Original Article
MiR-124 effect in neurons apoptosis in newborn rat with thyroid hypofunction

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Abstract: Congenital thyroid hypofunction can cause a variety of developmental disorders. Hippocampus is an important structure participating in the cognitive activities. Neural function damage is able to induce hippocampal neuron apoptosis. As a miRNA expressed specifically and abundantly in brain tissue, miR-124 has protective effect to neuron apoptosis caused by cerebral apoplexy. However, its role in neuron apoptosis caused by thyroid hypofunction is still unclear. The rats were divided into four groups including normal group, thyroid hypofunction group, miR-124 negative control group, and miR-124 mimics group. Propylthiouracil (50 mg/d) was injected to the stomach to the rats with 15 d pregnancy till the newborn rats were born. Inducing the thyroid hypofunction rat model and then injecting miR-124 mimics to ventricle. Serum TSH, FT3 and FT4 were detected to confirm the model. Immunohistochemistry was carried out to calculate neuron number. Tunel assay was used to detect neuron apoptosis. Western blot was applied to detect apoptosis related protein Caspase-3, Bcl-2 and Bax expression. After brain injection miR-124 mimics, hippocampal neuron number and morphology both improved in 15 d newborn mice compared with thyroid hypofunction group. Tunel staining found positive neurons reduced, which indicated that miR-124 can inhibit hippocampal neuron apoptosis in thyroid hypofunction rats. Further Western blot results revealed that apoptosis inhibition might be related to down-regulating activated Caspase-3 and Bax levels, and up-regulating tumor-suppressor gene Bcl-2 expression. MiR-124 can protect neuron apoptosis in thyroid hypofunction rat.

Keywords: miR-124, thyroid hypofunction, hippocampal neuron, apoptosis

Introduction

MicroRNAs is a kind of non-coding single stranded small RNAs about 18-25 nucleotides in length [1]. Mature miRNA has regulating effect to mRNA by complementary binding with target mRNAs [2]. Recent research suggested that miRNA widely participated in various systems growth regulation including nervous system. Thyroid hormone plays an important role in the mammalian brain development. Hypothyroidism is a type of general endocrine disease caused by a variety of reasons that lead to thyroid hormone dyssynthesis, paracrisis or lack of biological effects. Among them, congenital thyroid hypofunction can cause a variety of developmental disorders [3], while hippocampal structure is an important center participating cognitive activities. Previous preliminary experiment data and other research results showed that thyroid hypofunction in the perinatal period may induce hippocampal neuron apoptosis in rats. As a miRNA expressed specifically and abundantly in brain tissue, miR-124 expression in central nervous system and nerve cells was 100 times higher than in other parts [4]. It has been confirmed that miR-124 could protect neuron apoptosis caused by cerebral apoplexy [5]. However, its role in neuron apoptosis caused by thyroid hypofunction is still unclear. At present, apoptosis regulation is often closely related to Caspase-3, Bcl-2 and Bax. In the preliminary experiment, we observed miR-124 down-regulation and neuron apoptosis in thyroid hypofunction of rats in the perinatal period but the relationship is unclear. This study aimed to clarify mechanism of miR-124 on protecting hippocampal neuron apoptosis on thyroid hypofunction rat model.

Materials and methods

Main reagents

MiR-124 mimics and negative control were both bought from Genepharma Biological co., LTD. (Shanghai, China). Brain stereotaxic apparatus was purchased from Stoelting Company (Shanghai, China). Rat thyroid stimulating hormone (TSH), FT3 and FT4 chemiluminescence immunoassay kits were got from Baoman Biological Technology co., LTD. (Shanghai, China). MiRNAs qPCR Quantitation Kit was bought in Invitrogen (CA). RNA extraction reagent was bought in Takara (Dalian, China). PVDF membrane and neuN antibody were got from Milipore (Hong Kong, China). Total protein extraction kit, BCA quantitative kit and other western blot reagents were all purchased from Beyotime co., LTD. (Shanghai, China). Immunohistochemistry kit was from Zsbio co., LTD. (Beijing, China). Tunel kit was bought from Roche (Shanghai, China). Caspase-3, Bcl-2 and Bax antibodies were purchased from Proteintech Company (Wuhan, China).

