miRNA-135b-5p Regulates GSK3β-TLR3 Signal Pathway to Inhibit Inflammatory Response with Cerebral Ischemia-Reperfusion Injury in Mice

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Abstract: Objective: The mechanism of cerebral ischemia-reperfusion injury is closely related to inflammation, and miRNA-135b-5p may play a key role in the inflammation of cerebral ischemia injury. In this study we investigate the effect of miRNA-135b-5p through GSK3β-TLR3 signal axis on inflammatory response in mice with cerebral ischemia-reperfusion injury. Methods: 48 mice were randomly divided into sham operation group (n = 12), model group (n = 12), negative control group (n = 12) and overexpression group (n = 12). After neurobehavioral score, the left brain of mice was killed and the water content of brain tissue was measured, the expression levels of TNF-α and IL-6 in brain tissue and serum were detected by ELISA method, the expression levels of TLR3, GSK3β and ERK protein in brain tissue were detected by Western Blot, and the mRNA expression levels of TLR3, GSK3β and ERK in each group were detected by RT-PCR. Results: The neurobehavioral scores in the model group, negative control group and overexpression group were significantly higher than those in the sham operation group (P < 0.05), and the neurobehavioral scores in the overexpression group were significantly lower than those in the negative control group and model group (P < 0.05). The expression level of miRNA-135b-5p in brain tissue of mice in model group and negative control group was significantly lower than that in sham operation group, while the expression level of miRNA-135b-5p in overexpression group was significantly higher than that in other three groups. The expression levels of TNF-α and IL-6 in brain tissue and serum of mice in model group and negative control group were significantly higher than those in sham operation group, while the expression levels of TNF-α and IL-6 in overexpression group were significantly lower than those in sham operation group, while the expression level of miRNA-135b-5p in overexpression group was significantly higher than that in other three groups. The expression levels of TNF-α and IL-6 in brain tissue and serum of mice in model group and negative control group were significantly higher than those in sham operation group, while the expression level of miRNA-135b-5p in overexpression group was significantly lower than that in sham operation group, while the expression level of miRNA-135b-5p in overexpression group was significantly higher than that in other three groups. The expression levels of TNF-α and IL-6 in brain tissue and serum of mice in model group and negative control group were significantly higher than those in sham operation group, while the expression level of miRNA-135b-5p in overexpression group was significantly lower than that in sham operation group, while the expression level of miRNA-135b-5p in overexpression group was significantly higher than that in other three groups. The expression levels of TNF-α and IL-6 in brain tissue and serum of mice in model group and negative control group were significantly higher than those in sham operation group, while the expression level of miRNA-135b-5p in overexpression group was significantly lower than that in sham operation group, while the expression level of miRNA-135b-5p in overexpression group was significantly higher than that in other three groups. Conclusion: Overexpression of miRNA-135b-5p could inhibit the expression of GSK3β-TLR3 signal axis related genes and reduce the inflammatory response during cerebral ischemia-reperfusion injury, which may be a new strategy for the treatment of cerebral ischemia-reperfusion injury.

Keywords: miRNA-135b-5p, GSK3β, TLR3, cerebral ischemia-reperfusion injury

1. Introduction

Cerebral ischemia after stroke can lead to severe brain injury, which can be aggravated during reperfusion [1]. The injury mechanism is mainly related to Ca2+ overload, inflammation, glutamate excitotoxicity, excessive production of reactive oxygen species (ROS) and apoptosis[2,3] caused by ischemia-reperfusion. Studies have found that in targeting specific miRNAs of cerebral ischemia-reperfusion injury (IRI), it has been shown to prevent neuronal injury both in vitro and in vivo[4]. MiR-135b-5p is a reported anti-apoptotic miRNA, involved in the regulation of inflammation and cell differentiation[5]. Our previous study found that in the OGD/R model[6], miR-135b-5p can down-regulate the expression of GSK3β and reduce the oxidative stress response to protect neurons from injury. GSK3β
is also a key mediator of inflammatory cytokines production during brain injury [7,8]. GSK3β can selectively regulate TLR3 to promote the release of inflammatory cytokines [9]. However, it is not clear whether miR-135b-5p can regulate the expression of GSK3β and its downstream inflammatory factors and reduce the inflammatory response during cerebral ischemia-reperfusion. Therefore, the purpose of this study is to explore the effect of miR-135b-5p on cerebral ischemia-reperfusion injury in mice through GSK3β-TLR3 signal axis, so as to provide a new idea for the treatment of ischemia-reperfusion injury.

