Original Article
Protective effects and mechanisms of dexmedetomidine on cerebral ischemia reperfusion injuries in rats

Ying Wang[1], Bishan Ou-Yang[1], Shuangfeng Li[1], Xiaohang Fan[2]

[1]Department of Anesthesiology, Hainan General Hospital, Haikou, Hainan, China; [2]Medical College of Hubei University of Arts and Science, Xiangyang, Hubei, China

Abstract: Objective: The aim of the current study was to explore the protective effects and mechanisms of dexmedetomidine on cerebral ischemia reperfusion injuries in rats. Methods: A total of 100 healthy male Wistar rats were employed. An ischemia reperfusion model of right middle cerebral artery occlusion was established using the modified thread embolism method. A total of 80 rats were divided into the control group (group N), model group (group A), dexmedetomidine group (group B), SP600125 group (group C), and dexmedetomidine + SP600125 group (group D) using the random number table method. Dosages of dexmedetomidine and SP600125 were 9 μg/kg and 10 μL/rat, respectively. They were given intravenously by single injections, immediately after ischemia. The remaining 20 rats were assigned to group N. Dexmedetomidine is a highly selective α2-adrenergic receptor (α2-AR) agonist associated with sedative and analgesic sparing effects. SP6000125 is a JNK-specific inhibitor. After 24 hours of successful modeling, changes in nerve function levels were observed. The rats were then killed by decapitation and water content levels of the brain tissues were detected. Activity levels of hippocampal neurons were measured using the CCK8 method. Levels of p-JNK, Bax, and Bcl-2 in rat hippocampus tissues were detected by Western blotting. IL-6, TNF-α, superoxide dismutase (SOD), and malondialdehyde (MDA) levels in rat hippocampus tissues were detected by ELISA. Results: There were no abnormalities in neurological function in group N. Zea-Longa scores and neurological function scores of groups A, B, C, and D were statistically different (P = 0.016). Groups A, B, C, and D showed higher brain water content and levels of p-JNK protein, TNF-α, MDA, and Bax, along with lower hippocampal neuronal cell activity and levels of SOD, compared to group N (both P < 0.001). Conclusion: Dexmedetomidine can ameliorate neurological dysfunction and neuronal damage. It can control inflammation and stress response and improve tissue edema by inhibiting activation of non-nuclear way of JNK pathways in rats with cerebral ischemia reperfusion.

Keywords: Dexmedetomidine, cerebral ischemia reperfusion injury, motor function, neurological function

Introduction

With the development of society and the economy, incidence of cerebrovascular disease has gradually increased [1]. Ischemic cerebrovascular diseases are common, including ischemic strokes. Ischemic cerebrovascular diseases can cause brain damage and dysfunction in patients with severe sequelae. They may even lead to death, seriously endangering the life and health of patients [2, 3].

Ischemia reperfusion is a secondary injury to tissues. During ischemia, tissues suffer from severe hypoxia and metabolic imbalance. When

blood and oxygen supplies recover, severe inflammation often occurs. This further promotes tissue damage [4, 5]. In recent years, many studies have suggested the protective effects of dexmedetomidine in brain injuries [6-9]. Post-treatment of dexmedetomidine can alleviate brain injuries in rats with hypoxic-ischemic brains by activating α2 adrenergic receptors and inhibiting inflammation [10]. Dexmedetomidine can also reduce the release of inflammatory mediators and neuroendocrine hormones, while maintaining intracranial function [11]. It can also inhibit activation of TLR 4/NF-κB pathways and NLRP 3 inflammasomes, inhibiting

early brain injuries induced by subarachnoid hemorrhaging [8]. At present, the mechanisms have not been fully explored. There are few reports concerning ischemic cerebrovascular diseases.

Therefore, the current study re-validated the protective effects of dexmedetomidine in cerebral ischemia reperfusion injuries, exploring the mechanisms of dexmedetomidine.

