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Sodium butyrate relieves cerebral ischemia-reperfusion injury in mice by inhibiting JNK/STAT pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate whether sodium butyrate (NaB) attenuated cerebral ischemia-reperfusion injury (IRI) in mice by inhibiting JNK/STAT pathway, thereby exerting a neuroprotective role.

MATERIALS AND METHODS: ICR mice were randomly assigned into five groups, including the sham group, the model group, the 1 mg/kg NaB group, the 5 mg/kg NaB group and the 10 mg/kg NaB group, respectively. IRI model was established in mice using the bilateral common carotid artery occlusion (BCCAO) method. Open-field test was performed to evaluate degree of IRI damage by recording central travel distance and central active time. The morphology of hippocampal neurons was observed by hematoxylin and eosin (HE) staining. TUNEL staining was conducted to detect apoptotic neurons in the brain of mice. Meanwhile, activities of superoxide dismutase (SOD) and malondialdehyde (MDA) in brain tissues of mice were determined by relative commercial kits. The expression levels of inflammatory factors in brain tissues of mice were accessed using enzyme-linked immunosorbent assay (ELISA). In addition, the protein expressions of Jak2 and STAT3 in brain tissues of mice were detected by Western blot.

RESULTS: 10 mg/kg NaB treatment remarkably alleviated impaired neurological defect and hippocampal neurons, as well as significantly improved neuronal survival. Mice in the 10 mg/kg NaB group showed significantly lower central travel distance and shorter central active time than those in the sham group. In addition, 10 mg/kg NaB treatment markedly increased SOD activity, whereas significantly decreased MDA activity in IRI mice. Mice in the NaB treatment group showed significantly lower levels of IL-1β, TNF-α and IL-8. Meanwhile, TUNEL-positive neurons in mice of the NaB treatment group were remarkably fewer. In addition, the protein expression levels of Jak2 and STAT3 were obviously upregulated in IRI mice, which were significantly downregulated after 10 mg/kg NaB treatment.

CONCLUSIONS: Sodium butyrate exerts neuroprotective effects on cerebral ischemia-reperfusion injury by preventing oxidative stress, inflammatory response and neuronal apoptosis through inhibiting JNK/STAT pathway.

Key Words:
Sodium butyrate, JNK/STAT pathway, IRI.

Introduction

Cerebral ischemia-reperfusion injury (IRI) is the aggravation of nerve damage and neuronal dysfunction after blood reperfusion in ischemic areas. IRI is a very complicated process involving multiple factors, such as oxidative stress, excitatory amino acid toxicity, inflammatory reaction and apoptosis[1-3]. These mechanisms interact with each other and form a complex network that results in a series of cascade reactions. This may eventually lead to neuronal apoptosis/death and neurological abnormalities. Among them, oxidative stress plays a major role in the pathological process of ischemic stroke[4]. It is also an important cause of the development of ischemic injury cascade[5]. In addition, inflammatory response is greatly involved in IRI progression. Scholars[6-8] have found that pro-inflammatory cytokines stimulate and aggravate brain damage. The expression levels of pro-inflammatory cytokines determine the damage degree of IRI[9]. Meanwhile, IRI may

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trigger a series of cellular changes, including cell necrosis and apoptosis[10]. Neuroprotective treatments for IRI have received widespread attention. Currently, neuroprotective drugs mainly include free radical scavengers, anti-inflammatory agents, and anti-apoptotic agents. However, due to side effects and drug resistance, the therapeutic efficacy of these drugs is far away from satisfactory. Therefore, researchers have made efforts on searching for natural products that can efficiently alleviate IRI without significant adverse reactions.

Sodium butyrate (NaB) is a metabolite of fermented non-digestible sugars in the intestines. It inhibits intestinal inflammatory response, and promotes the regeneration and repair of intestinal epithelial cells. Wu et al[11] have shown that NaB has anti-inflammatory and neuroprotective functions in neurodegenerative diseases. NaB can improve spatial learning and memory ability[12], and alleviate myocardial ischemia-reperfusion injury[13] as well as acute liver failure[14]. Meanwhile, NaB increases the protein expression level of BDNF in astrocytes, therefore protecting neurons[15]. Meng et al[16] have also demonstrated that NaB is capable of improving memory impairment, as well as reducing the proliferation and differentiation of mouse hippocampal dentate gyrus. After NaB treatment, the neurogenesis and behavioral improvement are greatly promoted in the ischemic model[17]. Although the neuroprotective effect of NaB has been confirmed in some neurological diseases, its protective effect on cerebral ischemia-reperfusion injury remain unclear.

