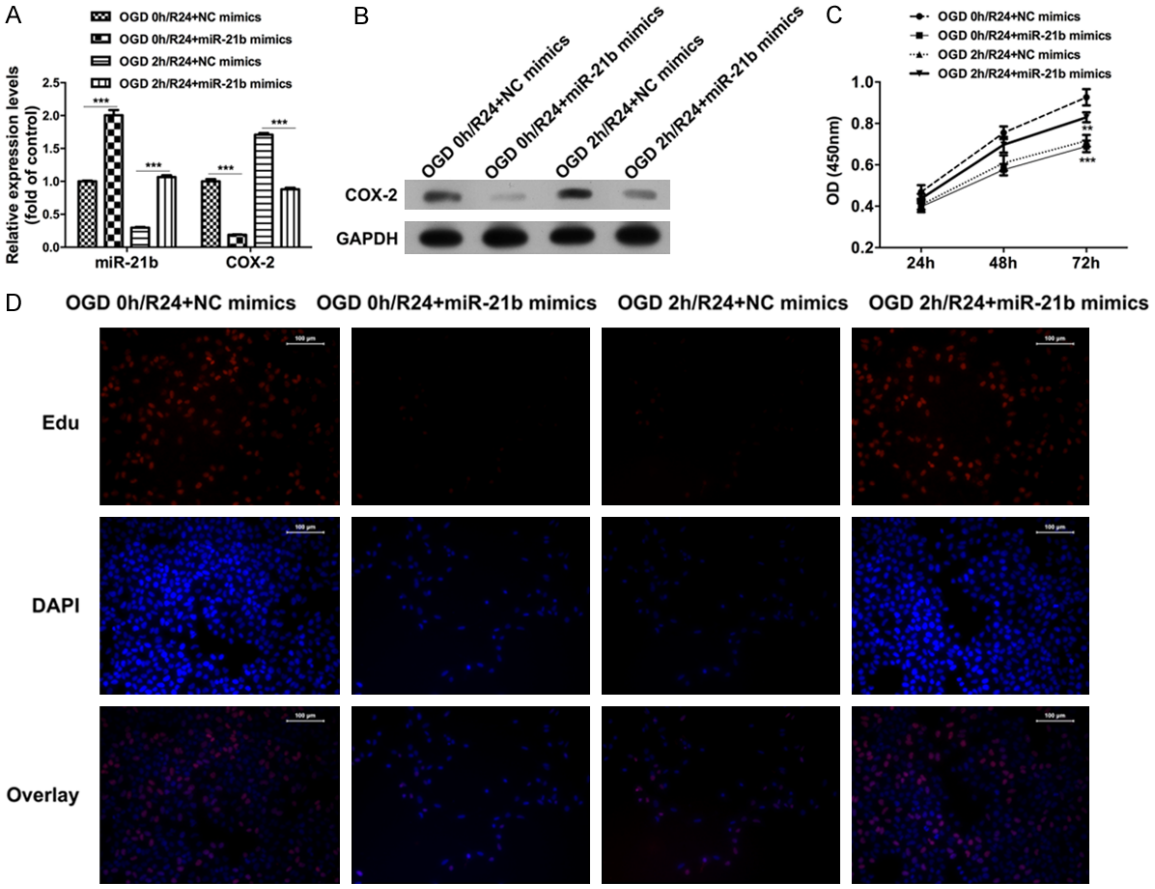

Figure 2. MiR-21b inhibited COX-2 expression and cell viability in OGD/R model. NS2OY cells were transfected with miR-21b mimics or NC mimics for 24 h and were exposed to OGD for 0 or 2 h followed by 24 h re-oxygenation. A. The expression levels of COX-2 and miR-21b were measured by qRT-PCR assay in each group (***P < 0.001). B. Western blot analysis was performed to measure the protein expression level of COX-2 in each group. C. The cell viability of NS2OY cells was measured by CCK-8 assay, and the OD was detected at 450 nm (**P < 0.01, ***P < 0.001). D. Edu staining (red) was used to measure the number of NS2OY cells during the S phases, DAPI (blue). Magnification 100 ×, Scale bar = 100 μm.