Materials and methods

Research subjects

A total of 100 healthy male Wistar rats were purchased from Beijing Witonglihua Laboratory Animal Technology Co., Ltd., with strain code 102 and production license No. SCXK (Beijing) 2012-0001. They were fed with common nutritional feed (Beijing Zhechengcheng Technology Co., Ltd.). Drinking water was acidified water with PH values between 2.5 and 3 after high pressure sterilization. The average age of the mice was (22.6 ± 2.4) days. The average body weight was (24.1 ± 1.5) g. The feeding temperature was 18-22°C and relative humidity was 40%-70%. The mice were kept in the feeding box, separately. The bedding was changed regularly, every morning and evening. Environmental noise was lower than 85 dB. Ammonia concentrations were less than 20 ppm and ventilated 8-12 times per hour. Nests were changed 1-2 times per week, as well as cleaned and disinfected. Noise was no more than 60 dB. Ammonia concentrations were no more than 14 ppm. They were ventilated no less than 15 times per hour and fluorescent lamps circulated light at 12-hour intervals.

Modeling method

All rats were fed adaptively for one week. They were then fasted overnight and allowed to drink water freely. A total of 100 rats were selected using the random number table method. An ischemia reperfusion model of right middle cerebral artery occlusion was established using the modified thread embolism method [12]. After routine abdominal disinfection, the rats were anesthetized with 10% chloral hydrate (350 mg/kg body weight) (Wuhan Yuancheng Science and Technology Development Co., Ltd.) by intraperitoneal injections. The distal end of the external carotid artery was ligated and disconnected, while the pterygopalatine artery was exposed and ligated. The arteriolar artery

was clamped at the proximal end of the internal carotid artery and the proximal end of the common carotid artery. A small opening was made with small scissors at the proximal end of the external carotid artery. A wire plug was inserted into the anterior cerebral artery through the internal carotid artery (release the artery clamp) by the small opening, blocking the blood flow of the middle cerebral artery. The average insertion depth of the suture was (18.5 ± 0.5) mm. The opening of external carotid artery did not ooze, suggesting that the middle cerebral artery was completely embolized and the model was successfully prepared. After 120 minutes of ischemia, the wire was retracted into the internal carotid artery, restoring blood flow. In this process, the surgical site was covered with saline gauze to prevent drying and contamination. After the operation, the rats were placed in a clean and ventilated cage. They were kept in a single cage with a temperature of 25°C. Afterward, 80 modeled mice were randomly divided into the model group (group A), dexmedetomidine group (group B), SP600125 group (group C), and dexmedetomidine + SP600125 group (group D). The remaining 20 rats were used as the control group (group N).

Dexmedetomidine was administered at 9 μg/kg (No. T2524, Nanjing Saihongrui Biological Technology Co., Ltd.). SP600125 (No. T3109, Target Mol, China) was administered at 10 μL/rat via tail vein injections, immediately after surgery.

Outcome measures

After 24 hours of successful modeling, changes in nerve function levels were observed. The rats were then killed by decapitation and water content levels of brain tissues were detected. Activity levels of hippocampal neurons were measured using the CCK8 method and apoptosis levels of neurons in brain tissues were detected by flow cytometry. Levels of p-JNK, Bax, and Bcl-2 proteins in rat hippocampus tissues were detected by Western blotting. IL-6, TNF-α, superoxide dismutase (SOD), and malondialdehyde (MDA) levels in rat hippocampus tissues were detected by ELISA.

Detection methods

Nerve function

According to the Zea-Longa [4] 5 grade quadruple method, neurological impairment scores of

rats in each group were evaluated 24 hours after reperfusion, as follows: 0 points, no nerve injury symptoms; 1 point, the left forelimb could not fully extend when crawling; 2 points, crawling to paralysis side rotation; 3 points, crawling to the opposite side of the lesion; 4 points, having conscious disturbance and could not walk spontaneously.

Water content in brain tissues

Infarcted hemispheres of the brains were separated. They were immediately weighed. They were baked in an electric oven at 100°C for 24 hours, then quickly weighed. Brain water content = (wet weight-dry weight)/wet weight * 100%.

