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Association of the TLR4-MyD88-JNK signaling pathway with inflammatory response in intracranial hemorrhage rats and its effect on neuronal apoptosis

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Abstract. – OBJECTIVE: To investigate the association of Toll-like receptor 4-myeloid differential protein-88- c-Jun N-terminal kinase (TLR4-MyD88-JNK) signaling pathway with inflammatory response in intracranial hemorrhage (ICH) rats and its effect on neuronal apoptosis.

PATIENTS AND METHODS: The autologous blood was drawn and injected into the brain to establish the rat model of ICH (model group), and the control group was set up. The neurological behavior Longa score was given. The blood and brain tissues of rats were then collected to detect the serum indexes, including glucose (GLU), creatinine (CR), K^+ and Na^+, and the content of interleukin-6 (IL-6), IL-1β and tumor necrosis factor-α (TNF-α) in each group. The neuronal apoptosis of brain tissues was detected *via* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Moreover, the expressions of apoptosis- and TLR4-MyD88-JNK pathway-related genes and proteins were detected *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting. Finally, the association of TLR4-MyD88-JNK signaling pathway with the inflammatory response in ICH rats and its effect on neuronal apoptosis were completely observed.

RESULTS: MiR-23b was dramatically down-regulated in CC and the low miR-23b expressions were associated with the poor prognosis and worse OS of CC patients. Additionally, the functional assays demonstrated that miR-23b overexpression obviously repressed CC cell proliferation, invasion and migration abilities through the regulation of the AKT/mTOR pathway and the epithelial-to-mesenchymal transition (EMT) progress. Moreover, the luciferase reporter assay indicated that six1 was one functional target for miR-23b in CC cells, indicating that the inhibitory functions of miR-23b in CC cells were partially regulated by six1. Moreover, miR-23b restoration could prominently repress tumor growth *in vivo*.

CONCLUSIONS: The TLR4-MyD88-JNK signaling pathway can facilitate the inflammatory response in ICH rats, thereby promoting the neuronal apoptosis.

Key Words
TLR4-MyD88-JNK signaling pathway, Intracranial hemorrhage rats, Inflammatory response, Neurons, Apoptosis.

Introduction

Intracranial hemorrhage (ICH) is an important clinical type of stroke and a common clinical disease. Stroke is one of the most common diseases of the central nervous system, and ICH accounts for only one-third of all stroke cases, but its prognosis is poor. Currently, there have been no effective drug therapies that can improve the survival rate or quality of life of survivors[1]. ICH can induce neuronal apoptosis, edema around the hematoma, inflammatory lesions, neurological impairment and energy metabolism disorder[2], and lead to initial and delayed brain injury. The initial injury is mainly due to dilated hematoma compression, while the delayed injury is due to the direct toxic effect of blood-derived factors released by the hematoma in brain tissues and

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blood vessels. The occurrence of ICH is related to cerebral amyloid angiopathy, seriously affecting the quality of life[3]. Moreover, inflammatory mediators are produced from blood lysates in the neuroinflammation caused by ICH, which activates the residual gliocytes and infiltrates blood-derived immune cells, thereby destroying the blood-brain barrier, forming the edema and deteriorating the neurobehavioral function. Such neuroinflammation caused by ICH plays a crucial role in the complex pathophysiological process of subacute-stage ICH[4-6]. ICH remains a great challenge and there have been no effective treatment methods currently[7]. Therefore, it is of great importance to deeply understand its molecular regulatory network for the treatment of ICH, and designing drugs targeting these genes or proteins will provide new ideas for the treatment of ICH. As a result, searching for new targets for ICH has become a problem urgently to be solved.

