Expression and related mechanism of miR-744 in ischemia-reperfusion rat model

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ABSTRACT

To study the expression of miR-744 in the rat model of ischemia-reperfusion (I-R) and its related mechanism. Seventy-five Wistar rats were randomly divided into 3 groups: sham operation group (SOG), model group (MG) and miR-744 group, with 25 in each group. The expression levels of IL-1β, IL-6 and TNF-α were observed by Western Blot after the model preparation, while miR-744 expression was detected by reverse transcription polymerase chain reaction (RT-PCR). The cerebral infarction volume of rats in the MG was significantly larger than that in the miR-744 group (P<0.05). The MG exhibited a markedly higher brain tissue water content than the SOG and the miR-744 group (P<0.05). When compared within the latter two groups, the brain tissue water content in the SOG was significantly lower than that in the miR-744 group (P<0.05). As to miR-744 expression, the relative expression of miR-744 in the brain tissue of the MG was the lowest among the three groups. When compared within the remaining two groups, the miR-744 expression of the miR-744 group was remarkably higher than that of the SOG (P<0.05). In terms of the expression levels of inflammatory factors, the expressions of IL-1β, IL-6 and TNF-α in the brain tissue of the SOG and the miR-744 group were significantly lower than those in the MG, and those of the SOG were significantly lower than that of the miR-744 group. MiR-744 may be involved in the development and progression of I-R in rats, and its mechanism may be related to the regulation of inflammatory response.

Introduction

Cerebrovascular disease ranks the third leading cause of death in human beings in the world and is the first cause of death among Chinese nationals. The prevalence, morbidity and mortality of stroke are increasing year by year, bringing huge social and economic burdens (1-3). About 60%-80% of the annual case of cerebrovascular disease is ischemic cerebrovascular disease (ICVD), which is characterized by high prevalence, high mortality, high recurrence rate and disability rate (4, 5).

Cerebral ischemia is induced by cerebral blood flow reduction and brain damage caused by vascular occlusion. The longer the cerebral blood flow reduction lasts, the more severe the brain damage will be (6, 7). Its pathogenesis is often accompanied by the production of various inflammatory mediators, the infiltration and accumulation of a large number of inflammatory cells in damaged brain tissues, the destruction of the blood-brain barrier and the secretion of inflammatory factors, as well as the up-regulation of adhesion molecules (8,9). Guan (10) showed that the down-regulation of NF-kB-mediated inflammatory response could protect the brain from ischemic injury caused by MCAO, indicating that the inflammatory response played an important role during cerebral ischemia and I-R injury. MicroRNAs (miRNAs) are a group of short sequences of RNAs widely existing in organisms that do not encode proteins. They can bind to the 3' non-coding region of the mRNA of a specific gene, hinder its translation or directly degrade it, so as to regulate the target gene (11, 12). It is well established that miRNAs are closely related to ischemic cerebrovascular disease (13, 14). Among them, miR-744 is one of the early discovered members of the miRNA family, and the current research on miR-744 mainly focuses on malignant tumors (15).

At present, the clinical treatment of stroke is mainly focused on the following aspects: neuroprotection, anticoagulation, thrombolysis, increased blood flow and blood pressure (16,17). However, due to the complexity of the pathophysiological process of ischemic stroke, no completely effective treatment drugs have been found, so exploring the relevant molecular mechanism of cerebral I-R injury has important clinical application value (18). In this study, rat models of complete cerebral ischemia and cerebral I-R were established to study the neuronal injury and its inflammatory response mechanism caused by cerebral ischemia and I-R.