Modeling

Pregnant SD rats were provided by Harbin Medical University experimental animal center. Research were approved by the Animal Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. The rats were divided into four groups including normal group, thyroid hypofunction group, miR-124 negative control group, and miR-124 mimics group. Propylthiouracil (50 mg/d) was injected once a day to the stomach to the rats with 15 d pregnancy till the newborn rats were born. Inducing the thyroid hypofunction rat model and then injecting miR-124 mimics to ventricle [6]. Newborn rats' eye open time, weight and activity were observed.

Serum TSH, FT3 and FT4 detection

Serum TSH, FT3 and FT4 were detected after birth, 1st, 5th, 10th, and 15th day to confirm the model.

Real-time PCR

After anesthetized by 2% pentobarbital sodium abdominal injection, the rats were fixed on the brain stereotaxic apparatus. The skull was opened at 1.0 mm after the former fontanelle and 1.7 mm near the midline. A micro syringe was injected vertically for 3.8 mm at 15 μm/s, and 5 μL 1 nmol/L miR-124 mimics solution was injected [7]. Newborn rats were killed and hippocampus was collected at 15th after the birth. Total RNA was extracted from the tissue according to the manual. The cDNA was synthesized through reverse transcription. The primers used for reverse transcription were as follows: MiR-124'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCATT-3'; U6, 5'-AAAATATGGAACGCTTCACGAATTTG-3'. The primers used for PCR were as follows: miR-124, forward 5'-GCTAAGGCACGCGGTG-3', reverse 5'-GTGCAGGGTCCGAGGT-3'; U6, forward 5'-CTCGC-TTCGGCAGCACATATACT-3', reverse 5'-ACGCTTC-ACGAATTTGCGTGTC-3'. The cycling conditions for PCR consisted of an initial, single cycle of 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. PCR amplifications were performed in three duplicates for each sample. Gene expression levels were quantified relative to the expression of U6 using an optimized comparative Ct ($^{\Delta\Delta}$Ct) value method.

Immunohistochemistry

After conventional paraffin embedding, sectioning, dewaxing, and hydrating steps, hippocampal tissue received antigen repair by citric acid buffer (pH = 10.0). After natural cooling, neuN antibody was added for incubation. Brown represented positive staining. Neuron number was calculated.

Tunel assay

After conventional paraffin embedding, sectioning, dewaxing, and hydrating steps, hippocampal tissue section was treated with 0.3% Triton for 10 min and incubated with Tunel at 37°C for 1 h. After washed by PBS, the section was observed under fluorescence microscope.

Western blot

After transfection, the cells were digested with lysis buffer. Total protein was separated by denaturing 10% SDS-polyacrylamide gel electrophoresis. After incubated with Caspase-3, Bcl-2, and Bax primary antibodies, the membrane was detected with chemiluminiscence. Protein levels were normalized to β-actin and changes were determined.

Table 1. Serum TSH level (mU/L)

	1st day	5th day	10th day	15th day
Normal control	1.27±0.62	1.36±0.45	1.24±0.82	1.42±0.33
Model	3.88±0.23**	3.25±0.30**	3.73±0.71**	3.88±0.42**
miR-124 negative	3.17±0.52**	3.22±1.21**	4.56±1.23**	4.52±1.09**
miR-124 mimics	4.13±0.46**	4.21±0.62**	4.25±0.59**	4.71±0.79**

**P<0.05, compared with control; ##P<0.05, compared with thyroid hypofunction group.

Table 2. Serum FT3 level (pmol/L)

	1st day	5th day	10th day	15th day
Normal control	4.12±0.33	6.13±0.45	7.54±0.47	8.42±0.73
Model	2.11±0.54**	3.25±0.47**	4.73±0.72**	4.21±0.33**
miR-124 negative	2.17±0.43**	3.42±0.54**	4.56±0.57**	4.52±0.44**
miR-124 mimics	2.23±0.36**	3.12±0.72**	4.82±0.49**	4.71±0.72**