2. Material and methods

2.1. Experimental animals

A total of 48 C57BL/6 mice weighing 18-25 g were selected. The experimental animals were provided by the Animal Experimental Center of three Gorges University, license number: SCXK (E) 2020-0412. The mice were free to eat and drink before operation.

2.2. Model and groups

Mice were anesthetized by intraperitoneal injection of 3.5% chloral hydrate (350mg/kg), and the model of cerebral ischemia-reperfusion injury was established by thread occlusion method. The thread was sent through the internal carotid artery to the connection between the anterior cerebral artery and the middle cerebral artery to make the blood supply area of the middle cerebral artery ischemic. After 1 hour of ischemia, the thrombus was carefully removed and the stump of the common carotid artery was ligated and reperfused for 24 hours (OGD/R). 48 mice were randomly divided into sham operation group (only incision of neck skin, ligation of left common carotid artery without insertion of thrombus, Sham), model group (cerebral ischemia-reperfusion injury model in mice, injection of isotonic saline, OGD/R+Saline), negative control group (injection of miRNA-135b-5p negative control miRNA -135b-5p agomir NC, OGD/R+miRNA-135b-5p-NC), overexpression group (miRNA-135b-5p agonist miRNA-135b-5p-OE). There were 12 rats in each group.

2.3. Main instruments and equipment

Including electrophoresis instrument and film transfer instrument (Bio-Rad company), chemiluminescence imaging system (GE company), dehydrator and embedding machine (Changzhou Zhongwei Electronic equipment Co., Ltd.), paraffin slicer (Leica company of Germany), optical microscope (Olympus company of Japan), automatic enzyme labeling instrument (Thermo company of USA), fluorescence quantitative PCR instrument (Bio-Rad company), freezing centrifuge (Eppendorf company of Germany) and so on.

2.4. Main reagents

Including overexpressed miRNA-135b-5p and negative control virus (Shanghai Jikai Genochemical Technology Co., Ltd.), phosphate buffer (China Jinuo Biomedical Technology Co., Ltd.), Trizol (Japan TAKARA Co., Ltd.), A11-in-one miRNAqRT-PCR Detection Kit (GeneCoPoeia Co., Ltd.), gene mutation primers and quantitative PCR primers (synthesized by Shanghai Bioengineering Co., Ltd.), 5×PfimeScfiptRTMasterMix (Japan Takara Co., Ltd.), mouse TNF-α, IL-6ELISA kit (Xin Bosheng Biotechnology Co., Ltd.). The other reagents are homemade analytical purity.

2.5. Neurobehavioral scores of mice

The paralysis, standing, flexion and walking of the limbs of mice were observed. The neurological function was scored by 5-grade 4-point method: no obvious neurological defect was scored as 0, left forelimb extension disorder as 1, rotation to the left as 2, dumping to the left as 3, unable to walk spontaneously, loss of consciousness, coma as 4, animal death as 5. The mice with scores of 0, 4 and 5 were all removed, and the excluded mice were randomly selected in the follow-up experiment.

2.6. Water content of brain tissue measurement

The complete left brain tissue was immediately weighed in an electronic balance (wet weight), then
the brain tissue was baked in an oven at 70 ℃ for 72 hours, and the weighing (dry weight) was measured again. The formula for calculating the water content of brain tissue is as follows: water content of brain tissue (%) = (wet weight-dry weight) / wet weight × 100%.

2.7. ELISA analysis

The brain tissue was placed in a glass homogenizer and fully ground with isotonic saline in an ice bath according to the ratio of body weight to volume at 1:9 to make 10% brain homogenate, then 8000r/min centrifugation, centrifugal radius 6.9cm, centrifugal 10min, the supernatant was taken, and the expression levels of inflammatory cytokines TNF-α and IL-6 in brain tissue and serum of mice in each group were detected.