Materials and Methods

A total of 75 clean-grade Wistar rats were purchased from Shandong Lukang Pharmaceutical Co., Ltd., with a body mass of 220-240 g and a age of 3-7 weeks. All rats were fed in a well-ventilated environment with an indoor humidity of (50.00±5.00)% and a temperature of 22-26°C. They were given free access to food and water, with the chow provided by Jiangsu Xietong Pharmaceutical Bioengineering Co, Ltd. The experiment was approved by the Medical Ethics Committee of the hospital, and the experiment was conducted in accordance with the Guide for...
the Care and Use of Experimental Animals (19). Animal model preparation: According to the principle of similar body weight, all the rats were randomly divided into the SOG, MG and miR-744 group by random number table method after 1 week of adaptive feeding, with 25 rats in each group. In the MG and miR-744 group, modified nylon suture-occluded method was applied to model focal cerebral I-R in rats: Before model establishment, the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (35 mg/kg). Decreased muscle tone, no response to external stimuli, and a lethargic state of rats indicated that the anesthesia was effective. Then the rats were fixed on the operating table in the supine position, the skin was prepared, and the skin was cut longitudinally through the median cervical incision to separate the subcutaneous tissue. And then the right common carotid artery was ligated near the heart, the external carotid artery was ligated at the bifurcation of the common carotid artery and the internal carotid artery was temporarily closed with a micro-artery clamp. Next, small incision was cut 2 mm away from the bifurcation of the common carotid artery, and the paraffin-coated fish line was inserted into the internal carotid artery at a depth of about 18 mm from the bifurcation of the common carotid artery. Finally, fixed the suture occlusion and loosened the arteriole clamp. Rats in the SOG were performed the same basic operations as the MG and miR-744 group, apart from that the suture occlusion was not inserted and the blood flow was not blocked. After the blood flow was blocked for 120 min in rats in the MG and the miR-744 group, the suture occlusion was removed to restore blood perfusion. Twenty-four hours after reperfusion, the rats were scored for neurological deficit according to Longa scoring criteria (20), whose score and clinical manifestation were as follows: 0 point: normal nerve function and activity; 1 point: unable to straighten the left front paw; 2 points: rear-end turn to the left when crawling; 3 points: leaning to the left when walking; 4 points: difficulty in walking and consciousness. A score of 1-3 points indicated that the model was successfully prepared. The MG and miR-744 group each had 25 rats successfully modeled. After modeling, 80 mg/kg miR-744 was injected into the tail vein of rats in both the miR-744 group and the MG when the blood flow was blocked, while rats in the SOG did not receive any injection.

Indicator detection

Calculation of cerebral infarction area: Having completed the model preparation, the rats were anesthetized and executed by cervical dislocation. The brain tissue was taken out and stored in the refrigerator at -20°C for freezing. After 30 min, the coronal section (about 2 mm thick) was placed in a 2% TTC solution and incubated in a constant temperature water bath at 37°C in the dark for 30 min. Then the photos were taken, the area and thickness of the infarction were analyzed by computer, and the percentage of cerebral infarction volume was calculated. Cerebral infarction volume = infarction area \times thickness; Percentage of cerebral infarction volume = total volume of brain infarction/total brain volume \times 100%. The water content of the tissue was determined by the wet-dry weighting method. The rats were killed by rapid cervical dislocation to collect the brain tissue. Then, the ischemic lateral hemisphere was cut along the midline of the brain, and the liquid on the surface of the brain tissue was blotted with filter paper and immediately weighed on an electronic analytical balance. And then, it was placed in an oven at 110°C for 24 hours, and the dry weight was weighed to calculate the water content of the brain tissue. The water content of the brain tissue = (brain tissue wet weight - brain tissue dry weight)/brain tissue wet weight \times 100%.

The expression of IL-1β, IL-6 and TNF-α protein in tissues was detected by Western-Blot: Tissue samples preserved in liquid nitrogen were taken out and homogenized routinely, and the collected third-generation BMSCs cells were extracted by RIPA lysis method to extract total proteins and denatured proteins. The protein concentration was detected by the colorimetric method with Coomassie Brilliant Blue and separated by Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). After the electrophoresis, the protein was rinsed with deionized water and transferred to the nitrate cellulose film, which was then sealed in 5% skim milk powder at 4°C overnight. Next, the sealing solution was discarded and rinsed with TBST 2-3 times. The primary antibody diluted at 1:500 was added and incubated in a shaker for 2 h at room temperature. After TBST rinsing, horseradish peroxidase (HRP)-labeled sheep anti-rat secondary antibody was added and incubated at 37°C for 1 h. Finally, diaminobenzidine (DAB) staining was performed after TBST rinsing, and the results were measured and analyzed with Gelpro4.0 gel absorbance analysis software. The relative expression level of protein = target protein band gray value/β-Actin protein band grayscale value.