Results

OGD/R treatment increased COX-2 expression, but decreased miR-21b-3p expression in NS2OY cells

To determine the levels of COX-2 and miR-21b-3p expression in NS2OY cells after OGD/R treatment, we established four OGD/R groups: OGD 0 h/R24 group; OGD 1 h/R24 group; OGD 2 h/R24 group and OGD 4 h/R24 group. The cells were subjected to oxygen and glucose deprivation for 0, 1, 2 and 4 h, respectively, and followed by 24 h of reperfusion. The cells in OGD 0 h/R24 group were used as control. First, we examined the level of COX-2 mRNA in the four groups. The results showed that following OGD/R injury, COX-2 mRNA expression was significantly increased (**Figure 1A**, ***P < 0.001 *vs.* control group). In addition, Western blot and immunofluorescence analysis elucidated that the protein expression of COX-2 was higher followed by OGD/R treatment (**Figure 1B** and **1D**). However, OGD/R treatment resulted in significantly lower expression of miR-21b-3p (*P < 0.05 *vs.* control, **Figure 1A**). The results were also validated by miRNA FISH analysis (**Figure 1C**). Interestingly, the most dramatic expression changes of COX-2 and miR-21b-3p was observed in OGD 2 h/reperfusion 24 h group induced compared to control, and this was confirmed in repeated experiments. Thus, we chose OGD 0 h/R24 and OGD 2 h/R24 groups for the subsequent experiments to examine the function and mechanism of COX-2 and miR-21b-3p during OGD/R.

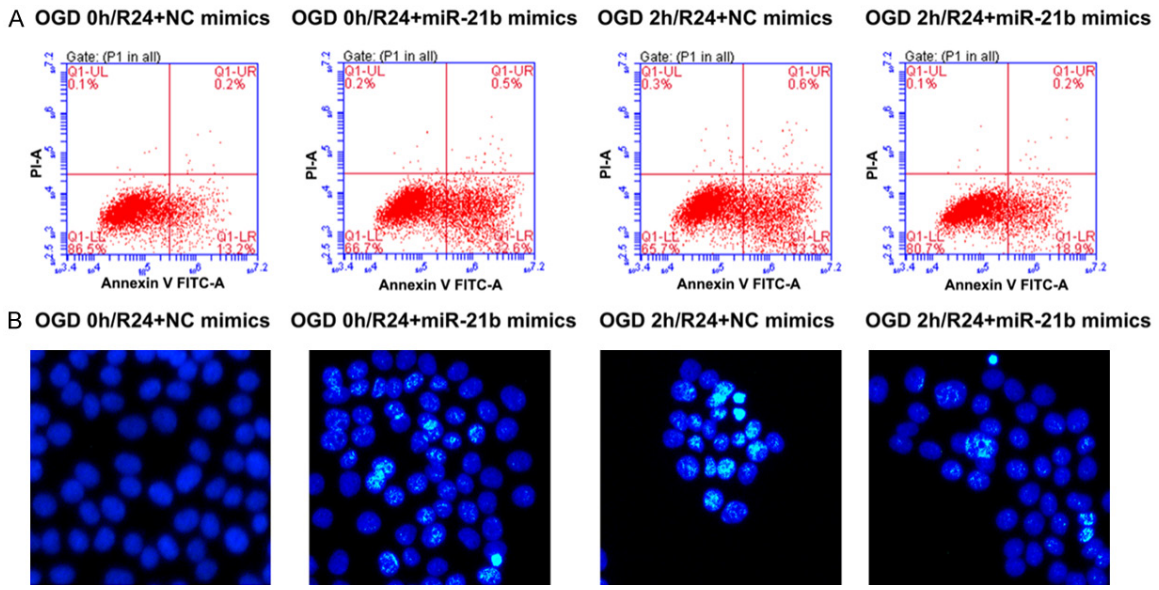

Figure 3. MiR-21b promoted the apoptosis of NS2OY cells in OGD/R model. NS2OY cells were transfected with miR-21b mimics or NC mimics for 24 h and were exposed to OGD for 0 or 2 h followed by 24 h re-oxygenation. A. Flow cytometry assay was used to assess the number of apoptotic tumor cells by staining with Annexin V-FITC/PI, the proportions of Annexin V-FITC$^-$/PI$^-$ cells, Annexin V-FITC$^+$/PI$^-$, Annexin V-FITC$^+$/PI$^+$ and Annexin V-FITC$^+$/PI$^-$ cells were shown. B. Hoechst staining was performed to measure the apoptosis, the number and color of positive cells were shown in each group Magnification 400 ×, Scale bar = 20 μm.

miR-21b-3p overexpression decreased COX-2 expression in NS2OY cells and increased cell viability after OGD/R

To examine the function of miR-21b-3p during OGD/R, the NS2OY cells were transfected with miR-21b-3p mimics or negative control (NC) mimics before OGD/R treatment. QRT-PCR results showed that miR-21b-3p mimics significantly induced miR-21b-3p overexpression compared to NC mimics (**Figure 2A**). However, transfection with miR-21b-3p mimics significantly downregulated the mRNA and protein expression of COX-2 in NS2OY cells (**Figure 2A** and **2B**).

