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Effect of atorvastatin on expression of TLR4 and NF-κB in stroke rats and its protective effect on brain

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Abstract. – OBJECTIVE: This study aimed at investigating the effect of atorvastatin on the expression of TLR4 and NF-κB in brain tissue of stroke rats and its protective effect on brain.

MATERIALS AND METHODS: Sixty SD rats were selected. The control group (n=20) was raised without treatment. MCAO was used in the experimental group (n=20) and model group (n=20) for stroke modeling. The experimental group was treated with atorvastatin intragastric administration, while the model group received no treatment. The blood lipid level was detected by a full-automatic biochemical analyzer. The expression levels of TLR4 and NF-κB proteins were detected by Western-Blot (WB), and the mRNA expression of NF-κB and TLR4 in brain tissues was detected by real-time quantitative fluorescence (qtPCR). The neurological deficit score was performed.

RESULTS: TC, TG, LDL-C in blood lipid indexes of the experimental group and the model group were remarkably higher than those of control group, while HDL-C was remarkably lower than that of control group ($p<0.05$). After treatment, TC, TG, LDL-C in the experimental group gradually decreased, while HDL-C concentration gradually increased ($p<0.05$). The expression of TLR4 and NF-κB protein in the experimental group was significantly lower than that in the model group ($p<0.01$) but significantly higher than that in the control group ($p< 0.01$). The expression of TLR4 and NF-κB mRAN in the experimental group was also considerably lower than that in the model group ($p<0.01$) but higher than that in the control group ($p<0.01$). After atorvastatin treatment, the neurological deficit score of the experimental group was remarkably lower than that of the model group ($p<0.001$).

CONCLUSIONS: Atorvastatin can effectively reduce the expression of TLR4 and NF-κB in brain tissue of stroke rats, and has a certain protective effect on cerebral nerve function, which is expected to be the first choice for stroke treatment in the future.

Key Words:
Atorvastatin, Stroke, TLR4, NF-κB, Brain protection.

Introduction

Stroke, also known as apoplexy, is brain tissue damage caused by sudden rupture or blockage of cerebral vessels. There were various causes for this disease, including ischemic stroke and hemorrhagic stroke; mostly ischemic stroke. Patients often suffer from different degrees of sensory and motor dysfunction after onset. It is a common clinical cerebrovascular disease and has become the main cause of death and disability worldwide[1]. Stroke is characterized by high incidence rate, high mortality rate and high disability rate. Survivors are often accompanied by stroke sequelae. According to a research by Boehme et al[2], the incidence of stroke is expected to increase by 3.4 million between 2012 and 2030. Along with the high incidence, up to 50% of survivors are chronically disabled[3]. In addition, the incidence of stroke has increased significantly among young adults in low-income and middle-income countries[4]. At present, stroke has become a public health problem, posing a serious threat to human health and quality of life. Due to the high incidence, high mortality and high disability of stroke, it has become a hot research topic in clinical practice.

Stroke, as the main cause of death from disease, has a complicated pathogenesis. Some studies show that inflammatory reactions are involved in the incidence and development of stroke[5]. In addition, dyslipidemia and hypertension are recognized risk

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factors for stroke. Several clinicians and researchers have paid attention to the prevention of stroke by controlling blood pressure and reducing blood lipid[6]. At the same time, some studies also suggest that cognitive impairment after stroke is bound up with brain tissue damage in stroke patients[7]. The use of statins began in the 1980s. Among them, atorvastatin could stably and rapidly reduce blood lipid level. At the same time, it can prevent degeneration of glial progenitor cells and promote their proliferation and differentiation, so as to repair the nerves[8]. Robertson et al[9] showed that atorvastatin can improve the recovery of neurological function in patients with mild craniocerebral injury. In addition, Chan et al[10] showed that atorvastatin can inhibit inflammation and promote vascular maturation and can effectively treat chronic subdural hematoma. However, at present, there is still little research on atorvastatin in the treatment of stroke at home and abroad.

Therefore, this study aims at providing a reliable reference for the future clinical treatment of stroke patients. We investigated the effect of atorvastatin on the expression of the inflammation-related toll-like receptor 4 (TLR4) and nuclear transcription factor-κB (NF-κB) on the brain and its protective effect on the brain.

