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Effect of NT-3 on infection-induced memory impairment of neonatal rats

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Abstract. – OBJECTIVE: To explore the effect of neurotrophin-3 (NT-3) messenger ribonucleic acid (mRNA) in the hippocampus on infection-induced memory impairment of neonatal rats.

MATERIALS AND METHODS: 80 female Sprague-Dawley (SD) rats in the neonatal stage were selected to establish memory impairment model by bacterial meningitis infection. Rats were randomly divided into experimental group (n=40) and control group (n=40). Rats in experimental group were injected with β-amyloid precursor protein 319-335 peptide APP17p into brain tissue to up-regulate the expression of NT-3, and the rats in control group didn't receive treatment. Behavioral changes of rats were observed in Morris water maze and passive avoidance experiment. Apoptosis of nerve cells was detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method and Fluoro-Jade B method. NT-3 mRNA expression level was measured via reverse transcription polymerase chain reaction (RT-PCR).

RESULTS: NT-3 expression level in experimental group was higher than that in control group ($p<0.05$). Apoptosis rate of nerve cells in experimental group was lower than that in control group, but the learning and memory ability of rats in experimental group was better than that in control group ($p<0.05$).

CONCLUSIONS: Reduced NT-3 expression level may be correlated with the occurrence of meningitis because NT-3 can suppress nerve cell apoptosis and ameliorate learning and memory impairment to a certain extent to exert neuroprotective effects.

Key Words:
NT-3, Neonatal rat, Bacterial meningitis infection, Memory impairment, Nerve cell apoptosis.

Introduction

Meningitis refers to the diffused inflammation around brain and spinal meninges. As the most severe type of meningitis, bacterial meningitis is characterized by the high incidence, rapid onset, high mortality rate and neurologic sequelae[1,2]. Although antibiotic therapy, corticosteroids and advanced intensive care are actively applied, mortality rate of bacterial meningitis is still as high as 15-35%. Besides that, bacterial meningitis also increases the risk of neurological deficits[3]. Effective treatment for most meningitis remains vacant. Therefore, the purpose of treatment is to relieve the symptoms. Neurotrophin-3 (NT-3) is mainly expressed in neurons and astrocytes of the nervous system. Low expression level of NT-3 can also be detected in cerebral endothelial cells as well as microvessels of the nerve plexus[4]. It has been reported that NT-3 can protect the neurons and stimulate the growth of neurites and formulation of myelin sheaths by activating the Schwann cells[5]. Researches[6] have shown that NT-3 can also promote and enhance the locomotor recovery of untrained vertebrates. Therefore, NT-3 may also have protective effects on neurological function in patients with meningitis, but the correlation between NT-3 and meningitis has rarely been reported. In this study, NT-3 expression in rat bacterial meningitis models at neonatal stage was upregulated by peptide APP17p of β-amyloid precursor protein 319-335, to observe its effects on the rats' memory functions. We provided references for the treatment of bacterial meningitis.

Materials and Methods

Research Objects

All the Sprague-Dawley (SD) rats were purchased from Wuhan Myhalic Biotechnology Co., Ltd., and were fed with Shoobree Specific-Pathogen-Free (SPF)-grade rat feeds (Jiangsu Xietong Organism Co., Ltd., Jiangsu, China) and were

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raised at room temperature (21±2°C) with 30-70% humidity. Rats were allowed to access food and water freely. Female SD rats aged 7-11 d, and weighed 16-25 g. This study was approved by the Ethics Committee of the Affiliated Jiangyin Hospital of Southeast University of Medical College.