To evaluate the functions of miR-21b-3p on cell viability, CCK-8 assay and Edu staining were carried out. After 72 h of transfection, the absorbance of NS2OY cells in miR-21b-3p mimics group was significantly different from NC group. After OGD 0 h/R24 treatment, the cell viability was decreased in miR-21b-3p mimics compared with NC mimics. In contrast, OGD 2 h/R24 treatment increased the cell viability in miR-21b-3p mimics (*P < 0.05, ***P < 0.001, **Figure 2C**). Results from Edu staining were consistent with those from CCK-8 assay (**Figure 2D**). These findings indicated that miR-21b-3p overexpression increased cell viability during OGD/R injury.

MiR-21b-3p overexpression decreased apoptosis during OGD/R

We also evaluated the percentage of apoptotic cells after OGD/R treatment using flow cytometry and Hoechst staining. In the OGD 0 h/R24 + NC mimics group, the percentage of apoptotic cells was ~13%. In OGD 2 h/R24 transfected with miR-21b-3p mimics group, the percentage of apoptotic cells increased to ~32%. However, in OGD 2 h/R24 + miR-21b mimics group, the percentage of apoptotic cells was decreased to ~18%. (**Figure 3A**). The results from Hoechst staining confirmed that the number of cells was significantly decreased in OGD 2 h/R24 + NC mimics group, with the most positively stained cells showing the condensed nuclei (typical characteristics of apoptosis). Transfection with miR-21b-3p mimics significantly recovered apoptosis induced by OGD 2 h/R24 treatment (**Figure 3B**). These results suggested the cytoprotective effect of miR-21b-3p against OGD injury.