Materials and Methods

Animal Model

Sixty SD rats were provided by Animal Experimental Center of Anhui Medical University. The rats aged 6-7 weeks. The feeding conditions were room temperature (26°C), 75% humidity, with 5 rats in each cage, fed with normal illumination and free drinking water. This investigation was approved by the Animal Ethics Committee of our hospital.

Modeling Method

All rats were randomly divided into three groups (all n=20). The control group was fed normally without treatment. The experimental group and the model group were treated with reforming longa method to prepare permanent middle cerebral artery occlusion (MCAO) to simulate ischemic stroke in rats. In addition, the experimental group received atorvastatin treatment. Rats in the experimental group were given atorvastatin (Beijing Jialin Pharmaceutical Co., Ltd., approval number: SFDA approval number H 20093819) 6 mg/kg d aqueous suspension daily for intragastric administration. Rats in the model group and control group were given the same dose of normal saline for intragastric administration for a total of 4 weeks. At the 5th week, all rats were killed by dislocation, and specimens were collected.

Detection Methods

Western-blot detection (WB)

After all rats were executed, rat brain tissues were obtained, and the expression of TLR4 and NF-κB proteins in brain tissues were detected by WB. The collected brain cells were extracted by radio immunoprecipitation assay (RIPA) lysis method, and the protein concentration was detected by bicinchoninic acid (BCA) method. The protein concentration was adjusted to 4 μg/μL, separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membrane. It was stained with Ponceau S and washed after soaking in Phosphate-Buffered Saline and Tween (PBST) for 5 min. It was blocked with 5% skimmed milk powder for 2 h. NF-κB, TLR4, β-Actin primary antibody (1:1000) (Baisinuo (Tianjin) Biotechnology Co., Ltd., article number: EL803353-50, Shanghai Hengfei Biotechnology Co., Ltd., article number: K003881P, K001342M) were added and blocked overnight at 4°C. The primary antibody was removed by washing membrane, and horseradish peroxidase (HRP) labeled goat anti mouse secondary antibody (1:5000, Nanjing Saihongrui Biological Technology Co., Ltd., batch number: 2) was added, incubated at 37 °C for 1 h, and then rinsed with phosphate-buffered saline (PBS) for 3 times, with 5 min each time. Excess liquid was absorbed from the membrane with filter paper. Enhanced chemiluminescence (ECL) was used to illuminate and develop in a dark room. Protein bands were scanned, and the grayscale value was analyzed in Quantity One software. Relative expression level of the protein = grayscale value of the target protein band/β-actin protein band.

PCR detection

The expression of TLR4 and NF-κB mRNA in brain tissue was detected by real-time quantitative fluorescence (qtPCR). A proper amount of brain tissue samples was taken. TRIzol (the kit was purchased from Shanghai Mingjing Biology Co., Ltd., item number: 5003050) was added to extract total RNA. The purity, concentration and integrity of

the extracted total RNA were detected for by ultraviolet spectrophotometer and agarose gel electrophoresis. TransScript® miRNA RT Enzyme Mix and 2×TS miRNA Reaction Mix were used in the reverse transcription of the total RNA (the kit was purchased from Beijing Baiaolaibo Technology Co., Ltd., item no: ALH266-PTO). The operation steps were strictly in accordance with the manufacturer's kit. Subsequently, PCR amplification experiment was carried out (the kit was purchased from Zhen Shanghai and Shanghai Industrial Co., Ltd., item no: HZ-051021). PCR reaction system: cDNA 1 μL, upstream primers 0.4 μL, downstream primers 0.4 μL, 2×TransTaq® Tip Green qPCR SuperMix 10 μL, Passive Reference Dye (50X) 0.4 μL. Finally, ddH_2O was added to make up to 20 μL. PCR reaction conditions were as follows: pre-denaturation at 94°C for 30 s, denaturation at 95°C for 5 s, annealing and extension at 60°C for 30 s, with a total of 40 cycles. Three replicate wells were set up for each sample, and the experiment was performed three times. In this study, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal reference, and 2-ΔCt was used to analyze the data. All primer sequences were designed and synthesized by Shanghai Sangon Biotech Co., Ltd., as shown in Table I.

