MiR-210 inhibits apoptosis of cranial nerves in preeclampsia rats through suppressing the TGF-β signaling pathway

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Abstract. – OBJECTIVE: To explore the protective effect of micro ribonucleic acid (miR)-210 on cranial nerves in rats with preeclampsia (PE) by regulating the transforming growth factor-β (TGF-β) signaling pathway.

MATERIALS AND METHODS: A total of 36 pregnant Sprague-Dawley rats were randomly divided into normal group (n=12), model group (n=12), and miR-210 mimics group (n=12). The rats were fed normally in the normal group. In the latter two groups, the PE model was established, followed by injection of normal saline or miR-210 mimics via the caudal vein, respectively. The above intervention lasted until 20 d of gestational age in pregnant rats. Then, the systolic blood pressure of the caudal vein was measured. The relative levels of Caspase3, phosphorylated TGF-β (p-TGF-β), and miR-210 were detected via immunohistochemistry, Western blotting, and quantitative Polymerase Chain Reaction (qPCR). Cell apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

RESULTS: The systolic blood pressure of the caudal vein significantly increased in the other two groups compared with that in the normal group (p<0.05), while it significantly decreased in the miR-210 mimics group compared with that in the model group (p<0.05). The results of immunohistochemistry showed that the positive expression of Caspase3 significantly rose in the other two groups compared with that in the normal group (p<0.05), while it remarkably declined in miR-210 mimics group compared with that in the model group (p<0.05). The results of Western blotting revealed that the protein expression of p-TGF-β was evidently higher in the other two groups than that in the normal group (p<0.05), while it was evidently lower in the miR-210 mimics group than that in the model group (p<0.05). Moreover, it was found via qPCR that the other two groups had remarkably lower relative expression of miR-210 than normal group (p<0.05), while miR-210 mimics group had remarkably higher relative expression of miR-210 than the model group (p<0.05). According to the results of TUNEL assay, the apoptosis rate markedly increased in the other two groups compared with that in the normal group (p<0.05), while it markedly decreased in the miR-210 mimics group compared with that in the model group (p<0.05).

CONCLUSIONS: MiR-210 inhibits apoptosis via suppressing the TGF-β signaling pathway, thereby exerting a protective effect on cranial nerves in PE rats.

Key Words: Preeclampsia, MiR-210, TGF-β, Apoptosis.

Introduction

Preeclampsia (PE) is a kind of common pregnancy syndrome that has an important impact on both mother and fetus, which is mainly characterized by maternal proteinuria, hypertension, and edema. Currently, it is believed that PE is one of the most important causes of death of pregnant women, fetuses, and even newborns, and its maternal mortality rate is up to 15%. The incidence rate of PE in pregnant women can reach 3-5%, and PE can lead to oligohydramnios, epilepsy, stroke, cerebral hemorrhage in pregnant women and even fetal death. According to further research, PE also has an important impact on the fetus, which can cause severe consequences, such as premature birth, delayed growth and development, and even fetal death. Therefore, it is of great importance to study the pathological mechanism and effective treatment of PE.

One of the most important pathological mechanisms of PE is the excessive apoptosis...
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Proliferation and differentiation of brain neurons, resulting in cranial nerve damage and poor repair. Therefore, effectively inhibiting the excessive apoptosis of neurons is one of the most important strategies for the treatment of PE. It has been observed that the transforming growth factor-β (TGF-β) signaling pathway, one of the most important signaling pathways in the body, is closely related to pathophysiological reactions, such as cell proliferation, apoptosis, and necrosis. It is also involved in the pathological process of apoptosis via regulating the expressions of multiple downstream signaling pathways and substances. Therefore, the TGF-β signaling pathway is considered as one of the most important targets regulating apoptosis.

Micro ribonucleic acid (miR)-210 is an important member of the miRNA family, which has an important effect on regulating multiple downstream signaling pathways. Therefore, miR-210 exerts a protective effect on cranial nerves in PE rats by regulating the TGF-β signaling pathway.

Materials and Methods

Laboratory Animals

A total of 36 specific pathogen-free pregnant Sprague-Dawley (SD) rats aged 1 month old were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. [license No.: SCXK (Shanghai, China) 2014-0003]. The rats were fed with normal feed and sterile filtered water every day in the Laboratory Animal Center under a 12/12 h light-dark cycle, room temperature, and regular humidity. This study was approved by the Ethics Committee of Dongying People’s Hospital of Shandong.

Laboratory Reagents and Instruments

MiR-210 mimics (Sigma-Aldrich, St. Louis, MO, USA); lipopolysaccharide (Sigma-Aldrich, St. Louis, MO, USA); primary antibodies: anti-Caspase3 antibody, anti-phosphorylated TGF-β (p-TGF-β) antibody, and secondary antibodies (Abcam, Cambridge, MA, USA), immunohistochemistry kit, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis kit, and quantitative polymerase chain reaction (qPCR) kit (Vazyme, Nanjing, China), optical microscope (Leica, Wetzlar, Germany), fluorescence qPCR instrument (ABI, Applied Biosystems, Foster City, CA, USA).