Studies have demonstrated that the lesions of endothelial cells, inflammatory cells and local brain neurons and the activation of the Toll-like receptor (TLR) inflammatory response pathway are caused by ischemia and hypoxia around the lesions after the onset of ICH, which leads to secondary inflammatory injury[8,9]. Therefore, the prevention and treatment of secondary inflammatory injury and inflammation become increasingly attractive in the clinic. TLR4 is a cell surface sensor that interacts with multiple protein complexes and triggers the activation of downstream pathways, leading to phosphorylation and translocation of downstream factors[10]. Downstream TLR4 activates myeloid differential protein-88 (MyD88)-dependent or MyD88-independent pathway c-Jun N-terminal kinase (JNK)[11]. MyD88-mediated signals promote the early activation of nuclear factor-κB (NF-κB) and the production of many pro-inflammatory cytokines, inducing cell adhesion, proliferation, angiogenesis and apoptosis[12]. On the contrary, the MyD88-independent pathway involves JNK, which stimulates the TRIF signaling pathway, leads to the late activation of NF-κB and induces IRF3, thereby increasing the production of interferon (IFN) and IFN-induced gene products[13]. It is noteworthy that the significant inhibition on TLR4 expression inhibits MyD88, and the endogenous signals of microbiological products, such as urate crystals and adenosine triphosphate (ATP) as potential second signals[14], activate the NLRP3 inflammasome complexes. A single stimulus event triggers the pre-expression and release of interleukin-1β (IL-1β). The second signal is indispensable, because monocytes will release endogenous ATP and induce secretion of IL-1β after stimulus[15,16]. However, the definite correlation of the TLR4-MyD88-JNK signaling pathway with the inflammatory response in ICH rats and the concrete mechanism of its effect on neuronal apoptosis remain unclear. In this research, therefore, the effects of the TLR4-MyD88-JNK signaling pathway on inflammatory response and neuronal apoptosis in ICH rats were explored using molecular methods, to provide experimental and theoretical bases for the prevention and treatment of ICH through the TLR4-MyD88-JNK signaling pathway.

This study aims to investigate the effects of the TLR4-MyD88-JNK signaling pathway on inflammatory response and neuronal apoptosis in ICH rats. TLR4-MyD88-JNK is an important inflammatory regulator in various diseases, but whether it is involved in the pathogenesis of ICH and regulates the neuronal apoptosis is rarely studied. Therefore, the potential role of TLR4-MyD88-JNK in ICH was explored, and its effect on ICH was clarified through *in vivo* experiments and various molecular biological techniques in this study. The experimental results will enrich and improve the theoretical basis of the effects of TLR4-MyD88-JNK on inflammatory response and neuronal apoptosis in ICH.

Materials and Methods

Animal Grouping and Modeling

After anesthesia, the rats were placed on the operating table in a supine position, and 15 μL of autologous blood was drawn from the femoral artery. After hemostasis, the rats were immediately placed on the stereotaxic apparatus in a prone position, and the autologous blood was injected into the brain at a rate of 1 μL/min according to the method of Sansing et al[17] to establish the ICH model (model group, n=15). The rats underwent no treatment in the control group (n=15). All the experimental methods in the scheme were approved by the Laboratory Animal Ethics Committee of our hospital, and all animal operations were performed in accordance with the NIH Laboratory Animal Guide. The blood was drawn from the eyeballs and centrifuged, and the serum was collected and stored at -80°C to detect the serum biochemical indexes. After anesthesia with pentobarbital sodium, an appropriate number of

brain tissues were carefully taken and divided into two parts, one for morphological detection and one stored at -80°C for detection of expression levels of genes and proteins.

Neurological Score of Rats in Each Group

Before the rats were sacrificed in each group, the neurological score (0-6 points) was given using the blind method according to the scoring criteria in Table I. The rats with the highest and lowest scores and those with other clinical symptoms during the experiment were eliminated, and new rats were supplemented.

Detection of ICH Indexes

To predict the occurrence of ICH in advance in clinic and provide important references for early diagnosis, the glucose (GLU), creatinine (CR), K^+ and Na^+ were detected. The serum stored in the low-temperature refrigerator was taken, thawed and placed into the centrifuge tube, followed by detection of changes in their content using the full-automatic biochemical analyzer.

Detection of Content of Inflammatory Factors Via Enzyme-Linked Immunosorbent Assay (ELISA)

The serum inflammatory factors are important indexes for brain injury, and they can reflect the rate of injury repair, so the content of serum inflammatory factors was detected *via* ELISA (R&D Systems, Minneapolis, MN, USA). The serum stored at -80°C was taken out, slowly thawed at 4°C and centrifuged at low speed, and the supernatant was collected to detect the changes in indexes according to the instructions of the kit. Finally, the absorbance of inflammatory factors in each group was detected using a microplate reader.

