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Research on mechanism of sevoflurane in alleviating cerebral ischemia-reperfusion injury in rats through JNK signaling pathway

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Abstract. – OBJECTIVE: To explore the specific mechanism of sevoflurane in alleviating cerebral ischemia-reperfusion injury (CIRI) in rats through the c-Jun N-terminal kinase (JNK) signaling pathway.

MATERIALS AND METHODS: A total of 60 male specific pathogen-free Sprague-Dawley rats were randomly divided into sham group (n=20), model group (n=20), and sevoflurane group (n=20). In the sevoflurane group, sevoflurane (2.5%) was inhaled for 60 min at 24 h before the blockage of cerebral blood supply. The CIRI model was established using the suture method in the model group and sevoflurane group, while the right common carotid artery and external carotid artery were separated and ligated only, without suture placement, in the sham group. At 24 h after reperfusion, the neurological deficit score in each group was calculated, the water content in brain tissues in each group was detected based on dry-wet weight ratio, the infarction volume of brain tissues in each group was detected *via* 2,3,5-triphenyltetrazolium chloride (TTC) staining, and the apoptosis rate of brain cells in each group was detected using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Moreover, the protein levels of JNK, p-JNK, B-cell lymphoma-2 (Bcl-2), and the Bcl-2 associated X protein (Bax) in brain tissues were determined using Western blotting, and the gene expressions of Bax and Bcl-2 in brain tissues were determined through fluorescence quantitative Polymerase Chain Reaction (qPCR).

RESULTS: It was found that the water content in brain tissues and the cerebral infarction volume were significantly increased in the model group compared with those in the sham group ($p<0.01$, $p<0.01$), while they were notably decreased in the sevoflurane group compared with those in the model group ($p<0.05$, $p<0.01$). The neurological deficit score was significantly higher in the model group than that in the sham group ($p<0.01$), while it was remarkably lower in the sevoflurane group than that in the model group ($p<0.01$). According to the results of the TUNEL assay, the model group had an evidently higher apoptosis rate of brain cells than the sham group ($p<0.01$), while the sevoflurane group had a lower apoptosis rate of brain cells than the model group ($p<0.05$). Besides, the results of Western blotting revealed that the model group exhibited remarkably increased protein levels of JNK, p-JNK, and Bax ($p<0.05$, $p<0.01$, $p<0.01$) and a remarkably decreased protein level of Bcl-2 ($p<0.01$) compared with the sham group. Sevoflurane group had decreased protein levels of JNK, p-JNK, and Bax ($p<0.05$, $p<0.01$, $p<0.01$) and an increased protein level of Bcl-2 ($p<0.05$) in comparison with the model group. In addition, the gene expression of Bcl-2 significantly declined ($p<0.01$), and that of Bax remarkably rose ($p<0.01$) in the model group compared with those in the sham group, while the contrary is the case in the sevoflurane group compared with those in the model group ($p<0.05$, $p<0.01$).

CONCLUSIONS: Sevoflurane can regulate the protein and gene expressions of Bax and Bcl-2 and reduce apoptosis in CIRI by regulating the JNK signaling pathway, thereby exerting a protective effect on brain tissues and improving the symptoms of neurological deficit.

Key Words:
Sevoflurane, JNK signaling pathway, Cerebral ischemia-reperfusion injury.

Introduction

Cerebral ischemia-reperfusion injury (CIRI) refers to the phenomenon that after ischemia of brain tissues for a certain period of time, the tissue injury is aggravated due to the recovery of