SD Rat Modeling

Sprague Dawley (SD) rats underwent the Morris water maze twice a day for one week. SD rats were allowed to find the security platform in the third quadrant within 2 min and stay there for 45 s. Those that failed to finish the task were guided by researchers. At 30 min after the training, SD rat bacterial meningitis models were established according to the method described by Liechti et al[7]. SD rats were injected intraperitoneally with pentobarbital sodium (50 mg/kg). Streptococcus pneumoniae (Shanghai Guduo Biotechnology Co., Ltd., Shanghai, China) suspension with a concentration of DH5α 1×10 CFU/mL was prepared, and 20 μL of the suspension were injected into brain tissues to establish SD rats bacterial meningitis models. SD rats were subjected to Morris water maze training and passive avoidance experiment 24 h after inoculation of bacteria. Experimental results beyond 2 min indicated the successfully established model. A total of 80 rat bacterial meningitis models were successfully established, and were randomly divided into experimental group (n=40) and control group (n=40). Rats in experimental group were subcutaneously injected with 10 μL APP17p, while the rats in control group didn't receive treatment. Indications were observed after 24 h.

Ribonucleic Acid (RNA) Extraction

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from brain tissues according to the instructions of the kit. Concentration and purity of the extracted RNA were analyzed via an ultraviolet spectrophotometer, and the integrity of the RNA was analyzed through 3% agarose gel electrophoresis.

Complementary Deoxyribonucleic Acid (cDNA) Synthesis

TaqMan® MicroRNA reverse transcription kit was purchased by Thermo Fisher Scientific Inc., (Waltham, MA, USA) and the cDNA was synthesized through reverse transcription in accordance with the instructions. Reaction conditions: 37°C for 45 min and 95°C for 5 min. The product was stored at -20°C.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reaction system was 25 μL: DNA template 2 μL, primer 0.5 mol/L, 2X dNTP 2.0 μL, buffer solution 2.5 μL, $MgCl_2$ 1.5 mol/L, Taq DNA polymerase 1.0 IU, nuclease-free water was added to make the final volume of 25 μL. Reaction conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 45 s and 72°C for 3 min, and then 72°C for 5 min. PCR products were stored at 4°C. Primers used in PCR reactions: NT-3: upstream primer: 5'-GTATCTCATGGAGGATTACGTGGG-3', and downstream primer: 5'-TGTTCTCTGAAGTCAGTGCTCGGA-3'; U6 [GenScript (Nanjing) Co., Ltd., Nanjing, China] was used as the endogenous control. Three replicate wells were set for each experiment, and $2^{-\Delta\Delta Ct}$ method was utilized to analyze the results.

Terminal Dexynucleotidyl Transferase (TdT)-Mediated dUTP Nick End Labeling (TUNEL) Method

Frozen brain tissue sections were fixed in 10% neutral formaldehyde at room temperature for 10 min. Tissue sections were washed twice with phosphate-buffered saline (PBS), 5 min for each. The tissue sections were treated with ethanol: acetic acid (2: 1) solution at -20°C for 5 min. Next, tissue sections were washed twice with PBS, 5 min for each. TUNEL kit was purchased from Shanghai China Wins Da Industrial Co., Ltd., (Shanghai China). Brain tissues of the SD rats were stained according to the instructions of the kit. After staining, TUNEL positive neuron cells showed brown or yellowish-brown nuclei. Image analysis software (Image-pro Plus 5.0) was used to count the number of TUNEL positive cells of 5 visual fields at × 400 magnifications. The number of TUNEL positive cells was presented as the integrated optical density value.

Fluoro-Jade B Method

The Fluoro Jade-B kit was bought from Seebio Biotech (Shanghai) Co., Ltd. (Shanghai, China) Fluorescence staining of the brain tissues was conducted according to the instructions of the kit. Green fluorescence emitted from the cell body with clear outline and the neuronal cell dendrites greater than or equal to 1 indicated positive signal. Positive cells of 5 visual fields of were counted under a microscope with high magnification.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) was

Table I. General information of the two groups of rats.