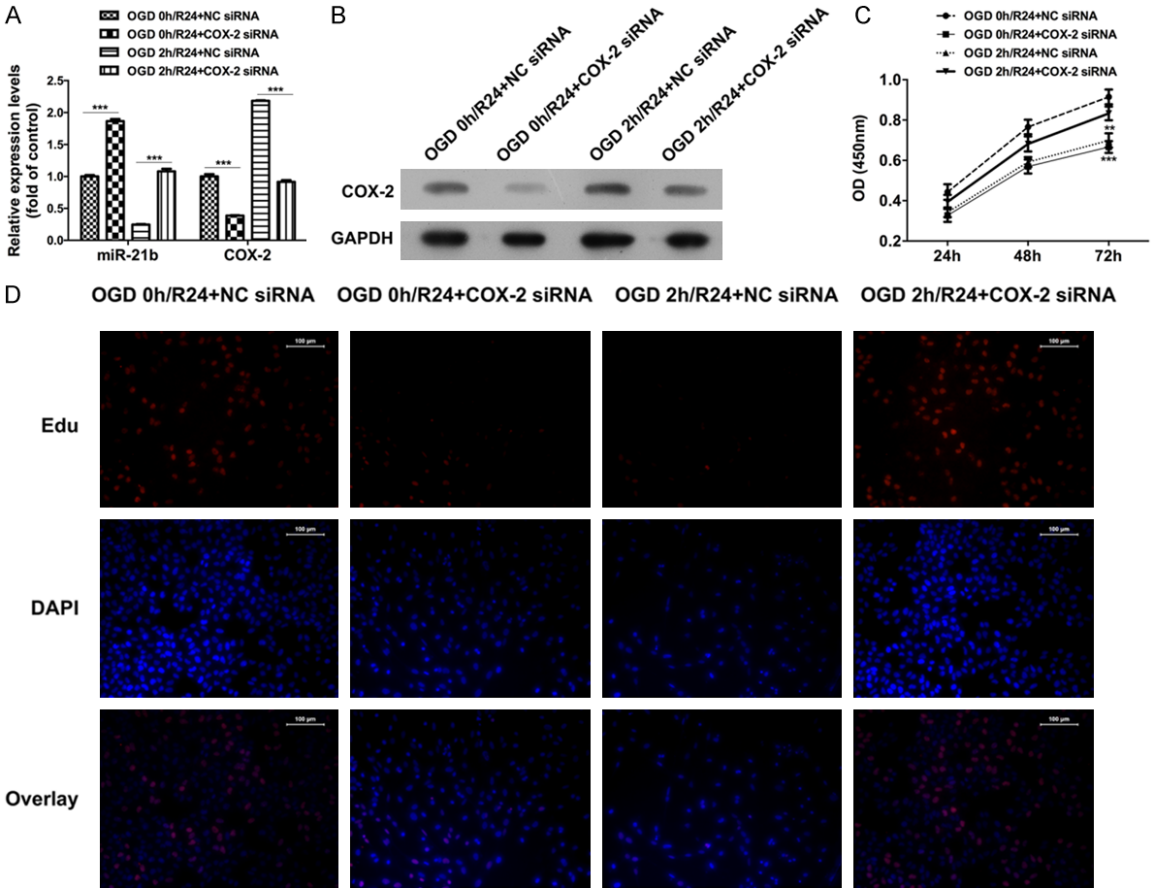

Figure 4. Downregulated COX-2 reduced miR-21b expression and cell viability in OGD/R model. NS2OY cells were transfected with COX-2 siRNA or NC siRNA before exposed to OGD. After 0 or 2 h OGD followed by 24 h re-oxygenation, the cells were harvested. A. The expression levels of COX-2 and miR-21b were measured by qRT-PCR assay in each group (***$P < 0.001$). The COX-2 expression was significantly downregulated in COX-2 siRNA group compared to NC group; however, the miR-21b expression was increased. B. Western blot analysis was performed to measure the protein expression level of COX-2 in each group. C and D. The cell viability of NS2OY cells was measured by CCK-8 assay and Edu staining **$P < 0.01$, ***$P < 0.001$, DAPI (blue), Magnification 100 ×, Scale bar = 100 μm.

Transfection NS2OY cells with COX-2 siRNA decreased COX-2 expression

To evaluate the effects of COX-2 during OGD/R injury, COX-2 siRNA was constructed and transfected to NS2OY cells to inhibit COX-2 expression. After transfection and OGD/R treatment, the COX-2 mRNA and protein expression in COX-2 siRNA group were significantly downregulated compared to NC groups (**Figure 4A** and **4B**). The results from qRT-PCR also indicated that transfection with COX-2 siRNA increased the expression of miR-21b-3p (**Figure 4A**).

Effect of COX-2 on cell viability and apoptosis during OGD/R injury

We next analyzed whether downregulated COX-2 expression by siRNA protected NS2OY cells against OGD/R injury. The results of CCK-8 assay and Edu staining showed that both COX-2 siRNA and OGD 2 h/R24 treatment significantly reduced NS2OY cells viability, while COX-2 siRNA recovered the cell viability mediated by OGD 2 h/R24 treatment (**$P < 0.01$, ***$P < 0.001$, **Figure 4C** and **4D**). The apoptotic ratio of NS2OY cells was determined by flow cytometry. The apoptotic ratio was significantly decreased to 19% in OGD 2 h/R24 + COX-2 siRNA group compared to OGD 2 h/R24 + NC siRNA group, indicating that COX-2 siRNA could effectively suppress apoptosis induced by OGD 2 h/R24 (**Figure 5A**). The results from Hoechst staining were consistent with those from flow cytometry (**Figure 5B**). These findings indicated that downregulated COX-2 expression exhibited protective functions in OGD/R injury.

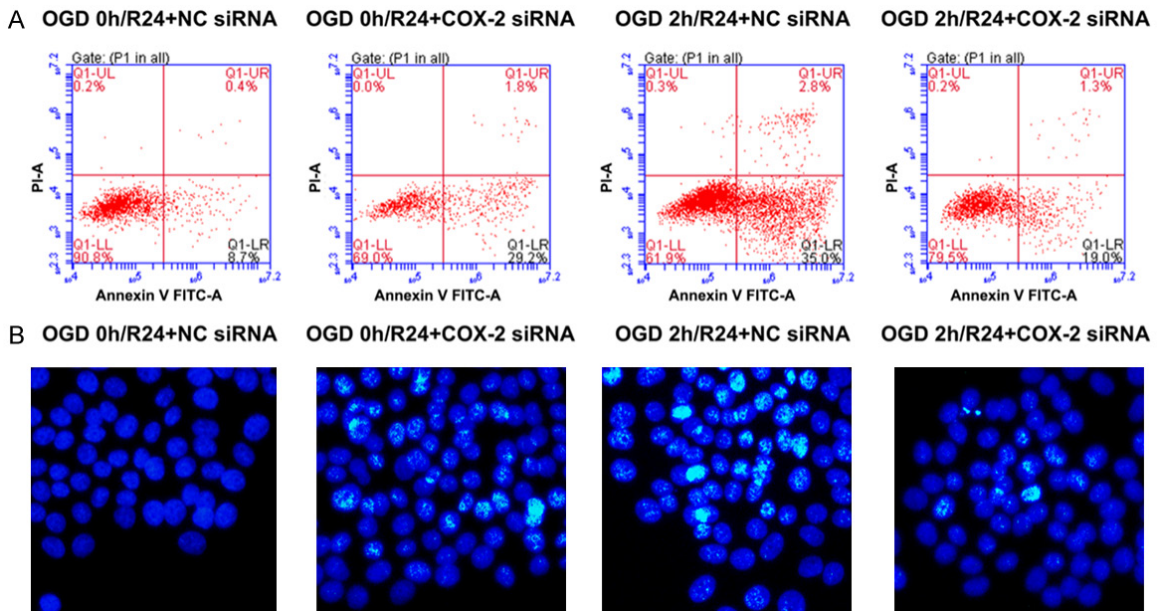

Figure 5. Downregulation of COX-2 facilitated NS2OY cells apoptosis in OGD/R model. NS2OY cells were transfected with COX-2 siRNA or NC siRNA before exposure to OGD. After 0 or 2 h OGD followed by 24 h re-oxygenation, the cells were harvested. (A, B) The number and percentage of apoptotic NS2OY cells in four groups were measured by flow cytometry (A) and Hoechst staining after different OGD/R treatments.