Detection of miR-744 expression in brain tissues

Twenty-four hours after the cerebral ischemia injury, the rats were decapitated after anesthesia, and the brain tissue of the rats was cut into pieces. After liquid nitrogen was added to the mortar, the cut tissue was ground into powder in the mortar. Then, 80 mg powder was taken to the EP tube, followed by the addition of 1 ml TRIzol lysate, mixed evenly, and then left for pyrolysis at room temperature for 5 min. After that, the EP tube was added with 200 μl chloroform, shaken up and down violently for 15 s, and centrifuged at 10000 r/min for 5 min at 4°C. Pipetted the upper aqueous layer into a new EP tube, extract RNA with 500 μl isopropanol, and gently invert up and down 10 times. The tube was then left standing at room temperature for 10 min, and centrifuged for 10 min at 10000 r/min. The supernatant was discarded, followed by the precipitation using 75% ethanol-suspended RNA, centrifuged at 10000 r/min for 15 min at 4°C, and the re-discarded the supernatant. Finally, the EP tube was placed on the ultra-clean table to air dry, and the extracted RNA concentration was determined by an UV spectrophotometer after the water suspension of diethylpyrocarbonate (DEPC) was added. The cDNA of miR-181a was synthesized by reverse transcription according to the instructions of the reverse transcription kit, and the level of miR-181a was detected by real-time fluorescence quantitative PCR.

Statistical analysis

Statistical analysis was performed using SPSS22.0 (Shanghai Yuchuang Network Technology Co., Ltd., Shanghai, China). The measurement data was expressed in the form of (x±sd), and the comparison was conducted by an independent sample t-test. While the counting data were expressed as percentages (%), and the inter-group...
comparison was carried out by a chi-square test, while the comparison between multiple time points was analyzed by repeated measures of variance. P<0.05 indicated a statistically significant difference.

Results

General information about the rats

No significant difference was observed among the SOP, MG and miR-744 groups in terms of general information, including gender, age, length, Glu, pre-modeling body mass, post-modeling body mass, indoor temperature, and indoor humidity, which indicated that there was no statistical significance (P>0.05). (Table 1)

Comparison of cerebral infarction volume and brain tissue water content among the SOG, MG, and miR-744 group

The cerebral infarction volume and brain tissue water content of the rats in each group identified significant differences (P<0.05). The cerebral infarction volume of the MG was significantly higher than that of the miR-744 group (P<0.05). The MG presented the highest brain tissue water content among the three groups (P<0.05), while when compared within the SOG and miR-744 group, the brain tissue water content of the former was markedly lower than that of the latter (P<0.05). (Table 2 and Figure 1)

Comparison of miR-744 expression in the brain tissue of rats in the SOG, MG and miR-744 group

The relative expression levels of miR-744 in the brain tissue of rats in the SOG and MG and miR-744 group were 1.12±0.04, 0.51±0.09 and 3.14±0.16, respectively, with significant differences (P<0.05). It was obvious that the relative expression of miR-744 in the brain tissue of rats in the MG was the lowest among the three groups, and that in rats of the miR-744 group was higher than the SOG (P<0.05). (Figure 2)

Table 1. General conditions of rats [n(%)]/(x±sd).

<table>
<thead>
<tr>
<th>Categories</th>
<th>The sham operation group (n=25)</th>
<th>The model group (n=25)</th>
<th>The miR-744 group (n=25)</th>
<th>F/χ² value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13 (52.00)</td>
<td>12 (48.00)</td>
<td>14 (56.00)</td>
<td>0.321</td>
<td>0.852</td>
</tr>
<tr>
<td>Female</td>
<td>12 (48.00)</td>
<td>13 (52.00)</td>
<td>11 (44.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (weeks)</td>
<td>8.31±0.38</td>
<td>8.25±0.29</td>
<td>8.39±0.34</td>
<td>1.075</td>
<td>0.347</td>
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<tr>
<td>Length (cm)</td>
<td>19.44±1.12</td>
<td>18.98±0.94</td>
<td>19.10±1.65</td>
<td>0.879</td>
<td>0.420</td>
</tr>
<tr>
<td>Glu (mmol/L)</td>
<td>71.27±4.08</td>
<td>72.16±4.37</td>
<td>72.12±4.14</td>
<td>0.358</td>
<td>0.700</td>
</tr>
<tr>
<td>Pre-modeling body mass (g)</td>
<td>223.75±13.62</td>
<td>222.46±14.18</td>
<td>225.18±14.05</td>
<td>0.238</td>
<td>0.789</td>
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<tr>
<td>Post-modeling body mass (g)</td>
<td>209.72±7.49</td>
<td>210.48±8.65</td>
<td>208.46±8.03</td>
<td>0.400</td>
<td>0.672</td>
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<td>Indoor temperature (°C)</td>
<td>24.18±1.05</td>
<td>23.86±0.82</td>
<td>23.92±1.10</td>
<td>0.727</td>
<td>0.487</td>
</tr>
<tr>
<td>Indoor humidity (%)</td>
<td>52.18±2.15</td>
<td>51.48±1.84</td>
<td>51.26±1.75</td>
<td>1.564</td>
<td>0.216</td>
</tr>
</tbody>
</table>