Blood lipid concentration

Blood lipid concentration was detected by AU5800 automatic biochemical analyzer, Beckman Coulter, USA.

Outcome Measures

1. Changes of blood lipid concentration in three groups of rats, including total cholesterol (TC), triacylglycerol (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C). 2. Expression of TLR4 and NF-κB proteins in brain tissue. 3. TLR4, NF-κB mRNA expression in brain tissue. Neurological defect score[11]: neurological defect score was performed before and after treatment, respectively. Three points mean that the rats had resistance to lateral pressure of front paw flexion and accompanied by rotation. Two points mean that the rats had resistance to lateral pressure of front paw flexion but did not rotate. One point means that the rats could not fully extend the opposite front paw, and 0 point means that the rats had no symptoms of nervous system injury.

Statistical Analysis

SPSS 24.0 statistical software (Shanghai Yuchuang Network Technology Co., Ltd.) was applied for statistical calculation of all experimental results. GraphPad5 was applied to draw all pictures. The results of this experiment were all expressed in the form of (mean±standard deviation). The comparison between multiple time points adopted repeated measurement ANOVA and Bonferroni post-hoc test. The comparison between multiple groups adopted one-way analysis of variance and LSD post-hoc test. Independent sample *t*-test was used for pairwise comparison. $p<0.050$ was considered statistically significant.

Results

Blood Lipid Test Results

TC, TG, LDL-C in the experimental group and model group were significantly higher than those in the control group, while HDL-C was significantly lower than that in control group ($p<0.05$). After treatment, TC, TG and LDL-C in the experimental group gradually decreased, while HDL-C concentration gradually increased ($p<0.05$), as shown in Table II.

WB Test Results

The expression of TLR4 protein in the experimental group was 0.78±0.16, which was significantly lower than that in the model group (1.24±0.32), $p<0.01$, and significantly higher than that in the control group (0.47±0.09), $p<0.01$. The expression of NF-κB protein in the experimental group was 0.72±0.11, which was significantly lower than that in the model group (0.99±0.19), $p<0.01$, and significantly higher than that in the control group (0.44±0.08), $p<0.01$, as shown in Figures 1 and Figure 2.

Table I. Primer sequences.

	Upstream primer	Downstream primer
TLR4	5'-CCCTGCCACCATTTACAGTTCG-3′	5'-GAGTCCCAGCCAGATGCAAGAG-3'
NF-κB	5'-AGA-GAAGCACAGATACCACTAAG-3′	5'-CAGCCTCATAGA AGCCATCC-3'
GAPDH	5'-ACAGCAACAGGGTGGTGGAC-3'	5'-TTTGAGGGTGCAGCGAACTT-3'

Table II. Blood lipid test results of two groups of rats (mmonl/L).

Control group	Experimental group				Model group
	Model group	Modeling for 12h	Treatment for 1 week	The treatment lasted 4 weeks	
TC	0.43±0.15	3.09±0.76*	2.32±0.57*#	1.16±0.38*#Δ	3.21±0.59*#Δ°
TG	0.71±0.20	1.84±0.35*	1.31±0.30*#	0.87±0.29*#Δ	1.86±0.36*Δ°
LDL-C	0.31±0.12	1.45±0.47*	0.94±0.23*#	0.46±0.19*#Δ	1.42±0.44*#Δ°
HDL-C	1.54±0.53	0.69±0.21*	0.89±0.33*#	1.23±0.41*#Δ	0.70±0.25*#Δ°

*denotes comparison with the control group, $p<0.05$.
#denotes comparison with 12 hours of modeling, and there is no significant difference between the model group and 12 hours of modeling ($p>0.05$).
Δdenotes comparison with 1 week of treatment, $p<0.05$.
°denotes comparison with 4 weeks of treatment, $p<0.05$.

PCR Detection Results

The expression of TLR4 and NF-κB mRAN in the experimental group were 1.66±0.41 and 1.58±0.46, respectively, which were significantly lower than those in the model group (2.67±0.59 and 2.36±0.48, respectively, $p<0.01$), but significantly higher than those of the control group (0.95±0.08, 1.14±0.17, $p<0.01$; Figure 3).