COX-2 is a target of miR-21b-3p in NS2OY cells

MiRDB analysis showed that miR-21b-3p could be a potential target of COX-2. The results from qRT-PCR and Western blotting (**Figure 2A** and **2B**) showed that in the NS2OY cells, miR-21b-3p mimics considerably increased miR-21b-3p expression, but decreased COX-2 expression compared to NC mimics. To confirm the correlation between miR-21b-3p and COX-2, luciferase reporter vectors, COX-2-Wt and COX-2-Mut, were constructed (**Figure 6A**). Dual luciferase activity assay showed that the luciferase activity in the miR-21b-3p mimics + COX-2-Wt group was about half of that in the NC mimics group. However, no significant difference was observed in the luciferase activity between the miR-21b-3p mimics + COX-2-Mut group and NC mimics + COX-2-Mut group (*$P < 0.05$, **Figure 6B**). Our study showed that miR-21b-3p could be a target for 3'-UTR of COX-2 and downregulate the expression of COX-2.

Discussion

Cerebral I/R could result in serious brain damage, which is associated with complex underlying mechanisms. Apoptosis and inflammation play crucial roles in I/R injury. COX-2, an inflammatory mediator, participates in the processes of I/R injury. Its role and modulated cascaded pathway in cerebral I/R are well-explored. It is reported that the number of immunostained cells with total mRNA of COX-2 are significantly increased after I/R [38]. The enhanced COX-2 expression could lead to neuronal death: prostaglandins mediated by the COX could result in inflammation and increased COX2 expression also induces higher levels of PGI2 and TXA2 [39]. Inhibition or gene deletion of COX-2 leads to a significant reduction in neuronal injury after I/R [40]. Neuronal overexpression of COX-2 results in increased brain damage after ischemia transgenic mice. Currently, many COX-2 inhibitors, such as anti-inflammatory drugs, are available for the treatment of chronic inflammatory diseases. However, long term usage of these drugs is associated with an increased risk of cardiovascular diseases.

In this study, we have showed that after OGD/R treatment, COX-2 expression in NS2OY cells was upregulated. However, the expression levels were not consistent with OGD time: the increasing level in OGD 4 h group was less compared to OGD 2 h group. It was opined that OGD/R treatment could also activate the immune response against the injury. Downre-