Table 2. Comparison of cerebral infarction volume and brain tissue water content of rats in the three groups (x±sd).

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Cerebral infarction volume (mm³)</th>
<th>Brain tissue water content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The sham operation group</td>
<td>25</td>
<td>71.19±11.63</td>
<td></td>
</tr>
<tr>
<td>The model group</td>
<td>25</td>
<td>34.74±3.43</td>
<td>91.13±10.17</td>
</tr>
<tr>
<td>The miR-744 group</td>
<td>25</td>
<td>23.21±2.51</td>
<td>81.35±9.17</td>
</tr>
<tr>
<td>F</td>
<td>-</td>
<td>1300.000</td>
<td>23.100</td>
</tr>
<tr>
<td>P</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Comparison of IL-1β, IL-6 and TNF-α expression in the brain tissue of rats in the SOG, MG and miR-744 group

The expression levels of IL-1β, IL-6 and TNF-α differed significantly among the SOG, MG and miR-744 groups (P<0.05). The expressions of IL-1β, IL-6 and TNF-α in the brain tissue of the SOG and the miR-744 group were significantly lower than those of rats in the MG, and the IL-1β, IL-6 and TNF-α in the brain tissue of rats in the SOG were significantly lower than those of rats in the miR-744 group. (Table 3 and Figure 3)

Discussion

The mortality and disability rate of ischemic stroke is relatively high, which greatly affects the patient's ability and quality. It is reported as one of the most important diseases in the world that endanger human life and health (21). Numerous clinical and experimental studies have shown that one of the principles for treating cerebral ischemia is to restore perfusion within an effective time window. However, it has been found that cerebral ischemia can lead to the loss of a large number of nerve cells in the central nervous system and the damage of neural networks. Restoring blood perfusion in a short period of time will further aggravate nervous system damage, which causes damage to the body far more than ischemic damage, and apart from that, it can cause cellular ischemia of ischemic tissue, eventually leading to cell necrosis and irreversible brain damage (22,23). Existing research suggests that this may be related to factors such as inflammatory response, apoptosis, and programmed necrosis, but the specific mechanism remains unclear (24, 25).

MiRNA is a class of single non-coding endogenous RNA, which can bind to some complementary sites in the 3'UTR region of target mRNA to induce the translation or degradation inhibition of target mRNA molecules, thereby regulating the expression of target genes (26, 27). In recent years, many a study has displayed that cerebral ischemia can cause significant changes in the expression profile of miRNAs in brain tissue, indicating that miRNAs are involved in the process of cerebral ischemia (28, 29). There are also studies such as Dharap (30) showing that the expression of miRNAs changed rapidly in the cerebral cortex following cerebral ischemia in rats. From 6h to 3d after ischemia, the expression of 20 miRNAs changed, at least 9 of which exhibited significant differences in expression. Lee (31) confirmed that the expression of the miR-200 family and miR-182 family was up-regulated 3 hours after cerebral ischemia and that 24 hours after cerebral ischemia, the expression levels of MIR-19 and miR-681 were up-regulated, while the expression of miR-468 was down-regulated. Located at the 17p12 site of the genome, miR-744 has been shown to be abnormally highly expressed in head and neck tumors, prostate cancer and nasopharyngeal carcinoma, and targeted to inhibit ARHGAP5 (Rho GTPase-activating protein 5) in nasopharyngeal carcinoma to promote tumor proliferation (32, 33). However, little is known about its role in cerebral I-R. In this study, a rat model of cerebral I-R was successfully established to compare the expression of miR-744 in the SOG, MG and miR-744 group. The relative expressions of miR-744 in the brain tissue of rats in the SOG, MG and miR-744 group were 1.12±0.04, 0.51±0.09 and 3.14±0.16, respectively, with significant differences among the three groups (P<0.05). From the above data, it was obvious that the relative expression of miR-744 in the brain tissue of rats in the SOG and the miR-744 group was significantly higher than that of rats in the MG, and that of the miR-