	Experimental group	Control group	*t*-value	*p*-value
Age (d)	8.2±1.3	9.8±1.6	0.542	0.899
Sex (male/female)	24/16	19/21	0.669	0.645
Weight (g)	22.4±3.8	26.7±4.2	0.452	0.785
Morris water maze test				
Swimming time (s)	141.2±31.3	142.4±42.1	0.297	0.985
Swimming distance (cm)	334.5±128.6	342.7±133.1	0.502	0.842
Passive avoidance experiment				
Number of error	9.2±2.7	8.7±3.3	0.693	0.796
Latent time (s)	74.3±34.6	77.2±41.5	0.515	0.877

used. χ^2-test was used for comparison of Qualitative data. Measurement data were expressed as MEAN ± S.E.M, and nonparametric Kolmogorov-Smirnov (K-S) test was conducted for comparisons between two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

General Information

A total of 80 Sprague Dawley (SD) rat bacterial meningitis model were successfully established, 15 rats were dead, and 30 rats were disqualified, with a success rate at 64%. All rats in two groups allowed to access water and food freely. There were no differences in gender, age and weight between two groups ($p>0.05$). The general data was shown in Table I.

Results of Morris Water Maze Test of two Groups of Rats

Average swimming time [(112.4±32.7) s *vs.* (152.3±42.2) s] and swimming distance [(237.6±99.7) cm *vs.* (347.6±112.2) cm] of SD rats in experimental group were significantly decreased compared with those in control group ($p<0.05$), indicating that the memory function of SD rats in experimental group was stronger than that in control group (Figure 1 and Figure 2).

Results of Passive Avoidance Experiment of Two Groups of Rats

Results of passive avoidance experiment of SD rats showed that the average number of error [(4.7±1.6) *vs.* (9.9±2.5)] and latent time [(49.8±21.5) s *vs.* (76.7±42.1) s] of SD rats in experimental group were remarkably decreased compared with those in control group ($p<0.05$), which future confirmed that the memory function of SD rats in experimental group was stronger than that of control group (Figure 3 and Figure 4).

Apoptotic Neuron Cells Detected by TUNEL Test

TUNEL staining results showed that the number of apoptotic neuron cells [(1542.11±144.15) *vs.* (2745.46±155.25)] of SD rats in experimental

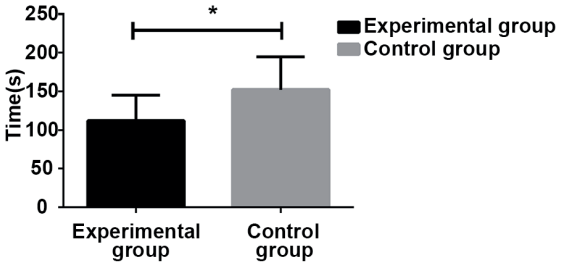

Figure 1. Average swimming time detected by Morris water maze test of two groups of rats. It is discovered from the results of Morris water maze test on the SD rats that the average swimming time [(112.4±32.7) s *vs.* (152.3±42.2) s] of SD rats in experimental group is significantly decreased compared with that in control group ($t=2.475$, $p=0.012$; $t=2.014$, $p=0.019$).

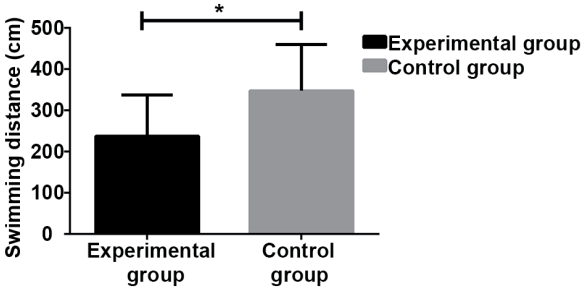

Figure 2. Average swimming distance of Morris water maze test of two groups of rats. It is discovered from the results of Morris water maze test on the SD rats that the swimming distance [(237.6±99.7) cm *vs.* (347.6±112.2) cm] of SD rats in experimental group is significantly decreased compared with that in control group ($t=2.014$, $p=0.019$).