**P<0.05, compared with control; ##P<0.05, compared with thyroid hypofunction group.

Table 3. Serum FT4 level (pmol/L)

	1st day	5th day	10th day	15th day
Normal control	5.12±0.42	10.23±0.75	17.53±1.23	19.26±1.34
Model	2.52±0.56**	2.64±0.65**	4.23±0.74**	2.54±0.56**
miR-124 negative	2.46±0.64**	2.78±0.48**	4.56±1.22**	2.64±0.74**
miR-124 mimics	2.56±0.85**	2.85±0.64**	4.69±1.24**	2.85±1.24**

**P<0.05, compared with control.
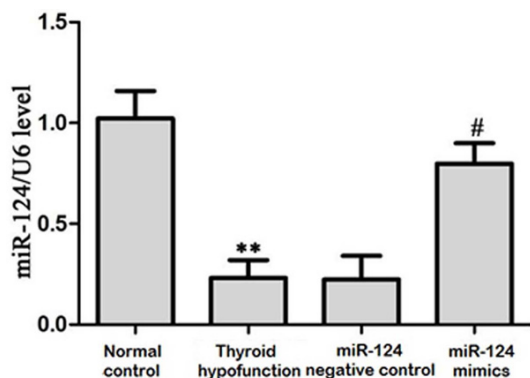
Figure 1. MiR-124 expression in hippocampus tissue. **P<0.05, compared with control; ##P<0.05, compared with thyroid hypofunction group.

Statistical analysis

All statistical analyses were performed using SPSS10.0 software. Numerical data were presented as means and standard deviation. Differences between multiple groups were analyzed by one-way ANOVA and SNK-Q test.

Results

Animal model

Newborn rats in normal group presented good activity with rapid weight gain and normal fur developing. Rats in other groups grew slower than the control with small figure, short tail, less activity, and sparse fur. Serum TSH, FT3, and FT4 expression in newborn rats at 1st, 5th, 10th, 15th day were listed in group 1, 2, and 3. Groups of young rats after the birth of one day, five days, ten days, fifteen days of serum TSH (**Table 1**), FT3 (**Table 2**) and FT4 (**Table 3**) are shown in **Tables 1**-3. Serum FT4 levels in each day was lower in experimental groups than in control (2.8 pmol/L); serum FT3 also decreased in different days; while TSH level increased significantly in experimental groups than in control.

MiR-124 expression in hippocampus tissue

MiR-124 is highly expressed in the brain and has been confirmed to regulate a variety of diseases. It is called the decision factor of ventricle region neurons [8]. To observe its correlation with neuron apoptosis in the rats with thyroid hypofunction, we used real-time PCR to detect its expression in newborn rats at 15 days (**Figure 1**). MiR-124 level decreased obviously in rats with thyroid hypofunction. After treated with miR-124 mimics, its expression increased significantly.