Table I. Scoring criteria.

Score	Behavior
0	Able to walk autonomously
1	Contralaterally rotate towards the lesion in autonomous walking
2	Contralaterally rotate towards the lesion in tail suspension
3	Decline in resistance to lateral pressure contralateral to lesion
4	The whole body bends contralaterally
5	No neurological defects

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Apoptosis Assay

The apoptosis in paraffin sections was detected using the "In situ cell death detection" kit (Roche, Basel, Switzerland), and the specific steps are as follows: the paraffin sections were deparaffinized, washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA), added with proteinase K working solution, soaked in blocking buffer, fixed, rinsed and permeated with 0.1% Triton X-100, followed by fluorescein isothiocyanate (FITC) end labeling of apoptotic DNA fragments using the TUNEL assay kit. Finally, the FITC-labeled TUNEL-positive cells were observed under a fluorescence microscope and counted in 10 fields of view.

Detection of Expressions of Related Genes Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

About 300 mg of sterile brain tissues were carefully taken under low temperature and added with lysis buffer (Invitrogen, Carlsbad, CA, USA), followed by homogenization under low temperature to extract the total RNA. After the purity and concentration of RNA were detected qualified, mRNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) and stored in a refrigerator at -80°C. Then, the primer amplification was performed using 20 μL of amplification system (2 μL of cDNA, 10 μL of qPCR mix, 2 μL of primer and 6 μL of ddH_2O, a total of 40 cycles), and the primer sequences of target genes and internal reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed according to the GenBank (Table II).

Table II. Scoring criteria.

Target gene	Primer sequence (5'–3')
GAPDH	F: 5'-TGACTTCAACAGCGACACCCA-3' R: 5'-CACCCTGTTGCTGTAGCCAAA-3'
TNF-α	F: 5'-CGCTACGACCGCCAG ATTG-3' R: 5'-ACACCGTTCACCAGCAAGTC-3'
Caspase3	F: 5'-CTACCGCACCCGGTTACTAT-3' R: 5'-TTCCGGTTAACACGAGTGAG-3'
TLR4	F: 5'-CTGAACCAGGGCATACCTGT-3' R: 5'-GAGAAGTCCATGTCCGCAAT-3'
MyD88	F: 5'-AGCTGGAGCAGACGGAGTG-3' R: 5'-GAGGCTGAGAGCAAACTTGGTC-3'
JNK	F: 5'-TTCCATTGTGGGTAGGTGG-3' R: 5'-CTTACAGCTTCCGCTTCAG-3'

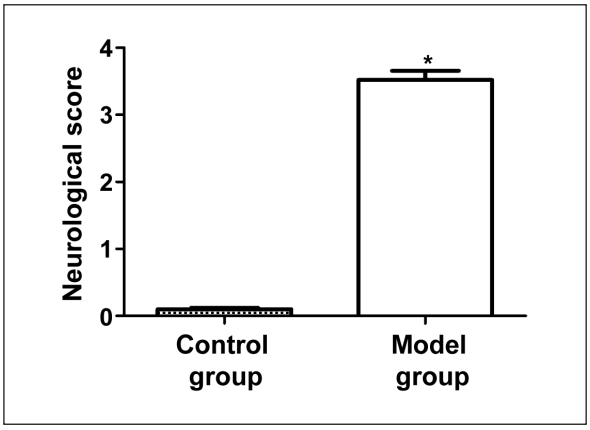

Figure 1. Neurological score. Some rats cannot walk and the whole body bends to the contralateral side in the model group, while there are no abnormalities in the control group ($p<0.05$; *$p<0.05$).

The expression levels of target genes were detected *via* qRT-PCR, and the relative expression levels of related genes in brain tissues in each group were calculated using $2^{-\Delta\Delta Ct}$.