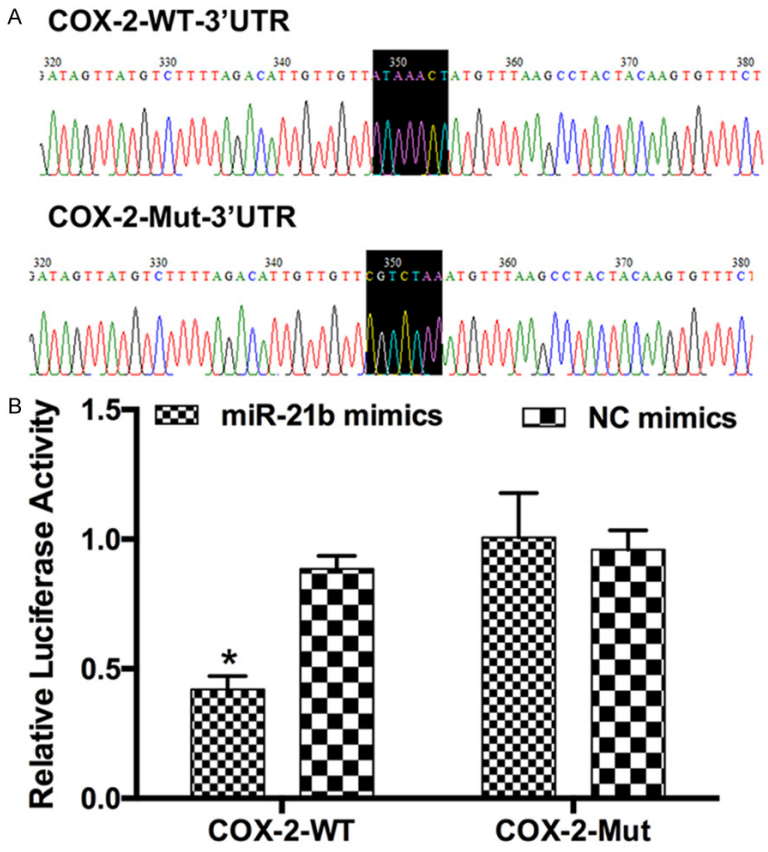

Figure 6. COX-2 is a target of miR-21b-3p in NS2OY cells. A. Upper panel shows the predicted miR-21b interaction sites in the COX-2 3'-UTR, lower panel shows the mutated sequence in the seed regions of COX-2. B. NS2OY cells were co-transfected with COX-2-WT or COX-2-Mut and miR-21b mimics or NC mimics. Relative luciferase activity measured by luciferase reporter assay (*$P < 0.05$).

gulation of COX-2 expression results in an increased cell viability, but decreased apoptosis when the cells were subjected to OGD 2 h/R24 treatment. However, it was worth noting that in cells cultured in normal conditions, downregulation of COX-2 expression decreased cell viability, but increased apoptosis. Therefore, a certain concentration of COX-2 is required to maintain the normal physiological activity.

MicroRNAs, a class of endogenous, noncoding RNAs, are usually the target of 3'-untranslated regions of target mRNAs, and inhibit mRNA translation or induce mRNA degradation. It is reported that over 20% of the miRNAs exhibit significantly changed expression profile in brain after I/R, which suggests that these play a critical role in I/R [41]. The regulation as well as the function of miR-21 has been extensively investigated. *In vivo*, miRNA-21 acts as a strong anti-apoptotic factor and is upregulated in most solid tumors and ischemic brain [42]. Functional analysis showed that miR-21 overexpression decreases cell apoptosis during oxidative stress [43]. Overexpression of miR-21 in cortical or N2A neuroblastoma cells significantly suppresses OGD-induced apoptotic cell death [44]. Many researchers have explored the molecular mechanisms of miR-21 involved in I/R injury. For example, *PTEN* and *FASL* are targeted genes of miR-21; miR-21 could target their 3'-UTR to downregulate their expression, which could efficiently reduce I/R injury [29].

Conclusion

In conclusion, upregulated COX-2 expression and downregulated miR-21b-3p expression were observed in NS2YO cells after OGD/R treatment. Both overexpressed miR-21b-3p and downregulated COX-2 expression decreased OGD/R injury. MiR-21b-3p could downregulate the expression of COX-2 by targeting its 3'-UTR. Given the important roles of COX-2 and miR-21b in cerebral ischemia reperfusion injury, our findings suggested that miR-21 inhibitors could be used as a novel treatment option for OGD/R.

Acknowledgements

XNW and KRP designed the experiments and collected the data, HH, ZSL, WX and TWD provided assistance on experiments, LL and WL analyzed the data, TZ provided administration supports. This work was supported by Guangdong Provincial Science and Technology Project [No. 2016A020215193]; National Natural Science Foundation of China [No.81601884]; Natural Science Foundation of Guangdong Province, China [No. 2015A030313607]; Guangdong Provincial Science and Technology Project [No. 2014A020215025]; Natural Science Foundation of Liaoning Province [No.

2015020420]; and Guangdong science and technology project [201607010288].

Disclosure of conflict of interest

None.

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References

[1] Eltzschig HK and Eckle T. Ischemia and reperfusion--from mechanism to translation. Nat Med 2011; 17: 1391-1401.

[2] Liu T, Zhang T, Yu H, Shen H and Xia W. Adjudin protects against cerebral ischemia reperfusion injury by inhibition of neuroinflammation and blood-brain barrier disruption. J Neuroinflammation 2014; 11: 107.

[3] Zheng JM, Chen XC, Lin M, Zhang J, Lin ZY, Zheng GY and Li KZ. Mechanism of the reduction of cerebral ischemic-reperfusion injury through inhibiting the activity of NF-kappaB by propyl gallate. Yao Xue Xue Bao 2011; 46: 158.

[4] Tobin MK, Bonds JA, Minshall RD, Pelligrino DA, Testai FD and Lazarov O. Neurogenesis and inflammation after ischemic stroke: what is known and where we go from here. J Cereb Blood Flow Metab 2014; 34: 1573-1584.

[5] Cho EY, Lee SJ, Nam KW, Shin J, Oh KB, Kim KH and Mar W. Amelioration of oxygen and glucose deprivation-induced neuronal death by chloroform fraction of bay leaves (Laurus nobilis). Biosci Biotechnol Biochem 2010; 74: 2029-2035.

[6] Jaeschke H and Woolbright BL. Current strategies to minimize hepatic ischemia-reperfusion injury by targeting reactive oxygen species. Transplant Rev (Orlando) 2012; 26: 103.