Activity levels of hippocampal neurons

Hippocampal neurons were isolated from cerebral infarction hemispheres of the rats. After digestion with 0.25% trypsin, 200 meshes were used to analyze hippocampal neurons. The cells were isolated by centrifugation at 1,200 r/min. Activity levels of neurons were then detected in 96-well plates. Specific steps referred to CCK8 kit instructions (No.GY025, Nanjing Guhe Biology Co., Ltd.).

Detection of JNK signaling pathway-related proteins

Western blotting was used to detect levels of p-JNK proteins in hippocampus tissues of rats. After repeated freezing and thawing, proteins in infarcted sides of the hippocampus tissues were extracted. They were separated by polyacrylamide gel electrophoresis. The initial voltage was 90 V. It then increased to 120 V, ensuring that the sample moved to the appropriate location of the separation gel. After electrophoresis, the membrane was transferred and 100 V constant pressure was kept for 100 minutes. It was sealed at 37°C for 60 minutes. The transfer membrane was then placed in 5% skimmed milk to be sealed. The immune reaction was then carried out. The membrane was incubated overnight with the first antibody at 4°C. The next day, it was washed three times with PBS for 5 minutes each time. It was incubated with the second antibody at room temperature for 1 hour. It was developed and fixed with ECL luminescent reagent. Quantity One software was used to analyze the bands scanned by film. Relative expression levels of proteins were equal to the band gray value/ internal reference gray value. The Western blot detection kit (No. JC-445) was purchased from Shanghai Youyu Biotechnology Co., Ltd. Moreover, p-JNK (No. IC153552) was purchased from Shanghai Yubo Biotechnology Co., Ltd. Bax and Bcl-2 monoclonal antibodies (No. 51003 and 51005) were purchased from Shanghai Kehui Biotechnology Co., Ltd. Sheep anti-mouse IgG secondary antibody (No. xyKS016) was purchased from Shanghai Xinyu Biological Technology Co., Ltd.

Detection of inflammatory and oxidative stress-related factors

ELISA was used to detect levels of IL-6, TNF-α, SOD, and MDA in the infarcted sides hippocampus tissues of rats. Specific detection steps referred to kit instructions. IL-6 and TNF-α detection kits (No. E-EL-H0102c and E-EL-H0109c) were purchased from Wuhan Elabscience Biotechnology Co., Ltd. SOD and MDA detection kits (No. JK-(a)-3861 and JK-(a)-35-60) were purchased from Shanghai Crystal Antibiotics Co., Ltd. Engineering Co., Ltd.

Statistical analysis

SPSS19.0 (Asia Analytics, Formerly SPSS China) was used to carry out statistical analysis. Measurement data are expressed by percentages and χ^2 tests were used for comparisons of rates. Count data are expressed by mean ± standard deviation (mean ± sd) and one-way analysis of variance (ANOVA). Post-hoc LSD tests were used for comparisons between groups. Repeated measures ANOVA with post-hoc LSD testing was used for comparisons at different time points in the group. P < 0.05 indicates statistically significant differences.

Results

Nerve function scores

Rats in group N showed no signs of abnormal neurological function. Results of neurological function evaluations showed significant differences in Zea-Longa scores between groups A, B, C, and D (P = 0.016). Chi-square tests and partition of χ^2 showed that nerve function scores in groups B, C, and D were lower than those in group A (P = 0.012, P = 0.017, P =

Table 1. Nerve function scores (n = 20)

	Group N	Group A	Group B	Group C	Group D	F/χ^2	P
0	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	10.315	0.016
1	0 (0.00)	0 (0.00)	3 (15.00)	2 (10.00)	10 (50.00)		
2	0 (0.00)	3 (15.00)	13 (65.00)	12 (60.00)	8 (40.00)		
3	0 (0.00)	16 (80.00)	4 (20.00)	6 (30.00)	2 (10.00)		
4	0 (0.00)	1 (5.00)	0 (0.00)	0 (0.00)	0 (0.00)		
mean ± sd	0 (0.00)	2.90 ± 0.44*	2.05 ± 0.59*,#	2.20 ± 0.60*,#	1.60 ± 0.68	17.044	< 0.001