Western Blotting

After 200 mg of tissues were placed into a 10 mL Eppendorf (EP; Hamburg, Germany) tube on ice, they were added with lysis buffer prepared proportionally and incubated in the refrigerator, so that the tissues were fully lysed to release tissue protein. The mixture was centrifuged and the supernatant was collected to detect the protein concentration according to the instructions of the bicinchoninic acid (BCA) kit (Pierce, Waltham, MA, USA). Western blotting was performed as follows: the protein was loaded, subjected to electrophoresis, transferred onto a membrane and incubated with the primary and secondary antibodies. Finally, the image was developed using the gel imaging system, the level of protein to be detected was corrected using GAPDH, and the gray value of the protein band was analyzed using Image Lab (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for the processing of raw experimental data, and multiple comparisons were performed for the data. The experimental results obtained were expressed as mean ± standard deviation ($\bar{\chi}\pm$SD), and $p<0.05$ suggested the statistically significant difference. The bar graph was plotted using the GraphPad Prism 7.0 (La Jolla, CA, USA).

Results

Neurological Score of Rats in Each Group

Some rats could not walk and the whole body bent to the contralateral side in the model group, while there were no abnormalities in the control group ($p<0.05$; Figure 1).

ICH Serum Detection Results

To predict the occurrence of ICH in advance in clinic, GLU, CR, K^+ and Na^+ were detected. As shown in Table III, GLU, CR and Na^+ were markedly increased, while K^+ was significantly decreased in the model group, indicating the occurrence and development of the disease. The changes in them were opposite in the control group ($p<0.05$).

Serum TNF-α, IL-1, and IL-6 Levels

As shown in Table IV, IL-1β, IL-6 and TNF-α were increased in the model group ($p<0.05$), while they declined in the control group ($p<0.05$).

Table III. Changes in content of GLU, CR, K^+ and Na^+.

Group	K^+ (mmol/L)	GLU (U/L)	Na^+ (mmol/L)	CR (μmol/L)
Control group	30.28±5.41	17.26±1.26	200.89±2.89	89.45±2.47
Model group	11.68±1.27[a]	6.68±2.64[a]	105.39±6.54[a]	27.36±4.58[a]

Note: GLU, CR and Na+ are markedly increased, while K^+ is significantly decreased in the model group ($p<0.05$; [a]$p<0.05$).

Table IV. Serum TNF-α, IL-1 and IL-6 levels.

Group	TNF-α (fmol/mL)	IL-6 (mg/L)	IL-1β (mg/L)
Control group	45.28±2.98	88.64±5.12	91.68±8.14
Model group	17.25±3.56[a]	25.38±6.87[a]	30.89±5.46[a]

Note: IL-1β, IL-6 and TNF-α are increased in the model group ($p<0.05$; [a]$p<0.05$).

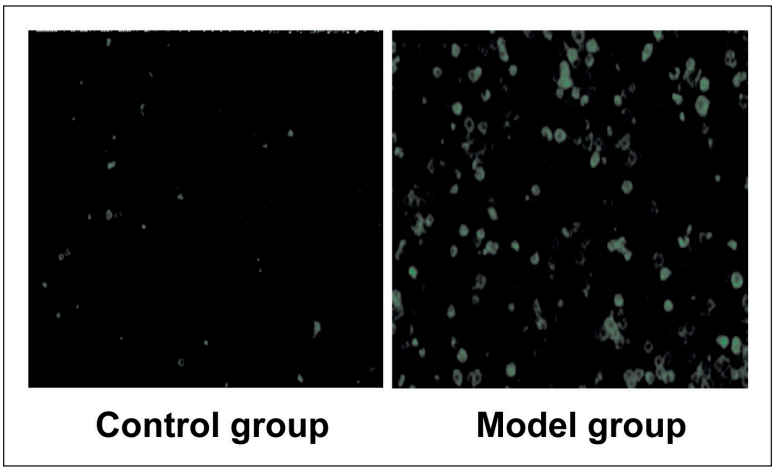

Figure 2. TUNEL staining. There are no significant positive cells in the control group, and the number of positive cells in the model group is markedly increased ($p<0.05$).