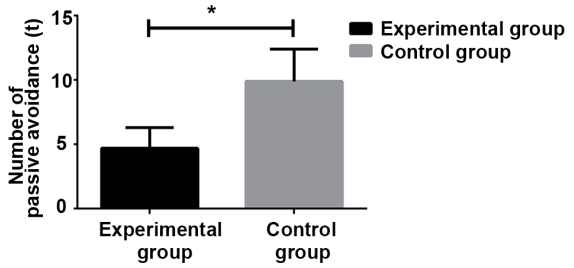

Figure 3. Average number of errors of passive avoidance experiment of two groups of rats. The results of passive avoidance experiment on SD rats show that the average number of error [(4.7±1.6) *vs.* (9.9±2.5)] of SD rats in experimental group is remarkably decreased compared with that in control group (t=2.334, p=0.014; t=2.467, p=0.017).

group was notably reduced compared with that in control group (p<0.05) (Figure 5).

Neuron Cells Degeneration Detected by Fluoro-Jade B Test

Through Fluoro-Jade B staining results showed that the number of degenerated neuron cells of SD rats in experimental group was significantly smaller than that in control group (p<0.05). The numbers of positive cells at ×400 magnifications in experimental group was 19.2±1.1, which was significantly smaller than that of control group (34.2±2.3) (Figure 6).

NT-3 miRNA Amplification of SD Rat Detected by RT-PCR

RT-PCR results showed that expression level of NT-3 mRNA of the SD rats in experimental group was remarkably elevated compared with that in control group [(0.61±0.02) *vs.* (0.31±0.01)] (p<0.05) (Figure 7).

Discussion

Bacterial meningitis affects more than 1.2 million new cases every year worldwide, and treatment of bacterial meningitis has attracted more and more attentions[8,9]. Mortality rate of acute bacterial meningitis is high, and mortality rate of patients with coma caused by bacterial meningitis can reach 62%. Risk factors of bacterial meningitis include impaired mental status, aging, complications induced by non-meningococcal causes and fulminant diseases[10,11]. In addition, 80% of the survived patients will develop sequelae of the nervous system[12,13]. Therefore, we aimed to investigate the effect of NT-3 on the memory function of bacterial meningitis rats by enhancing the expression of NT-3 with an expectation of providing references for the treatment of bacterial meningitis. The SD rat models with memory impairment induced by bacterial meningitis were established. Morris water maze and passive avoidance experiment showed that average swimming time, swimming distance, average number of errors and latency in experimental group were lower than those in the control group. RT-PCR results showed that expression level of NT-3 in experimental group was significantly higher than that in control group, indicating that increased expression level of NT-3 successfully improved memory function in experimental meningitis rats. Bacterial meningitis can result in permanent impairment of neurological function and usually include hearing loss, cerebral palsy and cognitive impairment, especially affecting learning and memory[14-16]. Bacterial meningitis caused by *S. pneumoniae* infection is one of the most serious types of meningitis. In patients with meningitis, neuronal injury is mainly located into cortical,

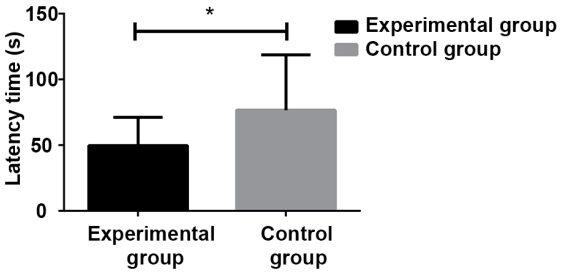

Figure 4. Average latency time detected by passive avoidance experiment of two groups of rats. The results of passive avoidance experiment on SD rats show that the latent time [(49.8±21.5) s *vs.* (76.7±42.1) s] of SD rats in experimental group is remarkably decreased compared with that in control group (t=2.467, p=0.017).