Cerebral ischemia-reperfusion injury involves numerous regulatory molecular mechanisms and signaling pathways. Haeusgen et al[18] have shown that the JNK pathway exerts an essential role in embryogenesis and neuronal differentiation. JNK is a subfamily of mitogen-activated protein kinases (MAPKs). It is also a multifunctional kinase involved in cell survival[19,20], apoptosis[21], pro-inflammatory cytokine production[22], and osmotic imbalance[23]. There are several transcription factors responsible for JNK phosphorylation, including c-Jun, ATF-2, Elk-1, p53, Klf-4, and STAT3[24-26]. STAT3 is involved in the JNK pathway induced CB1 receptor Gα I/O in mouse neuroblastoma cells[27]. However, whether NaB affects cerebral ischemia-reperfusion injury in mice through regulating JNK/STAT pathway remain unclear. Therefore, the aim of this work was to investigate the neuroprotective effects of NaB treatment on bilateral common carotid artery occlusion (BCCAO) induced IRI in mice, and to explore the possible underlying mechanism.

Materials and Methods

Experimental animals

Male ICR mice in SPF level (6-8 weeks old, 20-22 g in weight) were selected in this study. All mice were housed in an environment with 22-24°C of temperature, 45-55% of humidity and 12 h of light/dark cycle. Meanwhile, mice were given free access to drink and food. NaB (purity≥99%, Sigma-Aldrich, Co. Ltd., St. Louis, MO, USA) was dissolved in saline and administrated by gavage 3 hours after reperfusion. ICR mice were randomly assigned into five groups, including the sham group, the model group, the 1 mg/kg NaB group, the 5 mg/kg NaB group and the 10 mg/kg NaB group, respectively. This study was approved by the Animal Ethics Committee of Shandong University Animal Center.

Establishment of IRI Mouse Model

IRI model was established in mice using the BCCAO method. Before animal procedures, mice were kept in a fasting stage for at least 6 hours. Mice were anesthetized by intraperitoneal injection of 400 mg/kg chloral hydrate. The right common carotid artery was exposed and ligated with 5-0 nylon monofilament for 20 min. Subsequently, a transient occlusion of artery flow was reversed. After suturing, mice were maintained in a 37°C incubator until awake. After confirming the success of righting reflex, mice were sent back to the cage. Mice in the sham group were only cut open without ligation.

Open-field Test

Open-field test is an experimental method used to evaluate the independent activity and exploration ability of animals. Meanwhile, it can evaluate the degree of cerebral ischemic injury.

In the present study, the open-field area (25 cm × 25 cm × 45 cm) was made of plastic with a video tracking system (Smart2, Bio Research Center, Nagoya, Japan). Subsequently, the time spent and the walking distance in the center of the experimental device were recorded after 1 min of habituation.

Hematoxylin and Eosin (HE) Staining

Brain tissues of mice were harvested and paraffin embedded. The brain sections were dewaxed using

Xylene I for 10 min and Xylene II for 5 min. Gradient dehydration was performed with 100%, 95%, 90%, 80%, 70%, and 50% alcohol, respectively, with 3 min for each. Then the sections were stained with hematoxylin and eosin. Finally, the sections were sealed and observed under a microscope.

Determination of Superoxide Dismutase (SOD) and Malondialdehyde (MDA) Activities

Brain tissues of mice were harvested and homogenized on ice, followed by centrifugation at 3000 r/min for 10 min. The supernatant was then collected, and SOD and MDA activities were detected following the instructions of relative commercial kits (Thermo Fisher Scientific, Waltham, MA, USA).