2.8. Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from brain tissue and peripheral blood by Trizol method. CDNA, miR-135b-5p was obtained by reverse transcription with U6 as internal reference. SYBRGreenI real-time fluorescence quantitative PCR was used to detect the expression level of miR-135b-5p gene in each group. The primers were designed and synthesized by Shanghai Shenggong Company, and U6 was used as the internal control of miR-135b-5p normalization. The primers were designed as follows: miR-135b-5p F: 5'-GGTATGGGCTTTTCTTCATCTTCC-3' and R: 5'-ATTGGAACGATAACAGAAGATT-3' and R: 5'-GGAAACGGTTCAAGAATTTGG-3';GSK3βF: 5'-ATGGCAAGCAAGTAAACCACAG-3' and R: 5'-TCTCGGTTCCTTAAATCGTGTGTC-3'. The relative expression of miR-135b-5p gene in each group was calculated according to the formula (2^{-ΔΔCt}).

2.9. Western blot analysis

The total protein was extracted from brain tissue by adding cell lysis solution on ice, and the protein was quantified by BCA protein concentration determination kit. The protein sample was evenly mixed with the sample buffer solution and denatured in boiling water bath. The same amount of denatured protein sample was added to the sample hole and SDS-PAGE gel electrophoresis. After protein separation, transfer to PVDF membrane, seal the membrane in 5% skimmed milk powder for 1 hour, then add first antibody (diluted 1 / 15 500) to stay overnight at 4 ℃, and then add horseradish peroxidase labeled second antibody (diluted 1 / 3 000) after TBST washing membrane. After washing the membrane at room temperature for 1 hour, ECL chemiluminescence was used to take pictures in the dark room. β-actin was used as the internal standard protein. The total gray value of protein bands in each group was analyzed by Bandscan5.0 software. The relative expression levels of TLR3, GSK3β and ERK proteins in each group were detected.

2.10. Statistical analysis

Data were expressed as mean ± standard deviation. Student’s t-test and one-way analysis of variance (ANOVA) with Bonferroni post hoc test were used to determine the significance levels of differences using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA). Differences with p-values less than 0.05 were considered statistically significant.

3. Results

3.1. Neurobehavioral Scores

The neurobehavioral score in the model group, negative control group and overexpression group was significantly higher than that in the sham operation group (P<0.05), and the neurobehavioral score in the overexpression group was significantly lower than that in the negative control group and model group (P < 0.05) (Figure 1A). It is suggested that there is cerebral nerve function injury in cerebral ischemia-reperfusion model mice, and overexpression of miR-NA-126 can improve the nerve injury in mice.

3.2. Water content in brain tissue of mice

The water content of brain tissue in model group and negative control group was significantly higher than that in the sham operation group (P<0.05), and the neurobehavioral score in the overexpression group was significantly lower than that in the negative control group and model group (P < 0.05) (Figure 1A). It is suggested that there is cerebral nerve function injury in cerebral ischemia-reperfusion model mice, and overexpression of miR-NA-126 can improve the nerve injury in mice.
than that in sham operation group (P<0.05). The water content of brain tissue in the overexpression group was significantly lower than that in the model group (P<0.05) (Figure 1B). It is suggested that overexpression of miRNA-126 can reduce the water content of brain tissue in the model group.

Figure 1: Effect of miRNA-135b-5p on neurobehavioral scores and water content of brain tissue in cerebral ischemia-reperfusion model mice (x±s, n = 12)

3.2.1. Expression level of miR-135b-5p mRNA in brain tissue

The expression level of miR-135b-5p mRNA in brain tissue of mice in model group and negative control group was significantly lower than that in sham operation group, while the expression level of miR-135b-5p mRNA in overexpression group was significantly higher than that in other three groups. It is suggested that the mouse model of miR-135b-5p overexpression was successfully established. (Figure 2)

Figure 2: The expression level of miR-135b-5p mRNA in brain tissue of mice in each group (x±s, n = 12)