NeuN staining in rats at 15 days old

Hippocampus neuN staining clearly revealed the number and morphology of neuron. Compared with normal control, the positive neuron number reduced significantly in rats with thyroid hypofunction group, and the cell contour was unclear. After treated with miR-124 mim-
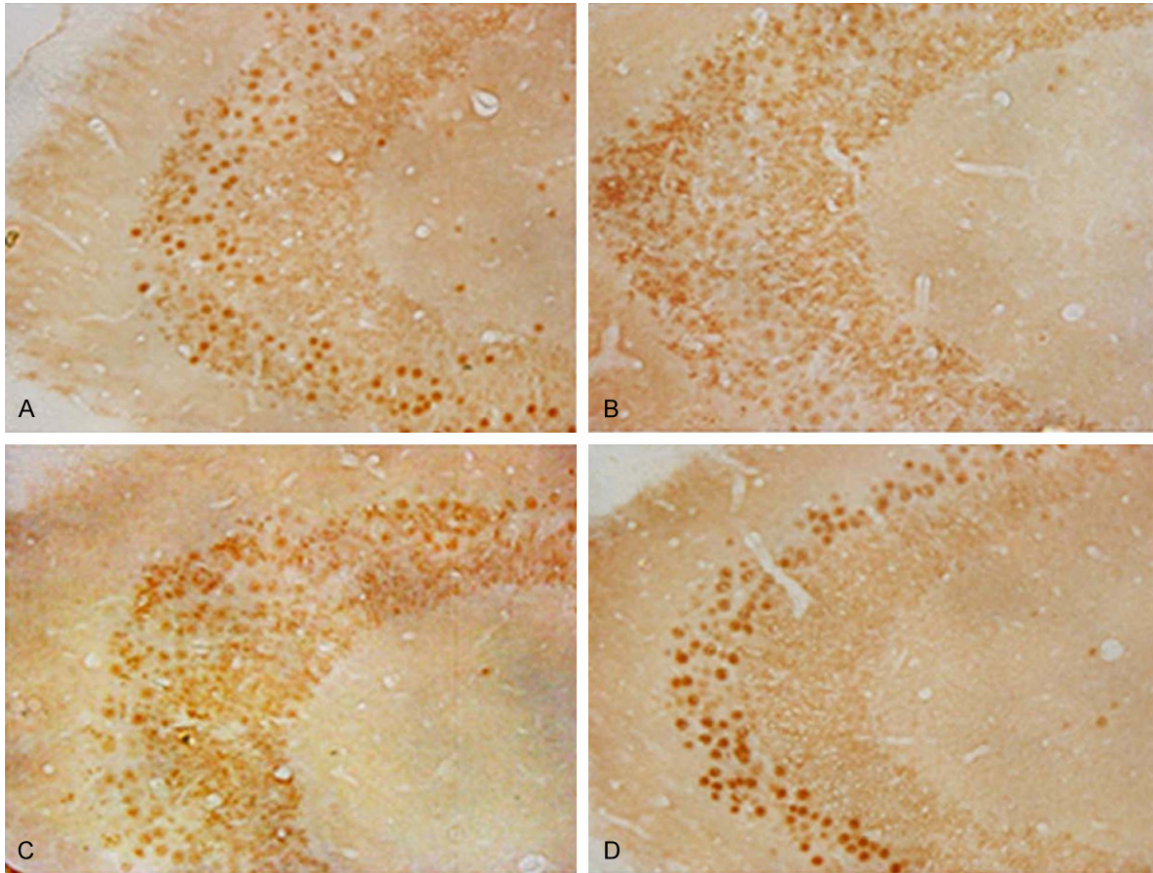
Figure 2. Hippocampus neuN staining in rats at 15 days old. A. Normal control; B. Thyroid hypofunction group; C. miR-124 negative control group; D. miR-124 mimics group.

ics, neuron number and morphology improved obviously (**Figure 2**).

Hippocampus Tunel staining in rats at 15 days old

Tunel staining revealed rat hippocampus neuron shrivel and positive cells scattered distribution. Granulosa cell apoptosis number at CA1 area was obviously larger in thyroid hypofunction group and miR-124 negative control group when compared with control, while apoptotic cell number declined after treated with miR-124 mimics (**Figure 3**).

Caspase-3, Bcl-2, Bax protein expression change

Activated caspase-3 expression increased significantly in thyroid hypofunction group and miR-124 negative control group compared with normal control, while it declined after treated with miR-124 mimics. MiR-124 mimics could

elevate Bcl-2 expression in model group and miR-124 negative control group. MiR-124 mimics also can reduce Bax expression (**Figure 4**).

