Down-regulated miR-21 promotes learning-memory recovery after brain injury

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Received December 10, 2018; Accepted January 20, 2019; Epub March 1, 2019; Published March 15, 2019

Abstract: Background: MicroRNA is an endogenous non-coding single strand RNA which consists of 22 nt. It post-transcriptionally regulates gene expression and development. MicroRNA 21 plays an important role in repairing injured brain tissues. Thus, in this research, we explored the function of miR-21 in learning-memory recovery after brain injury. Method: 3 days old newborn SD rats were separated into three groups: Sham operation group (Sham), inflammation-sensitized hypoxic-ischemic brain injury (LPS+HI) group and miR-21 inhibitor group. 28 days later, the learning and memory capability was assayed by water maze. H&E staining and Nissl's staining were used to assay pathologic changes and TUNEL was used to assay neuron apoptosis in brain tissue. Results: Water maze assay showed that the capability of positioning navigation in the IH-HI group was worse than in the Sham group and miR-21 inhibition group, and the Sham group was better than miR-21 group. Both of the comparisons had statistical significance (P < 0.05). H&E staining in the sham group showed that the neurons were arranged well in hippocampus. In LPS+HI group, some neurons in hippocampus had vacuolar degeneration and the neurons were not well arranged well. In the hippocampus of miR-21 inhibitor group, the neuron cell layers were decreased but the neurons were arranged better than in the LPS+HI group. Nissl’s staining in LPS+HI group showed neuronal edema, neurons decreased, and Nissl’s bodies decreased in the cytoplasm compared with the sham group. However, compared with the LPS+HI group, Nissl’s staining in miR-21 inhibitor group showed that the neuronal edema was alleviated and neurons were better arranged. TUNEL assay showed that the apoptosis rate of LPS+HI group was higher than in the miR-21 inhibitor group and miR-21 inhibitor group was higher than the sham group. Conclusion: Down-regulated miR-21 can alleviate LPS+HI injury in the brain.

Keywords: Brain injury, learning-memory recovery, miR-21

Introduction

Brain injury in premature infants can be induced by hypoxia/ischemia, or infection/inflammation [1]. Recently, the mortality rate of premature babies decreased significantly, but there are still 25%-50% premature babies that have various degrees of neurodevelopmental disorders, such as childhood cerebral palsy, epilepsy, and attention deficit. Currently, there is no effective treatment method for neurodevelopmental disorders [2].

miR-21 is widely spread in human tissues and involved in important physiologic processes, including cell growth, proliferation, differentiation, apoptosis and inflammation/immune [3]. miR-21 has an anti-inflammatory function. microRNA-21 can down-regulate inflammation and inhibits periodontitis [4]. Inhibition of microRNA-21 significantly reduces pancreatitis severity in wild-type (WT) mice [5]. These reports inspired us to speculate whether miR-21 has the function of protecting from LPS+HI induced brain injury.

In this study, we established an LPS+HI mouse model in order to evaluate miR-21 function in brain injury and try to get a clinical indication from our experiment.

Materials and methods

Animals

30 3-day old SD SPF rats were recruited in this project. The weight of each rat varied from
2.5-3 g. The animal experiment was approved by the Animal Care and Use Committee of Sichuan University. The rats were divided into 3 groups and each group contained 10 rats. All the groups were kept in the environment of 22-25°C, 55-60% relative humidity and 12 h/12 h light/dark cycle. In the LPS+HI and miR-21 inhibitor group, the rats were used to establish LPS+HI model. First, the rats were intraperitoneally injected with LPS 50 ng per gram according to each mouse weight. After 2 hours, the rats were anaesthetized by ether. After skin disinfection with alcohol, an incision was made in the anterior neck. Then, the right common carotid artery was ligated and the skin incision was sutured. After surgery, the mice were then placed in a chamber on the heating pad (36°C) and supplied with 8% oxygen for 1 hour. Finally, the rats were returned to their dams. In sham group, the rats were only performed surgery but were no injected with LPS and no ligated.

In miR-21 inhibitor group, 1 pmol antagomir-21 was injected by caudal vein from the second day to 28th day after modeling. The antagomir-21 sequence was 5'UCAACAUCAGUCUGAUAAGCUA-3' and was synthetized from Sangon Biotech (Shanghai) Co., Ltd.

**Morris water maze assay**

The apparatus was a black circular pool (200 cm in diameter) with walls 100 cm high. The pool was filled with water (50 cm depth) maintained at 25°C, which was made opaque by the addition of a nontoxic black paint. In the middle of the pool, there was a circular (10 cm in diameter) platform positioned 2 cm below the surface. The pool is divided into quadrants. Data were collected with a camera fixed to the ceiling of the room and connected to a computer.

**Place navigation**

Each rat was put into the 4 quadrants facing the pool wall respectively. Escape latency was defined from rats entering the water to climbing up the platform. If the escape latency time was over 120 s, the rats were pulled to the platform and the escape latency was recorded as 120 s. Every time, we let the rats rest on the platform for 20 s. The rats were trained for 3 days and on the 4th day, the escape latency of each rat was the average value of 4 times recorded.

**Spatial probe test**

on the 5th day, the platform was removed and we put each rat in the 4 quadrants respectively. We counted the times each rat came cross the area in which the platform was in 120 s. This was tested 4 times and we took the average value.

**H&E staining**

The rats were anaesthetized and then were fixed by 4% paraformaldehyde through cardiac route. The brains were harvested, embedded by paraffin and serially sectioned through the hippocampus. The slices were processed with H&E staining according to protocol.