TUNEL Apoptosis Assay Results

As shown in Figure 2, there were no significant positive cells in the control group, and the number of TUNEL-positive cells in the model group was markedly larger than that in the control group, and they were mainly distributed around the hemorrhagic foci and dominated by gliocytes ($p<0.05$). The above findings suggest that ICH promotes abnormal neuronal apoptosis.

Expressions of Inflammatory and Apoptotic Genes and Pathway Molecules

The mRNA levels of Caspase3 and TNF-α and pathway genes TLR4, MyD88 and JNK were increased in the model group, while they markedly declined in the control group ($^*p<0.05$; Figure 3 A-E), which suggests that the indicator genes play important roles in the process of ICH. The pathway genes and inflammatory factors are activated during the onset, indicating the further development of ICH.

Expression of Important Proteins and Pathway Proteins

The expressions of apoptosis protein Caspase3 and pathway proteins TLR4, MyD88 and JNK were remarkably increased in the model group, while they markedly declined in the control

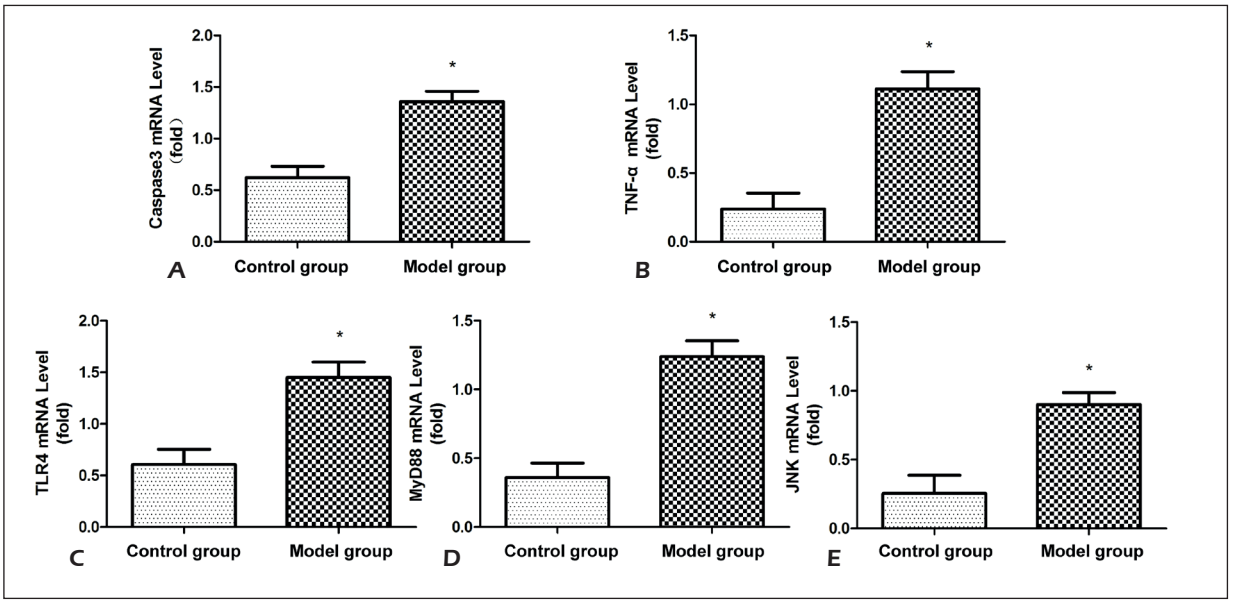

Figure 3. Expressions of key genes. The mRNA levels of Caspase3 ***(A)*** and TNF-α ***(B)*** and pathway genes TLR4 ***(C)***, MyD88 ***(D)*** and JNK ***(E)*** are increased in the model group ($p<0.05$; $^*p<0.05$).