Animal Grouping and Treatment

The above 36 SD rats were randomly divided into normal group (n=12), model group (n=12), and miR-210 mimics group (n=12) using a random number table. The rats were adaptively fed in the Laboratory Animal Center for 7 d before experiments.

The rats were fed normally without any treatment in the normal group. In the model group, the PE model was established, and then, normal saline was injected via the caudal vein every day. In miR-210 mimics group, the PE model was also established, and then, miR-210 mimics were injected (3 mM) via the caudal vein every day. The above intervention lasted until 20 d of gestational age in pregnant rats, followed by sampling.

Modeling

Lipopolysaccharide was injected (1 g/kg) via the rat caudal vein at 14 d after pregnancy, thus establishing the PE model. At 2 d after modeling, the systolic blood pressure of the caudal vein of pregnant rats was measured. The elevation of systolic blood pressure by 30 mmHg indicated the successful modeling of PE in rats.

Measurement of Systolic Blood Pressure of the Caudal Vein

At 20 d after pregnancy, the pregnant rats were successfully anesthetized via injection of chloral hydrate (5 mL/kg) into the caudal vein. The systolic blood pressure of the caudal vein was measured using a sphygmomanometer, followed by statistical comparison.

Sampling

After successful anesthesia, the newborn rats were taken from pregnant rats via cesarean section. The brain tissues were directly taken from 6 newborn rats in each group, which were washed with normal saline and stored in the Eppendorf (EP; Hamburg, Germany) tube at -80°C for Western blotting. Besides, the samples were directly taken from the remaining 6 newborn rats in each group, which were washed with normal saline and fixed with 4% paraformaldehyde for immunohistochemistry and TUNEL assay.

Immunohistochemistry

The paraffin-embedded tissues were sliced into 5 μm-thick sections, flattened in warm water at 42°C, and prepared into paraffin sections. Then, the sections were soaked and routinely deparaffinized in the xylene solution and gradient alcohol, placed in citric acid buffer, and heated in a
microwave for 3 times (3 min/time, braised for 5 min each time) for complete antigen retrieval. After the sections were washed, the endogenous peroxidase blocker was added dropwise for 10-min reaction. Then, the sections were washed again and sealed with goat serum for 20 min. After the goat serum was discarded, the anti-Caspase3 (1:200) primary antibody was added for incubation in a refrigerator at 4°C overnight. On the next day, the sections were washed, reacted with the secondary antibody for 10 min, fully washed, and reacted with streptavidin-peroxidase solution for 10 min. Color development was achieved using diaminobenzidine (DAB; Solarbio, Beijing, China). Finally, the nuclei were counterstained with hematoxylin, and the sections were sealed and observed.

Western Blotting
The cryopreserved brain tissues were lysed in lysis buffer in the ice bath for 1 h and centrifuged at 14,000 g for 10 min. The protein was quantified using bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The absorbance of protein was detected using a microplate reader and the standard curve was plotted, based on which the protein concentration was calculated. After protein denaturation, the protein in the tissue samples was separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the position of the Marker protein was observed. The electrophoresis was terminated when the Marker protein reached the bottom of the glass plate in a straight line. Then, the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and sealed with the blocking buffer for 1.5 h. Subsequently, the membrane was incubated with the anti-p-TGF-β primary antibody (1:1000) and secondary antibody (1:1000). After the membrane was washed, the image was fully developed using the chemiluminescent reagent for 1 min in the dark.

qRT-PCR
The total RNA was extracted from brain tissues using the RNA extraction reagent, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the reverse transcription kit. The qRT-PCR system (20 μL) was prepared and subjected to reaction at 53°C for 5 min, pre-denaturation at 95°C for 10 min, and 35 cycles for denaturation at 95°C for 10 s and annealing at 62°C for 30 s. The ΔCt value was calculated first, and then, the difference in the expression of the target gene was calculated. The primer sequences were shown in Table I.

Table I. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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<tr>
<td>MiR-206-3p</td>
<td>F: 5’-AATAGGGCCGATTAACCGTAAAAC-3’&lt;br&gt;R: 5’-TTATAATGGCGTAAGTGGCGCTTAG-3’&lt;br&gt;U6 F: 5’-GGCGCCTCGTGAAGCGTGTC-3’&lt;br&gt;R: 5’-GTGCAGGGTCCGAGGCT-3’</td>
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Statistical Analysis
Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Enumeration data were expressed as mean ± standard deviation. The t-test was used for the data with normal distribution and homogeneity of variance, and the corrected t-test was applied for the data with normal distribution and heterogeneity of variance. Comparison between groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). The non-parametric test was conducted for the data not in line with normal distribution and homogeneity of variance. Rank sum test was adopted for ranked data, and chi-square test for enumeration data. p-values < 0.05 were considered statistically significant.