Neurological Deficits Score

The control group had no neurological deficit symptoms before and after treatment. There was no considerable difference in neurological deficit score between the model group and the experimental group before treatment, $p>0.05$. There was no remarkable difference in neurological deficit score between the model group and the experimental group before and after treatment, $p>0.05$. It indicated that there was no remarkable recovery of neurological defects in the rats. Neurological deficit score in the experimental group after treatment was significantly higher than that before treatment, $p<0.001$, and significantly higher than that in the model group, $p<0.001$. It indicated that neurological deficit symptoms in the experimental group considerably improved after treatment (Table III).

Discussion

Stroke, as an acute and critical disease, is extremely common in clinic. Most patients suffer from brain function damage after onset, with the risk of death and disability. Stroke is the second

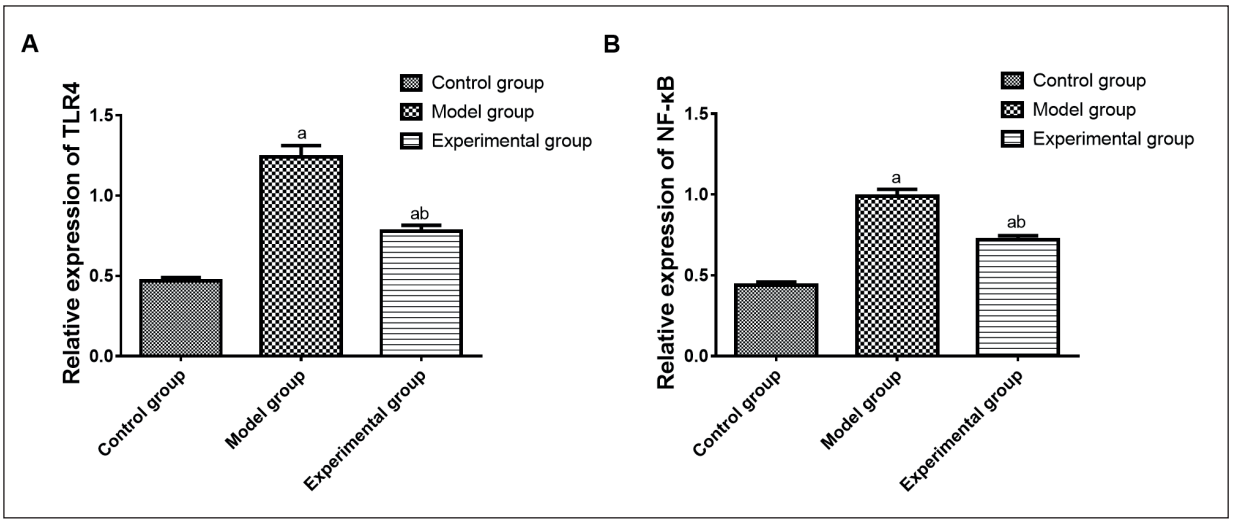

Figure 1. Comparison of TLR4 and NF-κB protein expression in three groups of rats. **A**, TLR4 protein expression in three groups of rats. **B**, NF-κB protein expression in three groups of rats. a denotes comparison with the control group, $p<0.01$. b denotes comparison with the model group, $p<0.01$.

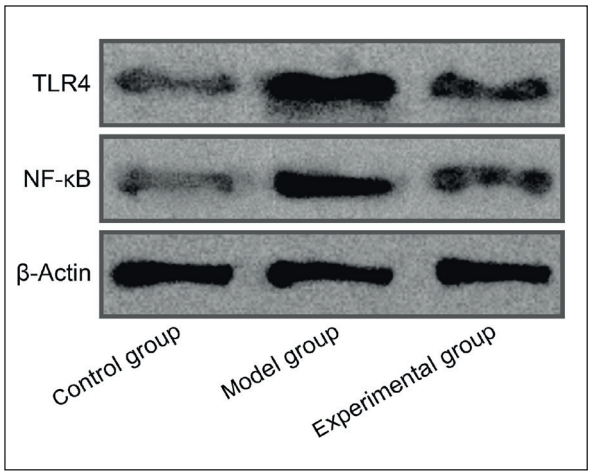

Figure 2. WB protein diagram.