TUNEL Assay

Tissue sections were dewaxed, washed, hydrated and fixed. Cell apoptosis was detected according to the instructions of TUNEL kit (Yeasen, Shanghai, China). Three randomly selected fields in each group were observed and captured. Finally, the number of apoptotic cells and total cells were counted under high magnification.

Enzyme-linked Immunosorbent Assay (ELISA)

Brain tissues of mice were homogenized, followed by detection of cytokine concentration using ELISA detection kit (BioLegend, San Diego, CA, USA). Optical density (OD) value at the wavelength of A450 nm was detected.

Western Blot

Tissues were lysed with radio-immunoprecipitation (RIPA) lysis buffer containing protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA), and total protein was extracted. The concentration of each protein was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Extracted proteins were separated by gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After incubation with primary and secondary antibodies, immuno-reactive protein bands were captured by the Tanon detection system using ECL reagent (Thermo, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Measurement data were compared using *t*-test. $p<0.05$ was considered statistically significant.

Results

NaB Treatment Improved IRI

Open-field test was first conducted after establishment of the IRI mouse model. Mice in the sham group were mainly concentrated in the surrounding area. Meanwhile, they barely walked into the central area, showing significant avoidance. Compared with the center of the experimental device, mice in the model group showed significantly less preference in the surrounding area ($p<0.01$). Moreover, NaB groups (1, 5, and 10 mg/kg) showed significantly shorter central travel distance in the experimental device. In particular, there was a significant difference found in the central travel distance between the 10 mg/kg NaB treatment group and the model group ($p<0.001$, Figure 1A). Central active time in the experimental device was remarkably shorter in NaB groups (1, 5, and 10 mg/kg) when compared with the model group ($p<0.05$, Figure 1B). The above data elucidated that NaB treatment markedly alleviated IRI, especially at the dose of 10 mg/kg. Hence, the following experiments were conducted with 10 mg/kg NaB.

NaB Treatment Protected Hippocampus of IRI Mice

Mice were sacrificed, and hippocampal tissues were collected. HE staining showed that hippocampal neurons in the sham group were well arranged with normal morphology and complete structure. On the contrary, hippocampal neurons in the model group were loosely arranged, manifesting as shrinkage of cell body, nuclear pyrosis and nuclear fragmentation. NaB treatment markedly alleviated pathological lesions in hippocampal neurons (Figure 2A). We further detected the activities of MDA and SOD in mouse brain tissues. Results showed that SOD activity in the model group was significantly decreased than that of the sham group, which was obviously elevated after NaB treatment (Figure 2B). However, MDA activity was remarkably increased in the model group compared with that of the sham group. Furthermore, NaB treatment significantly decreased MDA activity in mouse brain tissues (Figure 2C). These data elucidated that 10 mg/kg NaB treat-