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blood supply again. CIRI includes the primary injury during ischemia and secondary injury during reperfusion, which will lead to neurological dysfunction and cerebral infarction[1,2]. The apoptosis of brain tissues is an important pathological change in CIRI, and c-Jun N-terminal kinase (JNK) is closely related to CIRI. The JNK signal transduction pathway, one of the most important mitogen-activated protein kinase (MAPK) signal transduction pathways, widely exists in the brain, cerebellum, hippocampus, cortex, and striatum, which play an important regulatory role in apoptosis in the pathological process of CIRI[3]. Sevoflurane is a kind of commonly-used inhaled general anesthetic, and studies have found that sevoflurane preconditioning in cranial operation can effectively protect brain tissues and alleviate CIRI in brain tissues[4,5]. However, there has been no definite conclusion about whether sevoflurane preconditioning can affect the apoptosis of brain tissues in CIRI by regulating the JNK signaling pathway. In the present study, therefore, the sevoflurane preconditioning was performed before the establishment of rat model of CIRI. The effects of sevoflurane on the neurological deficit, water content in brain tissues, and cerebral infarction volume after CIRI were observed. At the same time, the apoptosis of brain tissues, the protein and gene expressions of JNK, p-JNK, B-cell lymphoma-2 (Bcl-2), and Bcl-2 associated X protein (Bax) were detected, and the specific mechanism of sevoflurane in mediating apoptosis of brain tissues in CIRI rats through the JNK signaling pathway was explored.

Materials and Methods

Laboratory Animals

Male specific pathogen-free (SPF) Sprague-Dawley (SD) rats aged 4 weeks old and weighing 200-250 g were provided by Shanxi Medical University Animal Center, and they were fed in the clean-grade laboratory according to the Guideline for Ethical Treatment of Laboratory Animal of the Ministry of Science and Technology in 2006. This study was approved by the Animal Ethics Committee of Shanxi Medical University Animal Center.

Laboratory Drugs and Reagents

SP600125 (MedChemExpress, Monmouth Junction, NJ, USA), sevoflurane (Shanghai Hengrui Pharmaceutical Co., Ltd., Shanghai, China), 4% paraformaldehyde (Sinopharm Chemical Reagent Beijing Co., Ltd., Beijing, China), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay kit (Beyotime, Shanghai, China), JNK, p-JNK, Bax, and Bcl-2 antibodies (Abcam, Cambridge, MA, USA), RIPA lysis buffer (strong), bicinchoninic acid (BCA) protein concentration assay kit (Beyotime, Shanghai, China), polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), Tris-Buffered Saline with Tween-20 (TBST), transfer buffer and electrophoresis buffer (Beijing APPLYGEN Co., Ltd., Beijing, China), AceQ quantitative Polymerase Chain Reaction (qPCR) SYBR Green Master Mix Kit (Vazyme, Nanjing, China), TRIzol (Life Technologies, Gaithersburg, MD, USA), and 2,3,5-triphenyltetrazolium chloride (TTC) staining fluid (Solarbio, Beijing, China).

Laboratory Animal Grouping and Intervention

A total of 60 male specific pathogen free (SPF) Sprague Dawley (SD) rats aged 4 weeks old were randomly divided into sham group (n=20), model group (n=20), and sevoflurane group (n=20). All rats were deprived of food for 12 h before operation. In the sevoflurane group, the rats were placed in a closed container connected to the evaporation jar of corresponding anesthetic gas. The container had one air inlet and one air outlet, the former was injected with experimental gases of 2.5% sevoflurane + 97.5% oxygen, and the latter was connected to the gas detector to measure the concentration of sevoflurane and oxygen in the container. After autonomous respiration for 60 min, the rats were eluted with pure oxygen for 15 min. After sevoflurane preconditioning, the rats were anesthetized *via* intraperitoneal injection of pentobarbital, and the right internal carotid artery was ligated using the nylon wire to block the cerebral blood perfusion in model group and sevoflurane group. In the sham group, the right common carotid artery and external carotid artery were separated and ligated only, without suture placement in the skull. After the blockage of cerebral blood perfusion for 2 h in the model group and sevoflurane group, the suture was removed to restore the blood perfusion. After reperfusion for 24 h, the neurological deficit score was given for all rats. Then, 4 rats in each group were randomly selected and quickly decapitated, and the whole brain tissues were taken to determine the water content. The brain tissues were

also taken from 4 rats randomly selected in each group to determine the cerebral infarction volume. Besides, 5 rats in each group were randomly selected and anesthetized *via* intraperitoneal injection of pentobarbital, from which the brain tissues were taken and fixed with 4% paraformaldehyde. Finally, the brain tissues were taken from the remaining 7 rats in each group and stored in liquid nitrogen for later use.