Note: Group N: normal control group, group A: model group, group B: dexmedetomidine group, group C: SP600125 group, Group D: dexmedetomidine + SP600125 group, * shows that compared with group D, P < 0.05. # shows that compared with group A, P < 0.05.
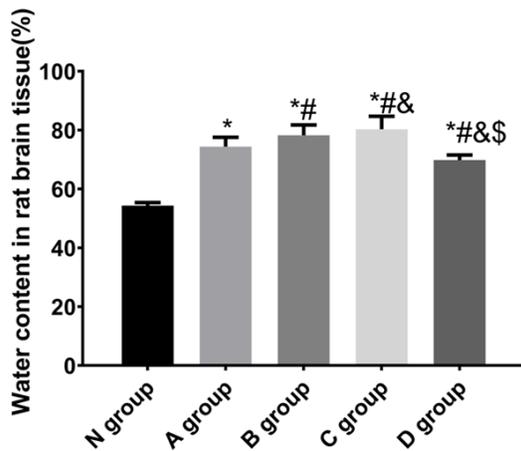

Figure 1. Changes in water content in brain tissues. * compared with group D, P < 0.05. # compared with group A, P < 0.05. & shows that compared with group B, P < 0.05. $ shows that compared with group C, P < 0.05.
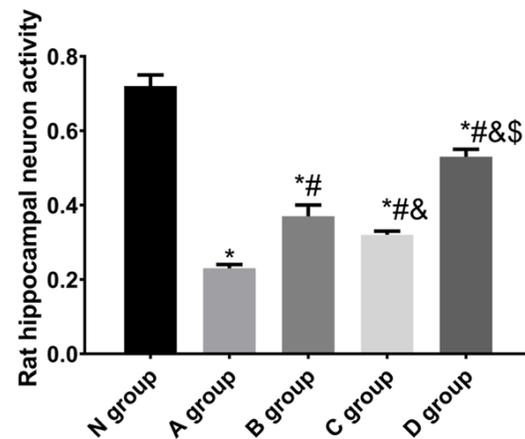

Figure 2. The Activity of hippocampal neurons in rats. * compared with group D, P < 0.05. # compared with group A, P < 0.05. & compared with group B, P < 0.05. & shows that compared with group B, P < 0.05. $ shows that compared with group C, P < 0.05.

0.003). There were no significant differences between groups B and C (P > 0.05). There were statistical differences in average scores of nerve function between the five groups. Average scores of nerve function in groups B, C, and D were lower than those in group A (P < 0.001, P = 0.002, P < 0.001). There were no differences between groups B and C (P > 0.05). However, average scores of nerve function in groups B and C were higher than those in group D (P = 0.017, P = 0.002) (**Table 1**).

Water content changes in brain tissues

Results of water content change testing in rat brain tissues showed significant differences between the five groups (P < 0.001). LSD post-tests showed that the water content in groups A, B, C, and D was higher than that in group N (P < 0.001). The water content in groups A, B, and C was higher than that in group D (P < 0.001). The water content in groups B and C

was higher than that in group A (P < 0.001). There were no significant differences between groups B and C (P = 0.055) (**Figure 1**).

Activity levels of hippocampal neurons

Results of hippocampal neuron activity testing in rats showed significant differences in between the five groups (P < 0.001). LSD post-tests showed that groups A, B, C, and D were lower than group N (all P < 0.001). Groups A, B, and C were lower than group D (all P < 0.001) and groups B and C were higher than group A (all P < 0.001). Group C was higher than group B (all P < 0.001) (**Figure 2**).