most common cause of death and the main cause of disability in the world[12]. According Lecoffre et al[13], stroke is the leading cause of death in women and the third leading cause of death in men in France. The age standardized rate of hospitalized patients with ischemic stroke increased by 14.3% from 2008 to 2014 among patients < 65 years old, indicating that the incidence rate of stroke is increasing among young people. According to Phan et al[14], global disease burden data in 2015 showed that there were about 15.2 million deaths of stroke and ischemic heart disease in the world, accounting for 85.1% of the total deaths of cardiovascular diseases. Stroke has not only an extremely high mortality rate, but also a high disability rate. Ojaghihaghighi et al[15] pointed out that about 15%-30% of stroke patients suffer from permanent disability. At the same time, the social cost of the disease is particularly high, costing up to US$ 50 billion per year (USA), bringing heavy burden to the country and families[16]. The specific pathogenesis of stroke is still not completely clear. It is known that in stroke diseases, inflammatory response is a physiological response to repair damaged brain tissues to protect the central nervous system. Large amounts of inflammatory cytokines and cell infiltration are usually observed in the damaged area[17].

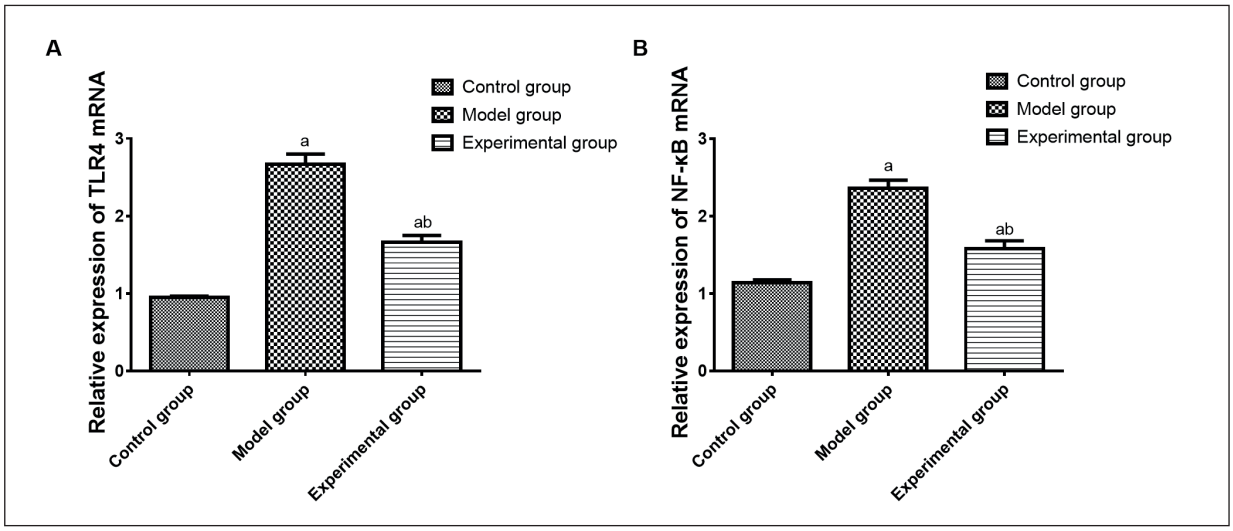

Figure 3. Comparison of TLR4 and NF-κB mRNA expression in three groups of rats. **A**, TLR4 mRNA expression in three groups of rats. **B**, NF-κB mRNA expression in three groups of rats. a denotes comparison with the control group, $p<0.01$. b denotes comparison with the model group, $p<0.01$.

Table III. Comparison of neurological deficit scores among three groups of rats.

Group	Before treatment	After treatment	*t*	*p*
Control group (n=20)	0	0		
Model group (n=20)	2.55±0.61	2.31±0.46	1.405	0.168
Experimental group (n=20)	2.63±0.68[c]	0.94±0.25[d]	10.430	<0.001

[c]denotes comparison with the model group, $p>0.05$.
[d]denotes comparison with the model group, $p<0.001$.