Neurological Deficit Score of Rats

0 point: there are no symptoms of neurological deficit. 1 point: the contralateral fore paw cannot be fully stretched in the case of tail suspension. 2 points: the body turns to the hemiplegic side while walking. 3 points: the body leans to the hemiplegic side while walking. 4 points: the level of consciousness declines and the rats are unable to walk spontaneously.

Detection of Apoptosis of Brain Cells Via TUNEL Assay

The brain tissue sections were routinely dehydrated and stained in strict accordance with the instructions of the TUNEL kit. The cells were counted and averaged in 4 non-repeated fields randomly selected in the cortex under an optical microscope (400×). The level of apoptosis was expressed by the apoptotic cell index (ACI). ACI = total number of apoptotic cells/total number of cells × 100%.

Determination of Water Content in Brain Tissues

After blood perfusion was restored for 24 h, the rats were decapitated, and the whole brain tissues were taken and washed with normal saline. The cerebellum and brainstem were removed, the surface water was sucked dry with the filter paper, and the wet weight of the brain was measured. Then, the brain tissues were baked dry in an oven at 100°C for 24 h, and the dry weight was measured. Finally, the water content in brain tissues was determined based on the dry-wet weight ratio. Water content in brain tissues (%) = (wet weight - dry weight) / wet weight × 100%.

Determination of Percentage of Cerebral Infarction Tissues

After blood perfusion was restored for 24 h, 4 rats in each group were randomly selected and decapitated, and the brain was taken. The olfactory bulb, cerebellum, and lower brainstem were removed, and the remaining brain tissues were equally divided into 5 pieces along the coronal plane. Then, the tissues were stained with 2% TTC staining fluid prepared with 0.1 mol/L PBS and incubated in the dark at 37°C for 30 min (gently shaken at 15 min to allow the tissues to fully contact with the staining fluid). The infarction area in each section was measured using the ImageJ 1.45. Percentage of cerebral infarction tissues (%) = infarction area / (infarction area + non-infarction area) × 100%.

Detection of Protein Expression Levels in Brain Tissues in Each Group Using Western Blotting

The total protein was extracted from the whole brain tissues in each group, according to the instructions of reagent, and the protein concentration was determined using the BCA method. Firstly, after denaturation, 20 μg of proteins were added into each well and subjected to 12% SDS-PAGE under the constant voltage of 60 V until the protein passed through the spacer gel, and then, the constant pressure was adjusted to 90 V till the end of electrophoresis. Secondly, the PVDF membrane was activated with absolute methanol for 10 min, and the PVDF membrane, glue, and filter paper were equilibrated with transfer buffer for 10 min. Thirdly, the sandwich clamp was made using the filter paper - glue - PVDF membrane - filter paper from bottom to top, followed by membrane transfer under the constant voltage of 100 V. After that, the membrane was washed with TBST, sealed with 5% skim milk powder at room temperature for 2 h, and incubated with the corresponding primary antibodies on a shaking table at 4°C overnight. After the membrane was washed with TBST for 3 times (10 min/time), it was incubated again with the corresponding secondary antibodies on the shaking table at room temperature for 1 h and washed again with TBST for 3 times (10 min/time). Finally, with the surface of protein on PVDF membrane upward, an appropriate amount of enhanced chemiluminescence (ECL) developing solution was added for reaction for 2 min, followed by exposure and image development. Finally, the gray value was analyzed using the ImageLab software, and the relative expression of protein was calculated.