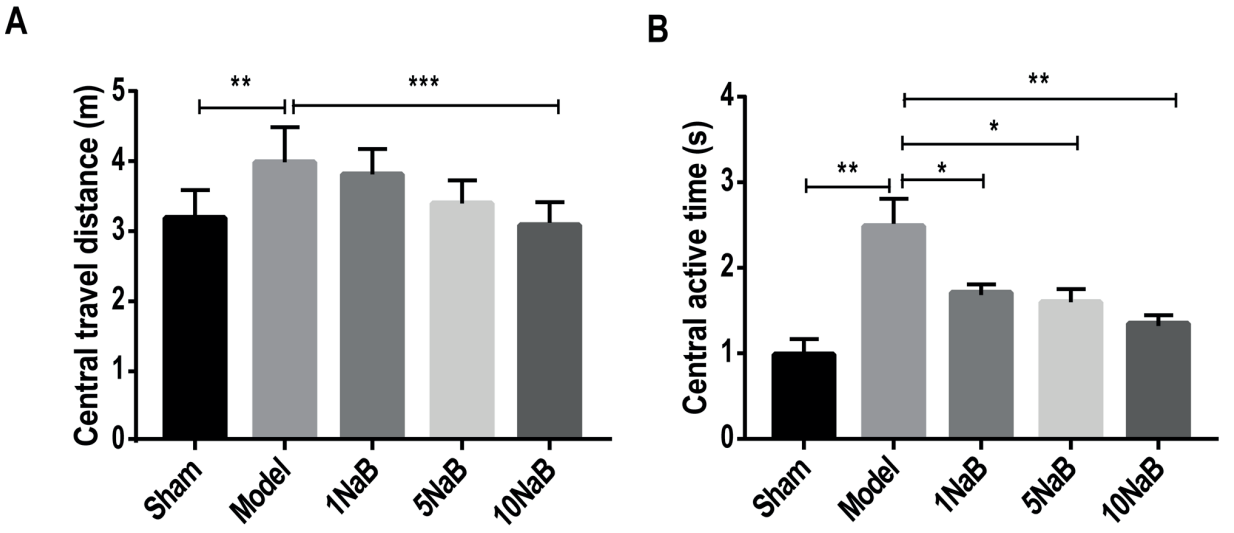

Figure 1. NaB treatment improved IRI. Open-field test was conducted after establishment of the IRI mouse model. ***A,*** Central travel distance (m) in the experimental device in the sham group, the model group, the 1 mg/kg NaB group, the 5 mg/kg NaB group and the 10 mg/kg NaB group. 10 mg/kg NaB group showed significantly lower central travel distance when compared with that of the sham group. ***B,*** Central active time (s) in the experimental device in the sham group, the model group, the 1 mg/kg NaB group, the 5 mg/kg NaB group and the 10 mg/kg NaB group. 10 mg/kg NaB group showed significantly shorter central active time when compared with that of the sham group. **p*<0.05, ***p*<0.01, ****p*<0.001.