[7] Song L, Yang H, Wang HX, Tian C, Liu Y, Zeng XJ, Gao E, Kang YM, Du J and Li HH. Inhibition of 12/15 lipoxygenase by baicalein reduces myocardial ischemia/reperfusion injury via modulation of multiple signaling pathways. Apoptosis 2014; 19: 567-580.

[8] Lin WH, Xu F, Bao L, Zheng SY and Shen WM. Microenviromental change after synthetic E-selectins interfere in ischemia-reperfusion in rats and its contribution to endogenetic/exogenous never stem cells. Int J Clin Exp Pathol 2015; 8: 11847.

[9] James G and David B. The role of inflammation and interleukin-1 in acute cerebrovascular disease. J Inflamm Res 2013; 6: 121.

[10] Gulati P, Muthuraman A, Jaggi AS and Singh N. Neuroprotective effect of gadolinium: a stretch-activated calcium channel blocker in mouse model of ischemia-reperfusion injury. Naunyn Schmiedebergs Arch Pharmacol 2013; 386: 255-264.

[11] Macrae IM. Preclinical stroke research--advantages and disadvantages of the most common rodent models of focal ischaemia. Br J Pharmacol 2011; 164: 1062-1078.

[12] Marnett LJ, Rowlinson SW, Goodwin DC, Kalgutkar AS and Lanzo CA. Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. J Biol Chem 1999; 274: 22903-22906.

[13] Kirschenbaum A, Liotta DR, Yao S, Liu XH, Klausner AP, Unger P, Shapiro E, Leav I and Levine AC. Immunohistochemical localization of cyclooxygenase-1 and cyclooxygenase-2 in the human fetal and adult male reproductive tracts. J Clin Endocrinol Metab 2000; 85: 3436-3441.

[14] Kim SF, Huri DA and Snyder SH. Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. Science 2005; 310: 1966-1970.

[15] Gilroy DW and Colvillenash PR. New insights into the role of COX 2 in inflammation. J Mol Med 2000; 78: 121-129.

[16] Seibert K and Masferrer JL. Role of inducible cyclooxygenase (COX-2) in inflammation. Receptor 1994; 4: 17.

[17] Hawkey CJ. COX-2 chronology. Gut 2005; 54: 1509.

[18] de Vries EF. Imaging of cyclooxygenase-2 (COX-2) expression: potential use in diagnosis and drug evaluation. Curr Pharm Des 2006; 12: 3847.

[19] Fu JY, Masferrer JL, Seibert K, Raz A and Needleman P. The induction and suppression of prostaglandin H2 synthase (cyclooxygenase) in human monocytes. J Biol Chem 1990; 265: 16737.

[20] Candelario J and Fiebich B. Cyclooxygenase inhibition in ischemic brain injury. Current Pharmaceutical Design 2008; 14: 1401-1418.

[21] Jia L, Chen Y, Tian YH and Zhang G. MAPK pathway mediates the anti-oxidative effect of chicoric acid against cerebral ischemia-reperfusion injury in vivo. Exp Ther Med 2018; 15: 1640.

[22] Pang L, Cai Y, Tang EH, Yan D, Kosuru R, Li H, Irwin MG, Ma H and Xia Z. Cox-2 inhibition protects against hypoxia/reoxygenation-induced