Detection results of JNK signaling pathway-related proteins

Western blot analysis showed significant differences in levels of p-JNK proteins between the five groups (P < 0.001). LSD post-tests showed
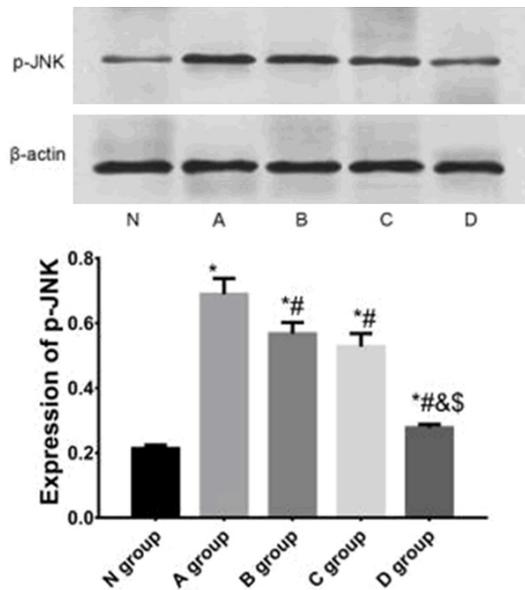
Figure 3. Western blot detection results of JNK signaling pathway related proteins. * compared with group D, P < 0.05. # compared with group A, P < 0.05. & compared with group B, P < 0.05. & shows that compared with group B, P < 0.05. $ shows that compared with group C, P < 0.05.

that groups A, B, C, and D were higher than group N (all P < 0.001). Groups A, B, and C were higher than group D (all P < 0.001). Groups B and C were lower than group A (all P < 0.001). Group C was higher than group B (P = 0.001) (**Figure 3**).

Detection results of inflammatory related factors

ELISA results of levels of IL-6 and TNF-α in hippocampus tissues showed significant differences between the five groups (all P < 0.001). LSD post-tests showed that levels of IL-6 (P < 0.001, P < 0.001, P < 0.001, P = 0.026) and TNF-α (all P < 0.001) in hippocampus tissues of rats in groups A, B, C, and D were higher than those in group N. Levels of IL-6 (P < 0.001, P = 0.007, P < 0.001) and TNF-α (P < 0.001, P = 0.025, P < 0.001) in hippocampus tissues of rats in groups A, B, and C were higher than those in group D. Levels of IL-6 (all P < 0.001) and TNF-α (P < 0.001, P = 0.014) in hippocampus tissues of rats in groups B and C were lower than those in group A. Levels of IL-6 and TNF-α in hippocampus tissues of rats in group B were higher than those in group C (P = 0.001, P = 0.021) (**Table 2**).

Relevant indicators of oxidative stress

ELISA results of levels of SOD and MDA in hippocampus tissues showed significant differences between the five groups (all P < 0.001). LSD post-tests showed that levels of SOD in hippocampus tissues of rats in groups A-D were lower than those in group N. Levels of MDA were higher than those in group N (all P < 0.001). Levels of SOD in hippocampus tissues of rats in groups A, B, and C were lower than those in group D. Levels of MDA were higher than those in group D (all P < 0.001). Levels of SOD in hippocampus tissues of rats in groups B and C were higher than those in group A. Levels of MDA were lower than those in group A (all P < 0.001). Levels of SOD in hippocampus tissues of rats in group C were lower than those in group B. Levels of MDA were higher than those in group B (all P < 0.001) (**Table 3**).

Detection of apoptosis related proteins

Western blotting results showed that expression levels of Bax and Bcl-2 in the five groups were significantly different (all P < 0.001). LSD post-tests showed that expression of Bax was higher in groups A-D than in group N (all P < 0.001). Expression of Bcl-2 was lower than that in group N (all P < 0.001). Expression of Bax was higher in groups A, B, and C than that in group D (all P < 0.001). Expression of Bcl-2 was lower than that in group D (all P < 0.001). Expression of Bax in groups B and C was lower than that in group D (all P < 0.001). Expression of Bcl-2 was higher than that in group D (all P < 0.001). Expression of Bax in group C was higher than that in group B (all P < 0.001). Expression of Bcl-2 was higher than that in group B (all P < 0.001) (**Table 4**; **Figure 4**).