Dyslipidemia increases the risk of stroke, especially in patients with high triglycerides and low high density lipoprotein-C, but the specific mechanism is still unknown[18]. Atorvastatin is a kind of statins belongs to 3-hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitor. It can catalyze the rate-limiting step of cholesterol synthesis, leading to up-regulation of low-density lipoprotein receptor[19]. As a third generation of statins with great curative effect and sufficient safety, atorvastatin has been fully proved to be effective in coronary heart disease, angina pectoris and other diseases[20,21], but there is still little research on its application value in stroke diseases. Therefore, by analyzing the effect of atorvastatin on inflammatory indexes and its protective effect on cranial nerves in stroke rats, this study tried to find an effective drug for clinical treatment of similar diseases, so as to improve the therapeutic effect and prognosis of stroke patients.

The results of this study showed that compared with the control group, the concentrations of TC, TG and LDL-C in the model group and the experimental group increased while the concentration of HDL-C decreased. After atorvastatin treatment, the concentrations of TC, TG and LDL-C in the experimental group were remarkably lower than those in the model group, and the concentration of HDL-C was significantly higher. It suggested that atorvastatin can effectively improve the dyslipidemia in cerebral tissue of stroke rats, which is similar to the research results of Zhang et al[22]. They also explored the effect of atorvastatin on improving the lipid level of atherosclerosis hyperlipidemia. Meanwhile, the study on inflammation-related factors TLR4 and NF-κB revealed that the expression of the two factors in the model group and the experimental group treated with atorvastatin was considerably higher than that in control group. Meanwhile, protein expression and mRNA expression of the two factors in the experimental group were considerably lower than that in model group, suggesting that atorvastatin can inhibit the expression of TLR4 and NF-κB to achieve the purpose of treating inflammation. TLR4 is a member of Toll-like receptor (TLR) family and is an important transmembrane receptor involved in inflammation. NF-κB is an inducible transcription factor that regulates a large number of genes involved in different processes of immune and inflammatory reactions[23,24]. NF-κB factor plays an important role in the central nervous system through Toll-like receptors. The release of proinflammatory mediators caused by neurological dysfunction may be helpful or harmful to the survival of normal cells. A wide range of cell signaling mediators and their interactions play a crucial role in neuroinflammation related to ischemia, brain trauma and age-related neurodegeneration[25]. In addition, the results of this study revealed that there was no significant difference in neurological deficit score between the model group and the experimental group before treatment. After atorvastatin treatment, the neurological deficit score of the experimental group was significantly lower than that of the model group, indicating that atorvastatin can effectively reduce the damage of nerve cells and protect brain function.

This investigation explored the effect of atorvastatin on the expression of TLR4, NF-κB and the cerebral nerve function in stroke rats by establishing a stroke rat model. However, due to the short experimental period, the long-term effect of atorvastatin on stroke cannot be observed. Moreover, the sample size of the study is small, we were not able to carry out big data statistical analysis. Moreover, this experiment only studies ischemic stroke, and it is impossible to know whether there is the same experimental result in hemorrhagic stroke. Inflammatory related factors correlated with stroke are not only limited to the two types detected in the literature. Due to the limited experimental conditions, we did not detect and analyze the other types. Therefore, we cannot exclude inflammatory related factors that may have more typical manifestations than TLR4 and NF-κB. In addition, there are certain differences between animal model and human body after all. It is not excluded that the effect of atorvastatin will deviate in human environment. We will carry out human experimentation as soon as possible, expand the experimental cycle and refine the experimental design to obtain the best experimental results.

Conclusions

At present, there is still little research about atorvastatin in the treatment of stroke; its exact mechanism on stroke is not yet clear. The incidence of stroke is increasing. If the treatment effect of atorvastatin can be confirmed, there will be a major breakthrough for the treatment of stroke in the future. This study indicated that atorvastatin can effectively reduce the expression of TLR4 and NF-κB in cerebral tissue of stroke rats and has certain protective effect on cerebral nerve function. In the future, atorvastatin is expected to be the first choice for stroke treatment and for the improvement of prognosis.

Conflict of Interests

The authors declare that they have no conflict of interest.

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