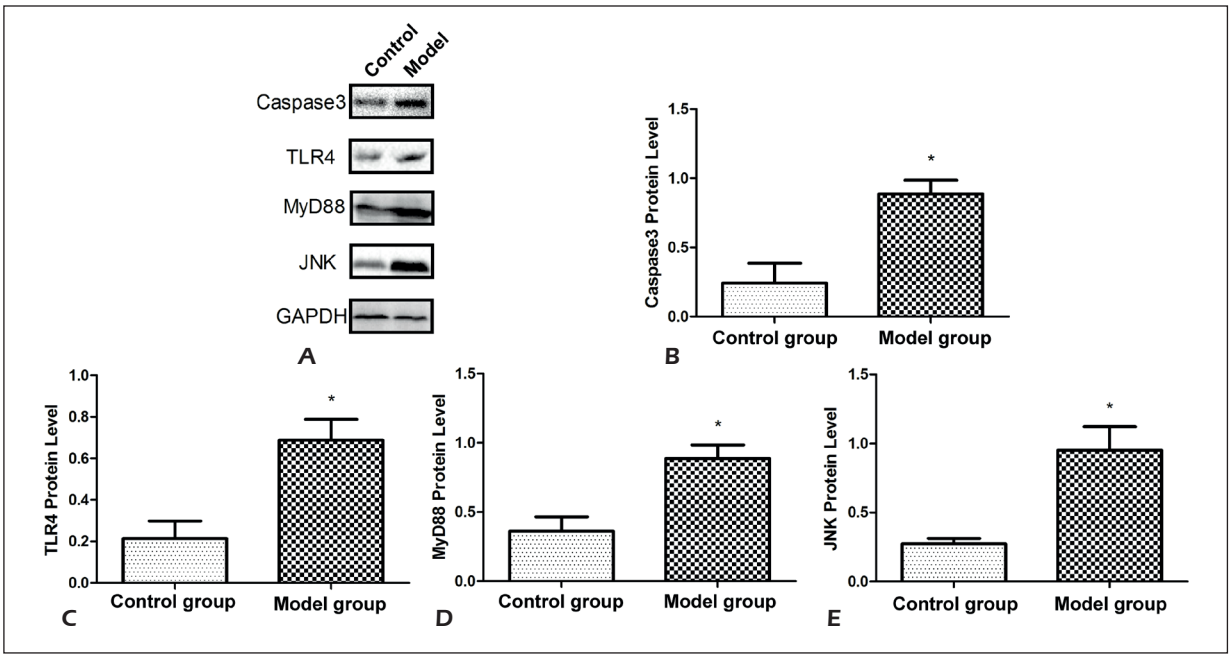

Figure 4. Protein expressions. The expressions of Caspase3 ***(A-B)*** and pathway proteins TLR4 ***(C)***, MyD88 ***(D)*** and JNK ***(E)*** are remarkably increased in the model group ($p<0.05$; $^{*}p<0.05$).

group ($p<0.05$; Figure 4 A-E), demonstrating that the activation of TLR4, MyD88 and JNK will promote the neuronal apoptosis and inhibit the recovery of ICH.

Discussion

ICH is a potentially fatal disease without effective clinical treatment methods, and it is also a common type of stroke, accounting for about 20-30% of the total[18,19]. Although the patients may survive after the initial attack, the hematoma size will increase and a series of life-threatening pathophysiological reactions may occur, such as accumulation of edema, the release of inflammatory cytokines and neurobehavioral defect. The morbidity rate of ICH has increased year by year, and a large number of neuropathological, neuroimaging and molecular studies have demonstrated that neuroinflammatory brain injury is a common pathological feature of ischemic and hemorrhagic stroke in the acute and subacute phase, in spite of the seemingly contradictory reasons for cerebral ischemia and ICH[20]. However, the neuroinflammatory mechanism of hemorrhagic stroke (a severer subtype) has not been studied as vigorously as that of ischemic stroke. The pathological changes in ICH are a complex process involving many factors. Zeng et al[21] showed that ICH is caused by arteriolar injury and rupture due to chronic hypertension or vascular amyloidosis. Moreover, other studies[22,23] have showed that inflammatory cell infiltration, edema and apoptosis of brain tissues in the lesions are an important predisposing factor for secondary brain injury after ICH, among which the theories of inflammatory response and apoptosis have sparked wide interest in clinical research. There is a close correlation between cerebral edema and brain injury: after ICH, the degree of cerebral edema is an important marker for secondary brain injury, as well as one of the important factors for the deterioration of brain injury[24]. The evidence so far suggests that infiltrating neutrophils are observed at 4 h in ICH rat model, and then inflammatory factors are increased[25]. In the present work, the ICH rat model was established, and the neurological score was given. The results revealed that the rats could not walk and the whole body bent to the contralateral side in the model group, while there were no abnormalities in the control group. To predict the occurrence of ICH in advance in clinic, GLU, CR, K^+ and Na^+ were detected. It was found that GLU, CR, and Na^+ were significantly increased, while K^+ was markedly decreased in the model group, indicating the occurrence and development of the disease. The changes in them were opposite in the control