Discussion

Dexmedetomidine is a highly selective α2 adrenergic agonist. It has been reported to alleviate cognitive impairment and neuronal apoptosis induced by isoflurane through JNK signaling pathways [13]. Whether JNK signaling pathways also provide protective mechanisms of dexmedetomidine in cerebral ischemia reperfusion injuries requires further examination. The current study explored this problem.

The Longa et al. improved thread embolism method was used to establish a focal cerebral

Table 2. Levels of IL-6 and TNF-α in rat hippocampus tissues (n = 20)

	Group N	Group A	Group B	Group C	Group D	F	P
IL-6 (μg/L)	59.73 ± 9.44	145.53 ± 24.94*	92.75 ± 18.94*,#	116.06 ± 27.30*,#,&	74.60 ± 18.92*,#,&,$	28.023	< 0.001
TNF-α (ng/mL)	0.57 ± 0.13	2.82 ± 0.73*	2.16 ± 0.33*,#	2.48 ± 0.37*,#,&	1.85 ± 0.36*,#,&,$	15.322	< 0.001

Note: Group N: normal control group, group A: model group, group B: dexmedetomidine group, group C: SP600125 group, Group D: dexmedetomidine + SP600125 group. * shows that compared with group D, P < 0.05. # shows that compared with group A, P < 0.05. & shows that compared with group B, P < 0.05. $ shows that compared with group C, P < 0.05.

Table 3. Levels of SOD and MDA in rat hippocampus tissues (n = 20)

	Group N	Group A	Group B	Group C	Group D	F	P
SOD (U/mg)	35.13 ± 1.45	9.13 ± 2.24*	18.42 ± 2.33*,#	15.83 ± 1.36*,#,&	24.02 ± 2.12*,#,&,$	505.266	< 0.001
MDA (nmol/mg)	3.67 ± 0.25	8.61 ± 0.69*	6.53 ± 0.54*,#	7.73 ± 0.56*,#,&	5.45 ± 0.52*,#,&,$	265.614	< 0.001

Note: Group N: normal control group, group A: model group, group B: dexmedetomidine group, group C: SP600125 group, Group D: dexmedetomidine + SP600125 group. * shows that compared with group D, P < 0.05. # shows that compared with group A, P < 0.05. & shows that compared with group B, P < 0.05. $ shows that compared with group C, P < 0.05.

Table 4. Detection of cell apoptosis-related proteins

	Group N	Group A	Group B	Group C	Group D	F	P
Bax	2.43 ± 0.17	14.13 ± 1.42*	8.37 ± 0.95*,#	11.12 ± 1.35*,#,&	5.26 ± 0.43*,#,&,$	116.713	< 0.001
Bcl-2	28.84 ± 1.33	9.14 ± 1.18*	13.14 ± 1.41*,#	11.33 ± 1.26*,#,&	15.84 ± 1.63*,#,&,$	42.226	< 0.001

Note: Group N: normal control group, Group D: dexmedetomidine + SP600125 group, group A: model group, group B: dexmedetomidine group, group C: SP600125 group. * shows that compared with group D, P < 0.05. # shows that compared with group A, P < 0.05. & shows that compared with group B, P < 0.05. $ shows that compared with group C, P < 0.05.
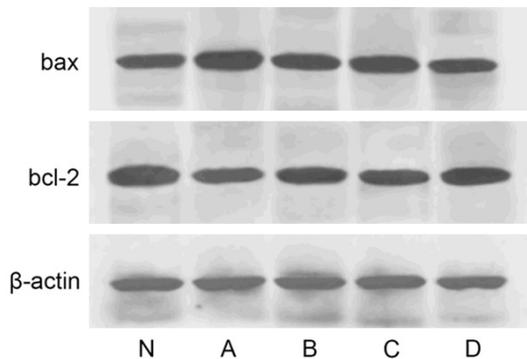

Figure 4. Protein expression levels of Bax and Bcl-2 detected by Western blot.