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>The sham operation group</td>
<td>25</td>
<td>0.13±0.05</td>
<td>0.16±0.03</td>
<td>0.18±0.05</td>
</tr>
<tr>
<td>The model group</td>
<td>25</td>
<td>0.75±0.21</td>
<td>0.37±0.08</td>
<td>0.62±0.18</td>
</tr>
<tr>
<td>The miR-744 group</td>
<td>25</td>
<td>0.25±0.08</td>
<td>0.24±0.05</td>
<td>0.33±0.09</td>
</tr>
<tr>
<td>F</td>
<td>-</td>
<td>153.000</td>
<td>85.970</td>
<td>87.270</td>
</tr>
<tr>
<td>P</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 3. Comparison of IL-1β, IL-6 and TNF-α expression in the brain tissue of rats in the SOG, MG, and miR-744 group. Comparison of IL-1β expression in the brain tissue of rats in the SOG, MG and miR-744 group (A): The expression of IL-1β in the brain tissue of rats in the SOG and the miR-744 group was significantly lower than that in the MG, and the expression of IL-1β in the brain tissue of rats in the SOG was notably lower than that of rats in the miR-744 group. Comparison of IL-6 expression in the brain tissue of rats in the SOG, the MG and the miR-744 group (B): The MG exhibited a markedly higher IL-6 expression in the brain tissue of rats than the rest two groups. When compared within the SOG and the miR-744 group, the IL-6 expression in the brain tissue of the former was significantly lower than that of the latter. Comparison of TNF-α expression in the brain tissue of rats in the SOG, the MG and the miR-744 group (C): The expression of TNF-α in the brain tissue of rats in the SOG and the miR-744 group was significantly lower than that of rats in the MG, and the expression of TNF-α in the brain tissue of rats in the SOG was significantly lower than that of rats in the miR-744 group.
miR-744 group was higher than the SOG (P<0.05), indicating that miR-744 was implicated in the occurrence and development of I-R. Due to the complexity of the cerebral I-R process, its molecular mechanism has not yet been clarified, but increasing evidence exhibits that the mechanism of I-R injury is mainly correlated with the intracellular inflammatory response (34). Therefore, the focus of the treatment of ischemic stroke is to inhibit inflammatory damage caused by I-R. IL-1β, IL-6, and TNF-α are common inflammatory cytokines with multiple functions and are inflammatory cytokines that are currently considered to produce a marked influence on cerebral ischemia and reperfusion reactions (35-37). For example, Chen (38) found that infusion of anti-IL-1β mAb could reduce short-term I-R-related histopathological tissue damage, and apoptosis, and decrease I-R-related caspase-3 activity in sheep fetal brain. Feng (39) demonstrated that IL-6 was able to alleviate cerebral I-R injury in rat models. Here in this study, compared with the MG, the expression levels of IL-1β, IL-6 and TNF-α protein in the brain tissue of rats were significantly increased than the SOG, while was significantly decreased than the miR-744 group, indicating that miR-744 might regulate inflammatory factor IL-1β, IL-6, TNF-α expression, which in turn inhibited I-R injury. Thus, it can be concluded that mir-744 may be involved in inhibiting the process of I-R by regulating inflammatory response and apoptosis.

In this study, the rat model of I-R was established to analyze the differences and changes in miR-744 expression levels in the three groups of rats. However, there are still some certain inadequacies due to limited experimental conditions, which underlies the differences between the animal model and human beings. Nevertheless, human experiments will be carried out as soon as possible to verify our experimental views and obtain the best experimental results.

In summary, miR-744 may be implicated in the development and progression of I-R in rats, and its mechanism may be related to the regulation of inflammatory response.

Conflict of Interests
The authors declared no conflict of interest.

References