**Nissl’s staining**

The sections were dewaxed in xylene, passed in alcohols with gradient concentrations, and placed in hematoxylin. Then, the slices were washed, decolorized, dehydrated, air-dried and cover-slipped.

**TUNEL assay**

5 μm slices were dewaxed in xylene, passed in alcohols with gradient concentrations, washed in PBS 2 times, and then stained according to the protocol of theTUNEL kit. Each slice was selected from 5 fields at ×400 and we calculated the apoptosis rate. Apoptosis rate = apoptosis number/total cell number.

**Statistical analysis**

ANOVA was used for two group comparison. One-way ANOVA was used for multi-group comparison analysis. SNK-q was used to analyze between comparisons. All the data were analyzed by using SPSS Statistical Package version 22. P < 0.05 was considered statistically significant.

**Results**

**miR-21 and the LPS+HI induced brain injury**

The expression of miR-21 decreased in the miR-21 inhibitor group and LPS+HI group (Figure 1A). Place navigation test showed that miR-21 group was better than the LPS+HI group but worse than sham group (Figure 1B, 1C). Spatial probe test had the same results as
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place navigation (Figure 1D). The difference had statistical significance $P < 0.05$ (Figure 1).

**H&E staining**

Compared with the control (Figure 2A), the CA1 region of hippocampus had pathological changes in LPS+HI group including neuron disseminated vacuolar degeneration, cell arrangement disorder, neuron decrease, and apoptosis (Figure 2B). In the miR-21 inhibitor group, the cell layers decreased (Figure 2C). The DG region of hippocampus had the similar pathologic changes (Figure 2D-F).

**Nissl's staining**

Compared with the controls (Figure 3A), The CA1 region of hippocampus showed pathologic changes in the LPS+HI group, including neuron edema, cell number decrease, cell gap increase and a decrease in Nissl's bodies in the cytoplasm (Figure 3B). In the miR-21 inhibitor group, the neuron edema was alleviated, cell number increased, and neurons arranged better than in the LPS+HI group. (Figure 3C). The DG region had similar pathologic changes (Figure 3D-F).

**Apoptosis in brain injury**

The apoptotic rate in three groups was assayed by TUNEL. Apoptosis rate of LPS+HI group (7.28±1.08%) was higher than in the miR-21 inhibitor group (2.98±0.09) ($P < 0.05$) and the apoptosis of miR-21 inhibitor group was higher than sham group (0.91±0.41) ($P < 0.05$). (Figure 4). This result indicated that down regulated miR-21 could alleviate the cell death of brain injury.

**Discussion**

Many harmful factors in the perinatal period may promote premature brain injury progres-
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Figure 2. Neurons in the right hippocampal CA1 region of inflammation-sensitized hypoxic-ischemic brain damaged rats. A: Sham-operation group; B: LPS+HI group; C: Anti-miR-21 group. Neurons in the right hippocampal DG region of inflammation-sensitized hypoxic-ischemic brain damaged rats. D: Sham-operation group; E: LPS+HI group; F: Anti-miR-21 group. Apoptotic cells are marked by arrows.

Figure 3. Neurons in the right hippocampal CA1 region of inflammation-sensitized hypoxic-ischemic brain damaged rats, Nissl’s staining. A: Sham-operation group; B: LPS+HI group; C: Anti-miR-21 group. Neurons in the right hippocampal DG region of inflammation-sensitized hypoxic-ischemic brain damaged rats. D: Sham-operation group; E: LPS+HI group; F: Anti-miR-21 group.

Figure 4. Apoptosis in the right hippocampal neurons of inflammation-sensitized hypoxic-ischemic brain damaged rats.
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Infection in the perinatal period not only may lead to premature birth, but also has a sensitization role on premature LPS+HI brain injury. However, the mechanism of sensitization is still unclear and there is no effective treatment method. Recent research showed that LPS+HI brain damage caused cell death including necrosis, apoptosis, autophagy, and necroptosis [6].

The Morris water maze is a good model which is used for researching hippocampus-related spatial learning and memory and can well reflect the spatial learning and memory ability. In this study, we used Morris water maze to test the recovery of LPS+HI brain damage. Our results showed that the miR-21 inhibitor group performed much better in Morris water maze test than the LPS+HI group. Considering the route of swimming, the miR-21 inhibitor group performance suggested downregulated miR-21 could help LPS+HI brain damage recover.

The limbic system of the brain is the physiologic basis of learning and cognition, especially the hippocampus, which is responsible for spatial learning and memory. Changes in neuron number and structure in the hippocampus will cause learning and memory ability changes [7]. H&E staining of brain slices on 3-day SD rats with LPS+HI brain damage showed that neuron swelling, and necrosis [8]. In our study, LPS+HI brain injury also showed pathologic changes in the hippocampus CA1 and DG regions, including neuron vacuolar degeneration, apoptosis, and damaged neuron structure. This provided the pathologic basis for the decreased learning and memory.

In the miR-21 inhibitor group, the hippocampal pathologic changes induced by LPS+HI were significantly alleviated. However, in glioma cells, the expression of miR-21 was upregulated. Inhibition of miR-21 promoted glioma cell apoptosis [9, 10]. The research on miR-21 and apoptosis seems to contradict our results. miR-21 expression is up-regulated in P. gingivalis LPS-stimulated macrophages. miR-21 mimic inhibits the pro-inflammatory cytokine production by macrophages. Combined with our results, we speculate that the protective function of downregulated miR-21 is due to preventing excessive inflammation. However, the molecular mechanism is still unclear and we will explore it in the future.

Acknowledgements

This research was supported by the National Science Foundation of China (81771634).

Disclosure of conflict of interest

None.

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