Results
Systolic Blood Pressure of the Caudal Vein in Each Group
As shown in Figure 1, the systolic blood pressure of the caudal vein significantly increased in the other two groups compared with that in the

Figure 1. Systolic blood pressure of the caudal vein in each group. Note: *p<0.05 vs. normal group, #p<0.05 vs. model group.
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Normal group, showing statistically significant differences ($p<0.05$). It significantly decreased in miR-210 mimics group compared with that in the model group, showing a statistically significant difference ($p<0.05$).

**Immunohistochemistry Results**

Caspase3-positive cells were stained dark brown. The positive expression of Caspase3 was lower in the normal group, but higher in the other two groups. According to the statistical results (Figure 2), the mean optical density of positive expression of Caspase3 significantly rose in the other two groups compared with that in the normal group, displaying statistically significant differences ($p<0.05$). However, it remarkably declined in miR-210 mimics group compared with that in the model group, displaying a statistically significant difference ($p<0.05$).

**Relative Protein Expression Detected Via Western Blotting**

The protein expression of p-TGF-β was lower in the normal group but higher in the other two groups (Figure 3A). According to the statistical results (Figure 3B), the relative protein expression of p-TGF-β was evidently higher in the other two groups than that in the normal group, displaying statistically significant differences ($p<0.05$). It was evidently lower in miR-210 mimics group than that in the model group, displaying a statistically significant difference ($p<0.05$).

**qRT-PCR Results**

It was found that the other two groups had remarkably lower relative expression of miR-210 than normal group, and there were statistically significant differences ($p<0.05$), while miR-210 mimics group had remarkably higher relative expression of miR-210 than model group ($p<0.05$) (Figure 4).

**TUNEL Assay Results**

Apoptosis cells were stained dark brown in TUNEL assay. There were fewer apoptosis cells in normal group, but more in the other two groups (Figure 5). As shown in Figure 5, the apoptosis rate markedly increased in the other two groups compared with that in normal group, and the differences were statistically significant ($p<0.05$). Nevertheless, it markedly decreased in miR-210 mimics group compared with that in model group, and the difference was statistically significant ($p<0.05$).

**Discussion**

PE is a clinically common pregnancy disease that often causes maternal and fetal death, which is one of the most critical diseases endangering the mother and fetus. Hypertension and severe proteinuria are major clinical symptoms of PE. As a result, controlling blood pressure and proteinuria is one of the most important symptomatic treatments for PE.$^{12,13}$ Apoptosis is one of the most important pathological mechanisms of PE.$^{14-16}$ In particular, the apoptosis of brain neurons is one of the most important pathological damages to the fetal cranial nerves. During the onset of...
PE, ischemia and hypoxia can cause pathological changes in a variety of organs and tissues, including cranial nerves, thus promoting the apoptosis of brain neurons. It has been found that there is massive apoptosis of brain neurons and abnormally high expression of the apoptosis effector molecule Caspase3 in cranial nerves of newborn rats of pregnant PE rats. It is suggested that neuronal apoptosis is involved in cranial nerve damage in infants with PE mothers. Apoptosis-associated genes are responsible for the massive apoptosis of brain neurons, harming the repair of cranial nerves of newborn rats of pregnant PE rats. Therefore, intervention and regulation of the pathological apoptosis during PE are effective strategy to repair cranial nerves of newborn rats of pregnant PE rats. Besides, among the signaling pathways closely related to apoptosis, the TGF-β signaling pathway is considered as an important one in the body. In the case of damage to the body, various damage factors, inflammatory factors and cytokines can further interact with TGF-β, a key molecule in the TGF-β signaling pathway, to phosphorylate TGF-β to form p-TGF-β. Consequently, the TGF-β signaling pathway and downstream transcription factors are activated, thereby controlling the downstream gene functions. As an important apoptosis effector molecule in the body, Caspase3 is a vital downstream target effector of the TGF-β signaling pathway, which can be regulated by the TGF-β signaling pathway. When activated, the TGF-β signaling pathway can upregulate Caspase3 expression, thus promoting apoptosis. In addition, miR-210 plays an important regulatory role in multiple signaling pathways, including TGF-β. The novelty of this study was that the TGF-β signaling pathway was activated and Caspase3 was upregulated in cranial nerves of newborn rats of pregnant PE rats, leading to the massive brain neurons. At the same time, the abnormally high expression of miR-210 was also observed in cranial nerves of newborn rats of pregnant PE rats. Furthermore, the overexpression of miR-210 could effectively inhibit the activation of the TGF-β signaling pathway and reduce the expression of Caspase3, thus suppressing apoptosis of brain neurons.

**Conclusions**

In this study it has been demonstrated that miR-210 inhibits apoptosis via suppressing the TGF-β signaling pathway, thereby exerting a protective effect on cranial nerves in PE rats.

**Conflict of Interest**

The Authors declare that they have no conflict of interests.

**References**