ischemia reperfusion injury model of middle cerebral artery occlusion [14]. This method has high repeatability, a simple operation, low mortality rates, and high success rates. Model rats were treated with dexmedetomidine and SP600125, a small molecule high selectivity inhibitor of JNK pathways. Intervention results were analyzed. Results showed that, compared with untreated model rats, rats treated with dexmedetomidine had lower scores of nerve function, lower water content levels in brain tissues, and less edema. They also had higher activity levels of hippocampal neurons, lower levels of IL-6, TNF-α, and MDA, and higher levels of SOD. Inflammatory and stress reactions were also controlled. Thus, dexmedetomidine can effectively improve neurological dysfunction and neuronal damage, as well as control inflammatory and stress response, in model rats. Further analysis of the protective mechanisms of dexmedetomidine suggested that expression levels of p-JNK in hippocampus tissues of rats after dexmedetomidine intervention were significantly lower than levels in rats without intervention. Results suggest that dexmedetomidine inhibits activation of JNK signaling pathways in rats with cerebral ischemia reperfusion injuries. This may be the protective mechanism of dexmedetomidine in cerebral ischemia reperfusion injuries. Numerous studies have shown that JNK signaling pathways are associated with neuronal apoptosis in cerebral ischemia reperfusion injuries [15, 16]. JNK pathways include nuclear and non-nuclear ways. Activated JNK phosphorylated nuclear substrate transcription factor c-Jun has led to increased transcriptional activity of activator protein-1. It regulates the transcription of apoptosis related genes [15, 16]. In contrast, activated JNK regulates the activation of non-nuclear substrates, including members of the

Bcl-2 family [16, 17]. Changes in expression levels of Bax and Bcl-2 were also detected. It was found that, after intervention with dexmedetomidine, levels of Bcl-2 in rat hippocampus tissues increased significantly. Levels of Bax decreased significantly. Results suggest that dexmedetomidine can inhibit activation of JNK signaling pathways through non-nuclear pathways, thereby inhibiting decreases of Bcl-2 and increases of Bax caused by injuries. In studies concerning ischemia reperfusion injuries of other tissues and organs, it has been reported that dexmedetomidine pretreatment could inhibit phosphorylation of JNK signaling pathways and reduce renal injuries [18]. However, the current study did not explore whether dexmedetomidine participates in the activation of the main JNK pathways in rats with cerebral ischemia-reperfusion. This will be analyzed in future studies.

Differences between dexmedetomidine intervention and SP600125 intervention were also analyzed. Improvements via dexmedetomidine on hippocampal neuronal cell activity, inflammatory factors, and stress response related factors were significantly better than those of SP600125, suggesting that inhibition of JNK signaling pathway activation was not the main form of protection for dexmedetomidine. According to some reports, dexmedetomidine can also reduce brain injuries through PI3K/Akt and ERK 1/2 pathways [19]. However, in the current study, improvement levels of the two intervention methods on neurological function and brain edema for model rats were similar. Further experimentation is necessary to fully understand the causes of this phenomenon. Future studies should expand the numbers of research subjects, adjust the dosages of two drugs, and further explore stricter experimental conditions. Although the middle cerebral artery occlusion ischemia-reperfusion model is a well-recognized method, it is still unable to completely simulate the complex pathological changes of ischemia-reperfusion injuries in human. There are species differences between rats and humans. Present results should be further confirmed by clinical trials.

In conclusion, dexmedetomidine can ameliorate neurological dysfunction and neuronal damage. Moreover, it can control inflammation and stress response and improve tissue edema by inhibiting the activation of non-nuclear way of JNK pathways in rats with cerebral ischemia reperfusion.

Disclosure of conflict of interest

None.

Address correspondence to: Xiaohang Fan, Medical College of Hubei University of Arts and Science, No. 296, Longzhong Road, Xiangcheng District, Xiangyang 441053, Hubei, China. Tel: +86-18271288329; E-mail: fanxhhh@163.com

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