cardiomyocyte apoptosis via Akt-Dependent enhancement of iNOS expression. Oxid Med Cell Longev 2016; 2016: 3453059.
[23] Nogawa S, Zhang F, Ross ME and Iadecola C. Cyclo-oxygenase-2 gene expression in neurons contributes to ischemic brain damage. J Neurosci 1997; 17: 2746.
[24] Wang LC, Huang CY, Wang HK, Wu MH and Tsai KJ. Magnesium sulfate and nimesulide have synergistic effects on rescuing brain damage after transient focal ischemia. J Neurotrauma 2012; 29: 1518.
[25] Candelariojalil E, Gonzálezfalcón A, Garcíacabrera M, Alvarez D, Aldalain S, Martínez G, León OS and Springer JE. Assessment of the relative contribution of COX-1 and COX-2 isoforms to ischemia-induced oxidative damage and neurodegeneration following transient global cerebral ischemia. J Neurochem 2003; 86: 545-555.
[26] Nakayama M, Uchimura K, Zhu RL, Nagayama T, Rose ME, Stetler RA, Isakson PC, Chen J and Graham SH. Cyclooxygenase-2 inhibition prevents delayed death of CA1 hippocampal neurons following global ischemia. Proc Natl Acad Sci U S A 1998; 95: 10954-10959.
[27] Ahmad M, Zhang Y, Liu H, Rose ME and Graham SH. Prolonged opportunity for neuroprotection in experimental stroke with selective blockade of cyclooxygenase-2 activity. Brain Res 2009; 1279: 168.
[28] Zhang Z, Gao Z, Hu W, Yin S, Wang C, Zang Y, Chen J, Zhang J and Dong L. 3,3'-Diindolylmethane ameliorates experimental hepatic fibrosis via inhibiting miR-21 expression. Br J Pharmacol 2013; 170: 649-660.
[29] Noetel A, Kwiecinski M, Elfimova N, Huang J and Odenthal M. microRNA are central players in anti- and profibrotic gene regulation during liver fibrosis. Front Physiol 2012; 3: 49.
[30] Yang Q, Yang K, Li A and Tan W. Anti-apoptosis and expression of microRNA-21 in rat myocardium during early ischemia-reperfusion injury. Zhong Nan Da Xue Xue Bao Yi Xue Ban 2013; 38: 483-489.
[31] Saikumar J, Hoffmann D, Kim TM, Gonzalez VR, Zhang Q, Goering PL, Brown RP, Bijol V, Park PJ, Waikar SS and Vaidya VS. Expression, circulation, and excretion profile of microRNA-21, -155, and -18a following acute kidney injury. Toxicol Sci 2012; 129: 256-267.
[32] Lu M, Jiang X, Tong L, Zhang F, Ma L, Dong X and Sun X. MicroRNA-21-regulated activation of the Akt pathway participates in the protective effects of H2S against liver ischemia-reperfusion injury. Biol Pharm Bull 2018; 41: 229-238.
[33] Kalthur G, Salian SR, Nair R, Mathew J, Adiga SK, Kalthur SG, Zeegers D and Hande MP. Distribution pattern of cytoplasmic organelles, spindle integrity, oxidative stress, octamer-binding transcription factor 4 (Oct4) expression and developmental potential of oocytes following multiple superovulation. Reprod Fertil Dev 2015; 28: 2027-2038.
[34] Culmsee C, Zhu C, Landshamer S, Becattini B, Wagner E, Pellecchia M, Pellechia M, Blomgren K and Plesnila N. Apoptosis-inducing factor triggered by poly (ADP-ribose) polymerase and Bid mediates neuronal cell death after oxygen-glucose deprivation and focal cerebral ischemia. J Neurosci 2005; 25: 10262-10272.
[35] Tu W, Xu X, Peng L, Zhong X, Zhang W, Soundarapandian MM, Balel C, Wang M, Jia N and Zhang W. DAPK1 interaction with NMDA receptor NR2B subunits mediates brain damage in stroke. Cell 2010; 140: 222-234.
[36] Andrabi SA, Kang HC, Haince JF, Lee YI, Jian Z, Chi Z, West AB, Koehler RC, Poirier GG and Dawson TM. Iduna protects the brain from glutamate excitotoxicity and stroke by interfering with parthanatos. Nat Med 2011; 17: 692-699.
[37] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.
[38] Yu L, Yang B, Wang J, Zhao L, Luo W, Jiang Q and Yang J. Time course change of COX2-PGI 2/TXA 2 following global cerebral ischemia reperfusion injury in rat hippocampus. Behav Brain Funct 2014; 10: 42.
[39] Yang JQ, Liu BZ, He BC and Zhou QQ. Protective effects of meloxicam on aluminum overload-induced cerebral damage in mice. Eur J Pharmacol 2006; 547: 52.
[40] Gaur V and Kumar A. Effect of nonselective and selective COX-2 inhibitors on memory dysfunction, glutathione system, and tumor necrosis factor alpha level against cerebral ischemia reperfusion injury. Drug Chem Toxicol 2012; 35: 218-224.
[41] Deng W, Wang Y, Long X, Zhao R, Wang Z, Liu Z, Cao S and Shi B. miR-21 reduces hydrogen peroxide-induced apoptosis in c-kit+ cardiac stem cells in vitro through PTEN/PI3K/Akt signaling. Oxid Med Cell Longev 2016; 2016: 5389181.
[42] Yang Q, Yang K and Li A. microRNA-21 protects against ischemia-reperfusion and hypoxia-reperfusion-induced cardiocyte apoptosis via the phosphatase and tensin homolog/Akt-dependent mechanism. Mol Med Rep 2014; 9: 2213-2220.
[43] Xu X, Kriegel AJ, Jiao X, Liu H, Bai X, Olson J, Liang M and Ding X. miR-21 in ischemia/reperfusion injury: a double-edged sword? Physiol Genomics 2014; 46: 789-797.
[44] Liu W, Chen X and Yu Z. Effects of microRNA-21 and microRNA-24 inhibitors on neuronal apoptosis in ischemic stroke. Am J Transl Res 2016; 8: 3179.