group ($p<0.05$). The above findings suggest that the model was successfully established and could be used for subsequent experiments.

TLR4 is a cell surface sensor that interacts with multiple protein complexes to activate MyD88-dependent or MyD88-independent pathway JNK[26]. It has been reported in many studies that TLR is able to regulate the pathway dependent on MyD88, a key adapter directly involved in TLR4-mediated expression of inflammatory genes[27,28]. TRIF, another receptor molecule of TLR, is responsible for regulating the MyD88-mediated pathway[29]. It is noteworthy that the significant inhibition on TLR4 expression inhibits MyD88, inducing immune responses, cell adhesion, proliferation, angiogenesis and apoptosis[12]. In this work, the content of IL-1β, IL-6, and TNF-α was increased in the model group ($p<0.05$), while that of TNF-α significantly declined in the control group. The levels of pathway genes and proteins TLR4, MyD88 and JNK were also increased in the model group, while they markedly declined in the control group ($p<0.05$), which, consistent with the above studies, suggests that the indicator genes play important roles in the process of ICH, and the pathway genes and inflammatory factors are activated during the onset, indicating the further development of ICH. Klionsky et al[30] has shown that neuronal apoptosis increases in ICH, and apoptosis, as a metabolic pathway, can remove the garbage produced during maintenance of vital activities in time. Moreover, apoptosis can eliminate the harmful substances in cells and respond to the damage in cells. Apoptosis reflex starts quickly in the case of fatal threats, which, as the body defender, supplies energy to the generation of subcellular structure and metabolism and keeps cells stable. At present, apoptotic autophagy has become one of the research hotspots in the biological field[31]. The mechanism of apoptosis in physiological metabolism has not been fully clarified, but the mechanism and pathway elucidated already can serve as important guidelines for various clinical diseases, such as tumors and ICH. In this work, the results of TUNEL staining showed that there were no significant positive cells in the control group, and the number of TUNEL-positive cells in the model group was markedly larger than that in the control group, and they were mainly distributed around the hemorrhagic foci and dominated by gliocytes ($p<0.05$). The above findings suggest that ICH promotes abnormal neuronal apoptosis. In addition, the expressions of apoptosis genes and proteins were remarkably increased in the model group, while they markedly declined in the control group ($p<0.05$), demonstrating that the activation of TLR4, MyD88 and JNK will promote the neuronal apoptosis and inhibit the recovery of ICH. Liu et al[32] also obtained consistent results with those in this experiment. The exact association of the TLR4-MyD88-JNK signaling pathway with the inflammatory response in ICH rats and the specific mechanism of its effect on neuronal apoptosis were verified using various molecular methods, which provides new potential targets for the gene therapy for ICH. However, there are still some deficiencies in this study. In the future, the specific regulatory mechanism of the TLR4-MyD88-JNK signaling pathway in inflammatory response and neuronal apoptosis in ICH rats will be further explored through *in vitro* cell culture experiments and various molecular biological methods.

Conclusions

We found that TLR4-MyD88-JNK may regulate the development of inflammation in ICH rats to further affect apoptosis, and it may play an important role in regulating the neuronal apoptosis in ICH. Therefore, the therapeutic effect and prognosis of patients can be evaluated through the TLR4-MyD88-JNK pathway. In the subsequent studies, more cell lines can be introduced and other possible mechanisms of action can be further verified and explored via gene knockout and flow cytometry.

Conflict of Interests

The authors declare that they have no conflict of interest.

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