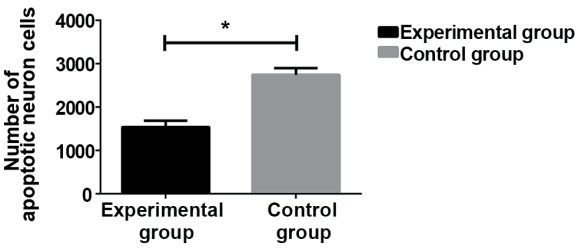

Figure 5. Neuronal apoptosis cell in SD rats detected by TUNEL method. It is found in the TUNEL staining results of neuronal cells of the SD rats that the number of apoptotic neuron cells [(1542.11±144.15) *vs.* (2745.46±155.25)] of SD rats in experimental group is notably reduced compared with that in control group (t=9.477, p=0.003).

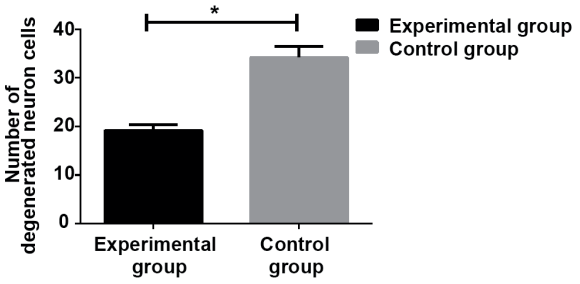

Figure 6. Detection of SD rat neuronal cell by Fluoro-Jade B method. Through Fluoro-Jade B staining of the SD rats' neuron cells, it is discovered in the results that the number of degenerated neuron cells of SD rats in experimental group is significantly smaller than that in control group (t=2.113, p=0.022). The numbers of positive cells under the field of vision at ×400 magnifications are 19.2±1.1 *vs.* 34.2±2.3 in experimental group *vs.* control group.

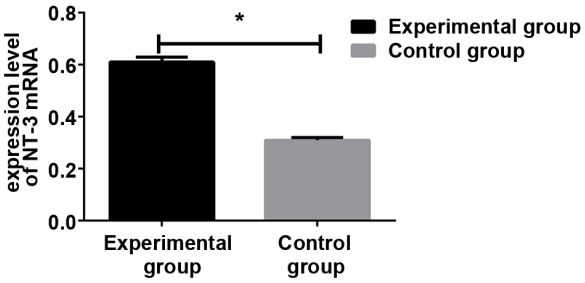

Figure 7. Amplification results of NT-3 miRNA by RT-PCR. It is revealed in the results of NT-3 mRNA amplification of the SD rats via RT-PCR that the expression level of NT-3 mRNA of the SD rats in experimental group is remarkably elevated [(0.61±0.02) *vs.* (0.31±0.01)] compared with that in control group (t=3.241, p=0.007).

subcortical and hippocampal regions[17]. Bacterial meningitis patients rarely have a cure for primary disease until the start of antibiotic treatment. Therefore, the treatment of bacterial meningitis-related secondary diseases is important to reduce the risk of bacterial meningitis[18]. Delgado et al[19] found that NT-3 could promote the survival of neuron cells, induce the endothelial cells to produce nitric oxide synthetase, thus promoting the neural stem cells to generate nitric oxide and maintaining the functions of the nervous system. TUNEL and Fluoro-Jade B staining showed that the numbers of apoptotic and degenerated neuron cells of the rats in experimental group were lower than those in control group, indicating that the increase in the NT-3 expression level is beneficial to neuron cells. Roy et al[20] reported that NT-3 can protect injured neurons, stimulate the proliferation of neuron cells and restore the injured functions of the neurons in the central nervous system caused by diseases, which is consistent with the findings in our study. Therefore, we suggest that upregulation of NT-3 level can inhibit the degeneration and apoptosis of neuronal cells, thereby protecting the neurological function of bacterial meningitis in rats.

Conclusions

Animal models used in this study may not fully reflect the disease conditions in human body. Therefore, clinical studies are needed to further confirm the conclusion. Thus, NT-3 expression level may be related to bacterial meningitis. NT-3 can reduce neuron cell apoptosis and degeneration, promote regeneration of nerve cells and alleviate memory impairment to some extent. NT-3 may be regarded as a therapeutic target for the treatment of memory impairment of patients with bacterial meningitis in future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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