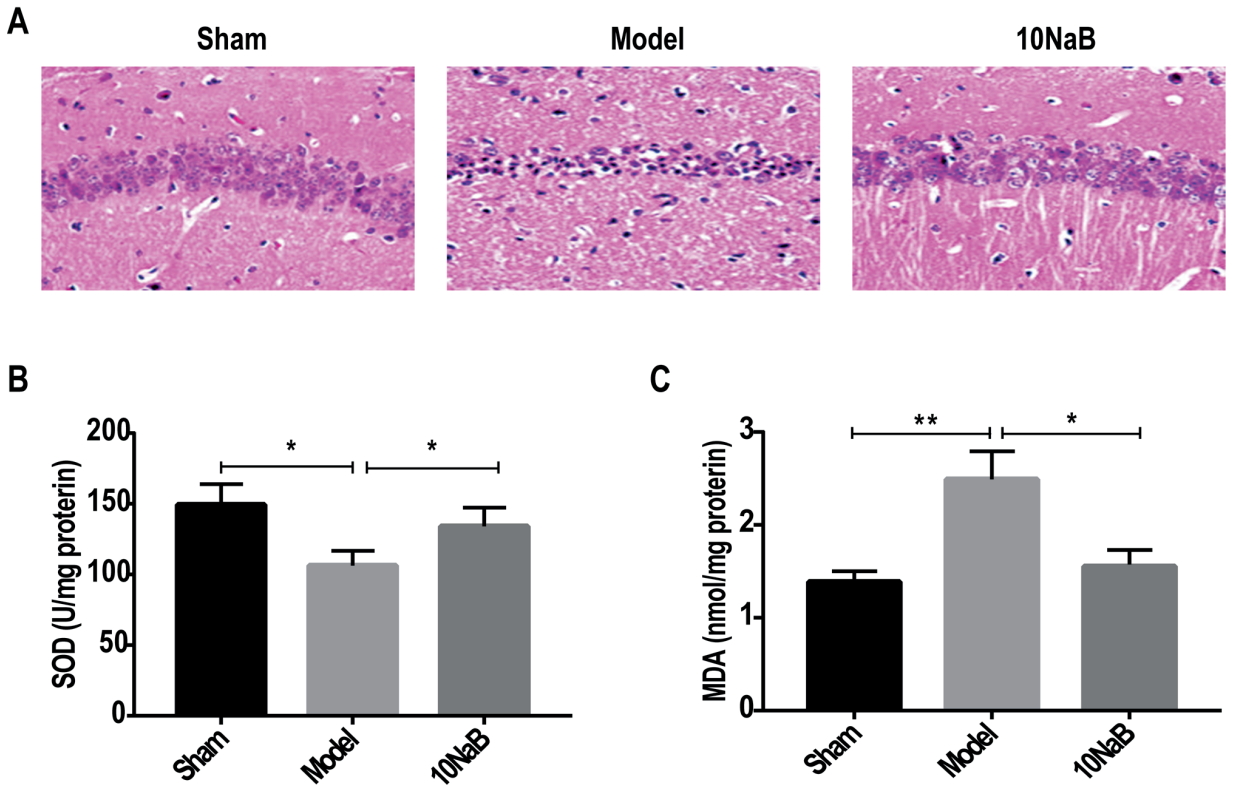

Figure 2. NaB treatment protected hippocampus of IRI mice. ***A,*** HE staining of hippocampal neurons in the sham group, the model group and the 10 mg/kg NaB group (Magnification*40). Hippocampal neurons in the model group were loosely arranged, with shrinking cell body, nuclear pyrosis and nuclear fragmentation. NaB treatment markedly alleviated pathological lesions in hippocampal neurons. ***B,*** SOD activity (U/mg protein) in the sham group, the model group and the 10 mg/kg NaB group. 10 mg/kg NaB treatment significantly increased SOD activity. ***C,*** MDA activity (mmol/mg protein) in the sham group, the model group and the 10 mg/kg NaB group. 10 mg/kg NaB treatment significantly decreased MDA activity. **p*<0.05, ***p*<0.01.