Discussion

Iodine deficiency in lactation is one of the important reasons that cause offspring irreversible central nerve damage. Thyroid hormone can directly act on neural base layer in chromosome and transcription level, which lead to brain morphological and biochemical changes, and brain function damage [9]. Iodine deficiency in fetal period or thyroid hypofunction after the birth is an important reason causing language, cognition, behavior, and movement disorder. Clinical phenomenon indicated that a lot of developmental delay and nervous system defects were caused by thyroid dysfunction [10]. At the critical moment of neural development, even a short time of thyroid hormone deficiency can lead to on hippocampal neurons function damage or irreversible injury
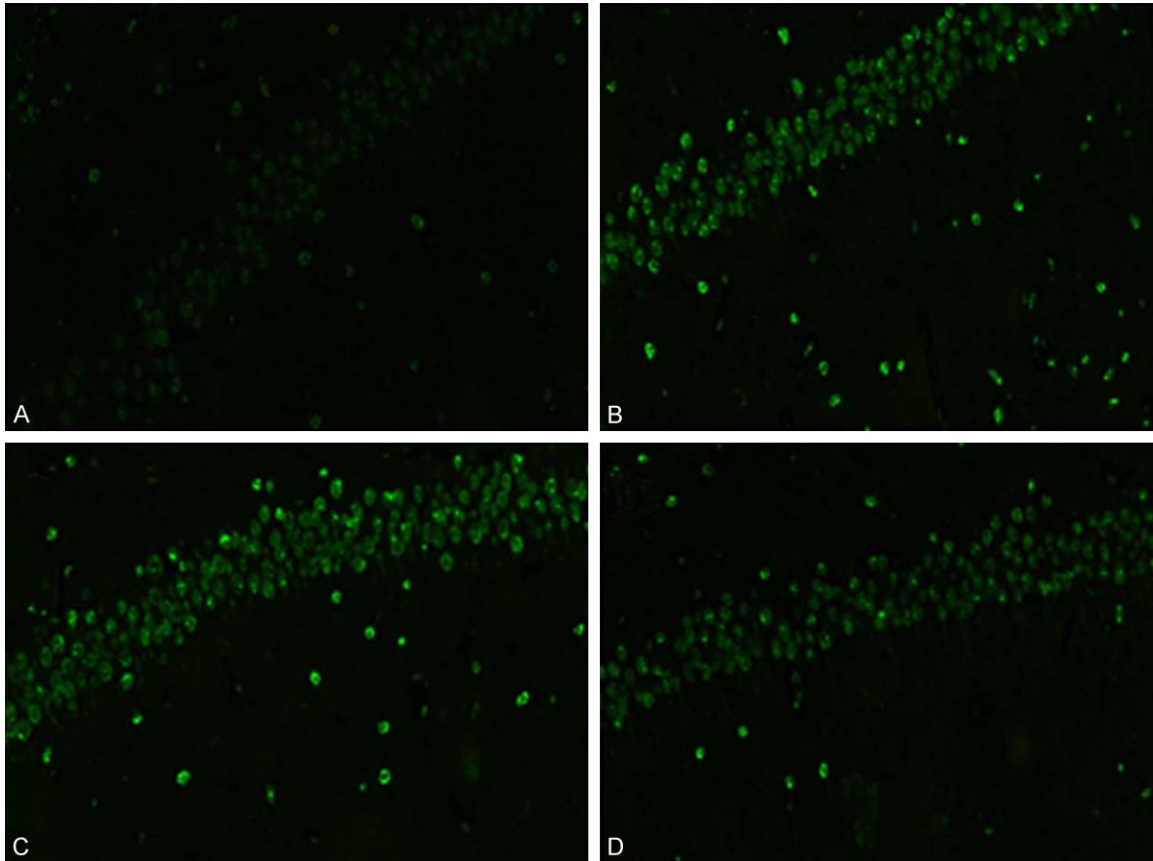
Figure 3. Hippocampus Tunel staining in rats at 15 days old. A. Normal control; B. Thyroid hypofunction group; C. miR-124 negative control group; D. miR-124 mimics group.
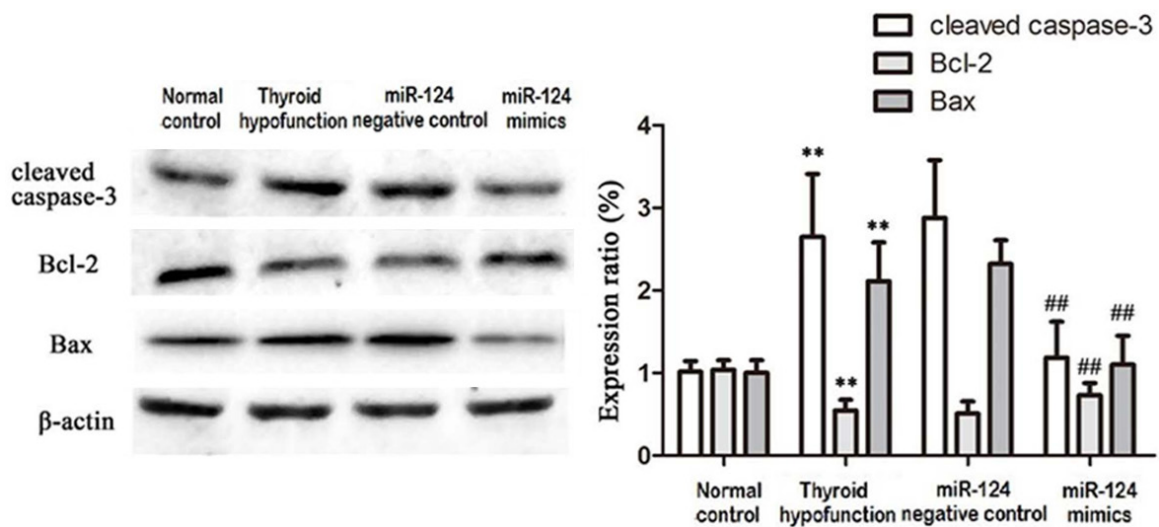
Figure 4. Caspase-3, Bcl-2, Bax protein expression in rats at 15 days old. **P<0.05, compared with control; ##P<0.05, compared with thyroid hypofunction group.