Detection of Gene Expression Levels in Brain Tissues in Each Group Using Fluorescence qPCR

The total RNA was extracted using TRIzol method in each group, and its concentration was

measured. Then, 2 μg of total RNA was taken for reverse transcription under the following conditions: 42°C for 60 min and 70°C for 5 min, and subsequently stored at 4°C. 2 μL of reverse transcription products were taken and loaded according to the instructions of the AceQ qPCR SYBR Green Master Mix Kit, and the fluorescence was collected using the fluorescence qPCR instrument. The primer sequences are shown in Table I. With GAPDH as the internal reference gene, the difference in gene expression among groups was detected using $2^{-\Delta\Delta Ct}$ method.

Table I. Primer sequences in fluorescence qPCR.

Gene	Sequence
GAPDH	F: GGTTGTCTCCTGCGACTTCA R: TGGTCCAGGTTTCTTACTCC
Bcl-2	F: TGTCACAGAGGGGCTACGAGT R: TCAGGCTGGAAGGAGAAGATG
Bax	F: GATGAACTGGACAGCAATATGG R: GCAAAGTAGAAGAGGGCAACC

Statistical Analysis

Statistical Product and Service Solution 19.0 (IBM Corp., Armonk, NY, USA) software was used for data analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The differences between the two groups were analyzed by means of the Student's *t*-test. Comparison among multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.01$ suggested the significant difference among groups.

Results

Effect of Sevoflurane Preconditioning on Neurological Deficit in Rats

The neurological deficit score was significantly higher in the model group than that in the sham group ($p<0.01$), while it was remarkably lower in the sevoflurane group than that in the model group ($p<0.01$) (Table II).

Neuronal Apoptosis Detected Using TUNEL Assay

The results of TUNEL staining showed that in the sham group, the cell morphology was intact, no evident pathological changes were found and there were fewer TUNEL-positive cells. In the model group, there were a large number of TUNEL-positive cells, the morphology of many cells was incomplete, and the cells were arranged disorderly. In the sevoflurane group, the number of TUNEL-positive cells declined compared with that in the model group, some cells had incomplete morphology, and they were arranged less orderly (Figure 1). It can be seen that the model group had an evidently higher apoptosis rate of brain cells than the sham group ($p<0.01$), while the sevoflurane group had a lower apoptosis rate of brain cells than the model group ($p<0.05$) (Table III).

Table II. Neurological deficit score in each group ($\bar{x} \pm s$).

Group	Sample size	Neurological deficit score
Sham group	10	0.00 ± 0.00
Model group	10	3.93 ± 0.29**
Sevoflurane group	10	1.52 ± 0.43##

**$p<0.01$ *vs*. sham group, ##$p<0.01$ *vs*. model group.

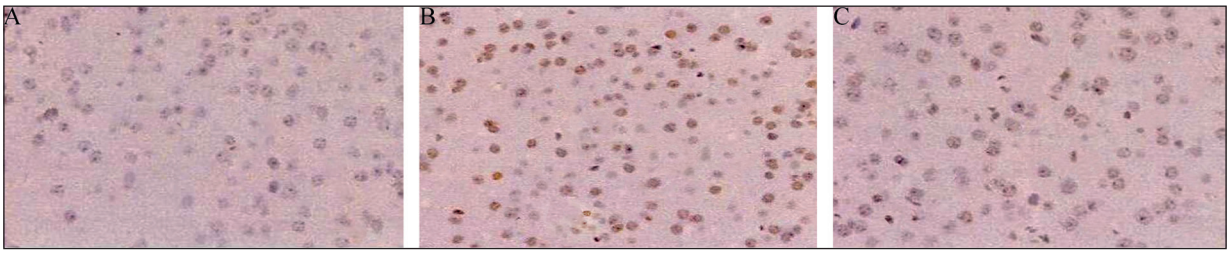

Figure 1. TUNEL staining of brain tissues in each group (×400). **A,** Sham group. **B,** Model group. **C,** Sevoflurane group.