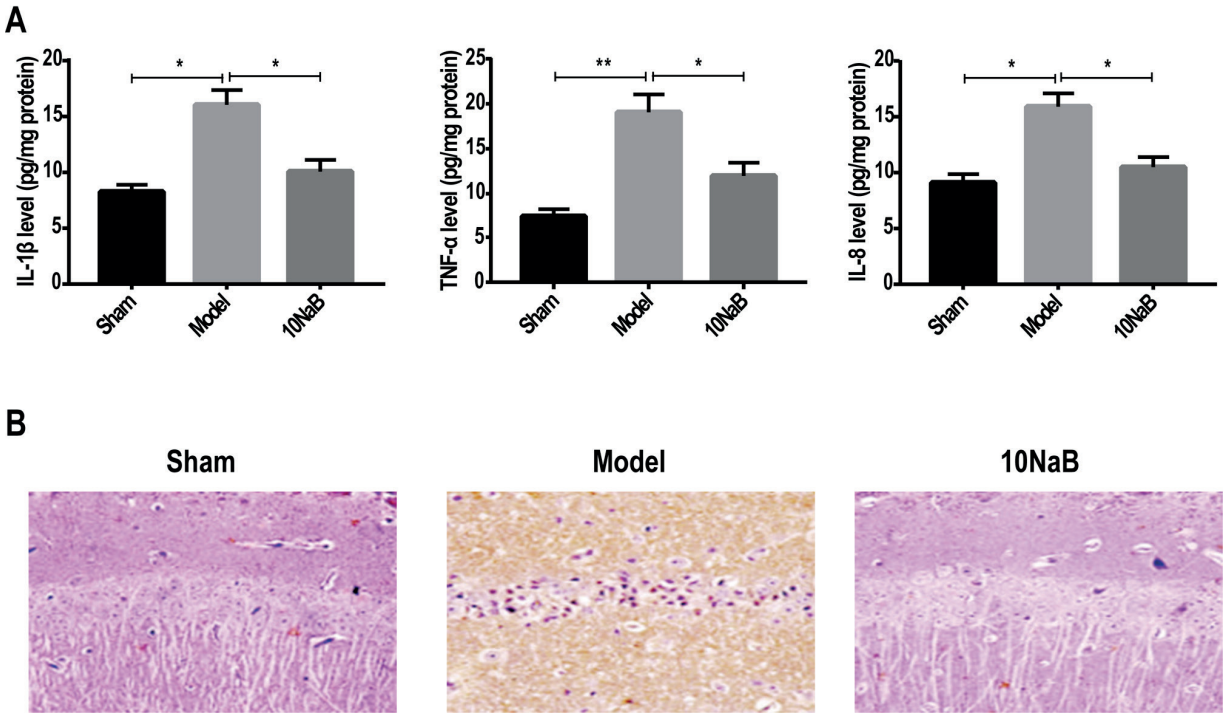

Figure 3. Effects of NaB treatment on inflammation and apoptosis after IRI. ***A,*** Levels of IL-1β, TNF-α and IL-8 (pg/mg protein) in brain tissues of the sham group, the model group and the 10 mg/kg NaB group. 10 mg/kg NaB treatment significantly decreased the levels of IL-1β, TNF-α and IL-8. ***B,*** Neuronal apoptosis in the sham group, the model group and the 10 mg/kg NaB group (Magnification*40). Large number of apoptotic cells were found in the model group. 10 mg/kg NaB treatment significantly decreased cell apoptosis. *p<0.05, **p<0.01.

ment efficiently alleviated oxidative stress in the brain tissues of IRI mice, exerting a neurological protective role.

Effects of NaB treatment on Inflammation and Apoptosis After IRI

The levels of IL- 1β, TNF-α, and IL-8 in hippocampal neurons after IRI were determined by ELISA. Results indicated that the levels of IL-1β, TNF-α, and IL-8 in the model group were significantly increased than those of the sham group. NaB treatment could markedly decrease the levels of the above molecules (Figure 3A). Furthermore, TUNEL assay was conducted to observe neuronal apoptosis in IRI mice. Results demonstrated that fewer TUNEL-positive neurons were found in NaB treatment groups when compared with the model group. This indicated the protective role of NaB in IRI-induced neuronal apoptosis (Figure 3B).

NaB Treatment Improved IRI Through JNK/STAT Pathway

To elucidate whether NaB treatment improved IRI *via* regulating JNK/STAT pathway, we detected the protein expressions of Jak2 and STAT3 in mouse brain tissues. Western blot results showed that the protein expression levels of Jak2 and STAT3 in the model group were significantly up-regulated when compared with the sham group. However, the protein expression levels of Jak2 and STAT3 were downregulated in the NaB group (Figure 4A and 4B). Our findings elucidated that NaB treatment improved IRI through inhibiting JNK/STAT3 pathway.

Discussion

Acute cerebral ischemia-reperfusion injury is one of the major diseases that seriously threatens human health[28]. In this study, we detected relative indicators of cerebral ischemia-reperfusion injury, inflammatory response and neuronal apoptosis. Meanwhile, the protective mechanism of NaB on cerebral ischemia-reperfusion injury was also investigated.

Oxidative stress is caused by ROS production during IRI, which in turn leads to cell death and even brain death after reperfusion[29,30]. Part of ROS can be cleared by antioxidants, such as