[11], which often characterized by hippocampal neuron apoptotic number elevation [12].

MiRNAs are small, non-coding RNA that can regulate mRNA expression [13]. MiR-124 is

highly expressed in nerve cells [14]. Numerous in vitro studies showed that miR-124 has a very important role in deciding neuronal fate [15, 16]. Experiments revealed that miR-124 has different roles in different tissue or physiological condition. For example, miR-124 showed inhibitory effect on colorectal cancer [17] and osteosarcoma [18], suggesting to be a widespread anticancer agent [19]. On the contrary, it showed opposite effect in many pathological conditions leading to apoptosis. For instance, it can protect liver cell apoptosis caused by stroke [20] or oxidative stress [21]. Previous results found that hippocampal neurons apoptosis appeared in rats with thyroid hypofunction in rats, while the role of miR-124 is still unclear.

Multiple methods could detect cell apoptosis, including morphology, biochemistry and flow cytometry, etc. The mainstream method is specific neuN staining observed by light or electron microscope. Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling (Tunel) assay is a common method to detect apoptosis specifically. Furthermore, we applied Western blot to detect apoptosis related proteins Caspase 3, Bcl-2, and Bax.

In this experiment, we found that miR-124 down-regulated in rats with thyroid hypofunction, along with neuron apoptosis number elevation. Combining with a variety of microRNAs protective role to nerve apoptosis in the literature [22] and miR-124 protective effect on neuron apoptosis caused by stroke [5], we speculated that miR-124 also plays a protective role in rats with thyroid hypofunction. Neurons neuN staining revealed neuron number reduction and cell contour changes in the thyroid hypofunction group, while miR-124 mimics can reverse such change to some extent. Similarly, Tunel assay showed that neuron apoptosis could be improved by miR-124 mimics in rats with thyroid hypofunction. Caspase-3 is the main terminal enzyme in the process of cell apoptosis, and its upregulation could be reversed by miR-124 mimics. MiR-124 mimics intervention can increase Bcl-2 and reduce Bax. We believed that miR-124 can up-regulate anti-apoptotic proteins expression, which may be the molecular mechanism of miR-124 protection on the neural apoptosis.

Taken together, our results suggested that miR-124 can protect neuron apoptosis in rats cau-

sed by thyroid hypofunction. As a new kind of antiapoptotic regulator, miR-124 could be treated as nerve protection material in thyroid hypofunction caused nerve damage. Similarly, it is still unclear about the role of miRNA in thyroid hypofunction and need further investigation.

Disclosure of conflict of interest

None.

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