3.2.2. Expression level of axis-related protein of GSK3β-TLR3 signal in brain tissue

The protein expression levels of TLR3, GSK3β and ERK in the model group and negative control group were significantly higher than those in the sham operation group, while the expression levels of TLR3, GSK3β and ERK in the overexpression group were significantly lower than those in the model group and negative control group. (Figure 3)
3.2.3. Expression levels of TLR3, GSK3β and ERK mRNA in brain tissue of mice

The expression levels of TLR3, GSK3β and ERK mRNA in brain tissue of mice in model group and negative control group were significantly higher than those in sham operation group, while the expression levels of TLR3, GSK3β and ERK mRNA in overexpression group were significantly lower than those in model group and negative control group. It is suggested that the GSK3β-TLR3 signal axis is activated in the model group, and overexpression of miR-135b-5p can reduce the protein and mRNA expression levels of GSK3β-TLR3 signal axis related genes. (Figure 4)

4. Discussion

During the reperfusion of the local tissue of cerebral ischemia, the inflammatory cells in the brain tissue are activated rapidly and express a large number of inflammatory cytokines, and these cytokines can release a large number of oxygen free radicals, cytokines and other inflammatory mediators outside the cell, which could aggravate brain tissue damage \([10,11]\). TNF-α and IL-6 play an important role in inflammatory response and cerebral ischemic injury, they can induce the production of other inflammatory mediators and initiate a variety of cytokine responses \([12]\). In cerebral ischemia-reperfusion injury, IL-1β can cooperate with other cytokines to promote the activation of B and T cells, and induce the production of inflammatory factor TNF-α. The expression of IL-6 began to increase 12 hours after cerebral ischemia-reperfusion in rats \([13]\). The results of this study suggest that the model is established successfully, and the inflammatory level of brain tissue in the model group is significantly increased, which is consistent with the above results.

As a multifunctional protein kinase, GSK3β participates in oxidative stress after cerebral ischemia and is also a key mediator of inflammatory cytokines, such as IL-6, TNF-α, p40 and IFN-γ \([14]\). At the same time, GSK3β is also involved in different TLR signal transduction \([15,16]\) and can regulate the activity of CREB in Myd88 dependent TLR pathway \([17]\). Other studies have shown that GSK 3β can regulate the activity of signal transducer and activator of transcription 3 (STAT3) induced by IFN-γ, and is necessary for the synergistic effect of LPS and IFN-γ on IL-6 cytokines \([18]\). In this study, it was found that the
expression levels of GSK3β, TLR3, ERK protein and mRNA in brain tissue of model group and negative control group were significantly higher than those of sham operation group, suggesting that GSK3β-TLR3 signal axis was activated in cerebral ischemia-reperfusion mice, which promoted the inflammatory level of cerebral ischemia-reperfusion.

miRNA-135b-5p is a reported anti-apoptotic miRNA. It has been reported that miRNA-135b-5p participates in the regulation of inflammation and cell differentiation\(^\text{[5]}\), and it also promotes tumorigenesis by inhibiting apoptosis, and protects osteoblasts from dexamethasone-induced apoptosis \(^\text{[19]}\). In addition, the down-regulated expression of miRNA-135b-5p in the hippocampus of rats with temporal lobe epilepsy can regulate the apoptosis of hippocampal neurons \(^\text{[20]}\). In mouse hippocampal neurons, miRNA-135b-5p is maladjusted under oxidative stress and has a neuroprotective effect in neurodegenerative diseases (Alzheimer's disease) \(^\text{[21]}\). The results of this study are similar to those mentioned above. And miRNA-135b-5p may down-regulate the secretion of inflammatory cytokines through GSK3β-TLR3 signal axis, so as to improve the inflammatory microenvironment of cerebral ischemia-reperfusion mice, which may be one of its mechanisms.

Anyway, miRNA-135b-5p may reduce the inflammatory response of brain tissue by targeting the expression of GSK3β-TLR3 signal axis-related genes, and protect against neurological dysfunction caused by cerebral ischemia-reperfusion injury.

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Declaration of interest

The authors declare that they have no conflict of interest.

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