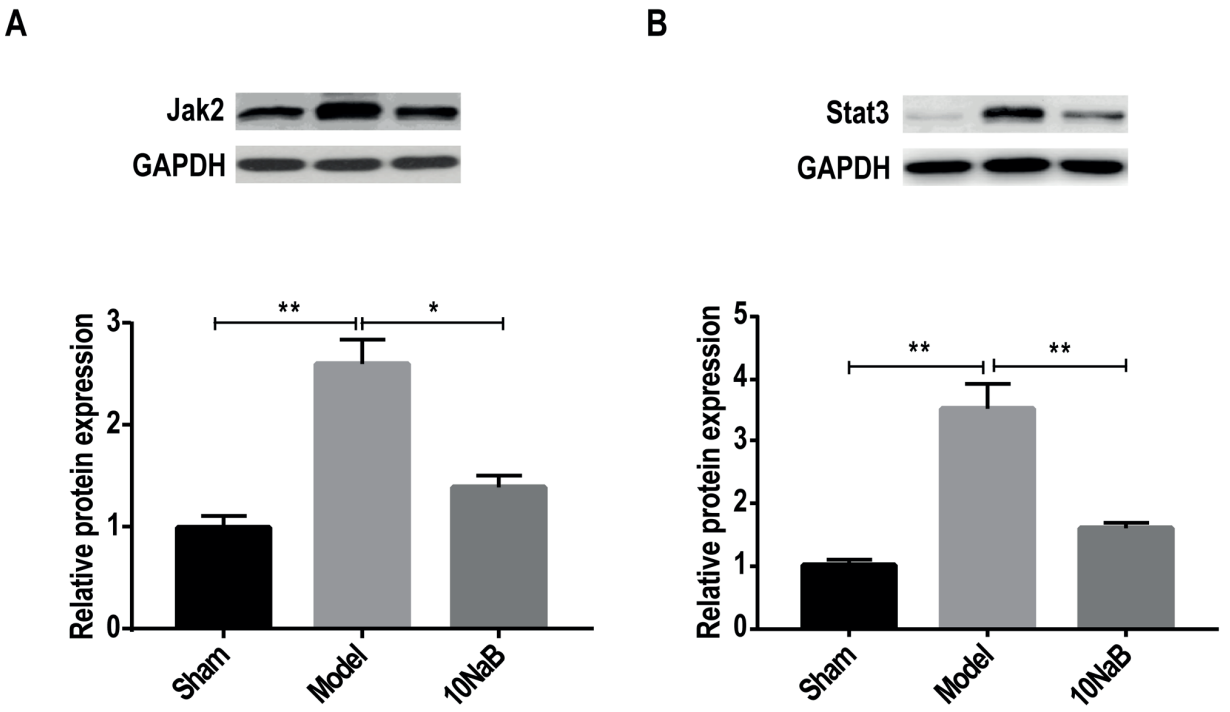

Figure 4. NaB treatment improved IRI through JNK/STAT pathway. ***A, B,*** Protein expression of Jak2 (***A***) and STAT3 (***B***) in brain tissues of the sham group, the model group and the 10 mg/kg NaB group. 10 mg/kg NaB treatment downregulated the protein expressions of Jak2 and Stat3. *p<0.05, **p<0.01.

SOD, catalase, glutathione peroxidase, vitamin C, and vitamin E[30]. However, their antioxidant capacity is limited. Particularly, additional antioxidant treatment is required during reperfusion[30]. Increasing evidences have shown that natural antioxidants (such as flavonoids, vitamins C, E, and β-carotene) may alleviate nerve damage caused by oxidative stress[31,32]. Therefore, antioxidant therapy is of great importance in the treatment of IRI. In addition to endogenous antioxidant enzymes, multiple biochemical molecules with antioxidant properties possess beneficial effects on IRI treatment[33,34]. A large number of studies have indicated that the level of MDA in brain tissues is significantly increased after cerebral ischemia[35,36]. Yavuz et al[37] have reported that 2-chloroadenosine treatment can decrease MDA level after IRI. Therefore, enhancing antioxidant capacity can effectively protect neurological damage. Our findings showed that NaB treatment could increase SOD activity and decrease MDA activity in brain tissues of IRI mice.

Inflammatory response is an important pathological step in cerebral ischemia-reperfusion injury. It can eventually lead to neuronal death in a very short time[38,39]. Pro-inflammatory factors, such as IL-1β, TNF-α and IL-8, are considered as important mediators of brain damage caused by local or global cerebral ischemia[40,41]. In this study, we found that the levels of IL-1β, TNF-α and IL-8 in the brain tissues of IRI mice were remarkably reduced after NaB treatment, indicating the anti-inflammatory effect in IRI[42]. Similar results were found in this paper. TUNEL-positive cells in the model group were significantly increased. However, after NaB treatment, TUNEL-positive cells were remarkably reduced, indicating that NaB could inhibit neuronal apoptosis.

We further explored the potential mechanism of NaB in cerebral ischemia-reperfusion, and found that NaB attenuated IRI by inhibiting JNK/STAT pathway. The protein expressions of Jak2 and STAT3 in the model group were significantly increased, which were significantly downregulated after NaB treatment. Our findings indicated that JNK/STAT pathway might be involved in the neuroprotective effect of NaB on IRI.

Conclusions

We found that sodium butyrate exerts neuroprotective effects on cerebral ischemia-reperfusion injury by preventing oxidative stress, inflammatory response and neuronal apoptosis through inhibiting JNK/STAT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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