Table III. Comparison of apoptosis rate of brain cells in each group ($\bar{x} \pm s$).

Group	Apoptosis rate (%)
Sham group	20.03 ± 1.35
Model group	76.26 ± 2.03**
Sevoflurane group	38.56 ± 1.56#

**$p<0.01$ *vs*. sham group, #$p<0.05$ *vs*. model group.

Water Content in Brain Tissues and Percentage of Infarction Tissues in Each Group

According to the determination results of water content in brain tissues, the water content in brain tissues was significantly raised in the model group in comparison with that in the sham group ($p<0.01$), indicating the severe edema in brain tissues, while it was notably reduced in the sevoflurane group compared with that in the model group ($p<0.05$), indicating the improved edema in brain tissues. Besides, the cerebral infarction volume was distinctly increased in the model group compared with that in the sham group ($p<0.01$), while it was significantly decreased in the sevoflurane group in comparison with that in the model group ($p<0.01$), suggesting that sevoflurane preconditioning can alleviate cerebral infarction in CIRI (Table IV).

Protein Expressions of JNK, p-JNK, Bax, and Bcl-2 in Brain Tissues in Each Group

The model group had remarkably elevated protein levels of JNK, p-JNK, and Bax ($p<0.05$, $p<0.01$, $p<0.01$) and a remarkably lowered protein level of Bcl-2 ($p<0.01$) compared with the sham group. Sevoflurane group displayed decreased protein levels of JNK, p-JNK, and Bax ($p<0.05$, $p<0.01$, $p<0.01$) and an increased protein level of Bcl-2 ($p<0.05$) compared with the model group (Figure 2, Table V).

Gene Expressions of Bax and Bcl-2 in Brain Tissues in Each Group

The gene expression of Bcl-2 significantly declined ($p<0.01$) and that of Bax remarkably rose ($p<0.01$) in the model group compared with those in the sham group, while the contrary is the case in the sevoflurane group compared with those in the model group ($p<0.05$, $p<0.01$) (Table VI).

Discussion

CIRI is a common pathological process in ischemic cerebral vascular diseases, mainly involving the energy metabolic disorder, local acidosis, release of inflammatory mediators, free radical damage, cellular Ca^{2+} overload, release of excitatory amino acid, and induction of apoptosis[6-8]. The above pathological links do not exist in isolation, but interact with each other, ultimately leading to cell death. In most research of CIRI in rats, time-points were chosen at 2 h and 24 h after reperfusion[9]. In this study, we selected the time-point based on previous evidence[10]. Apoptosis is a form of programmed cell death, an active cell death process regulated by genes[11]. In CIRI, the massive apoptosis of brain tissues directly results in impairment of neurological function[12,13]. JNK signaling pathway plays an important role in the occurrence and development of various diseases and pathological injuries, such as Alzheimer's disease, Parkinson's disease, diabetes, tumor, and IRI[14-17]. In neurological diseases, the JNK family can affect the neuron-specific functions by regulating neuronal death, such as synaptic plasticity and memory formation[18]. Moreover, the JNK signaling pathway can be activated by a variety of extracellular stimuli, including growth factors, cytokines, and stress (such as ultraviolet radiation, high osmotic pressure, and CIRI)[19]. After activation, the JNK signaling pathway can regulate the gene and protein expressions of Bcl-2 family, thus exerting a regulatory effect on apop-

Table IV. Water content in brain tissues and percentage of infarction tissues in each group.

Group	Water content in brain tissues (%)	Cerebral infarction volume (%)
Sham group	78.23± 0.35	0.00 ± 0.00
Model group	81.01 ± 0.13**	27.15 ± 1.98**
Sevoflurane group	79.06 ± 0.47#	18.14 ± 2.05##

**$p<0.01$ *vs*. sham group, #$p<0.05$ *vs*. model group, ##$p<0.01$ *vs*. model group.

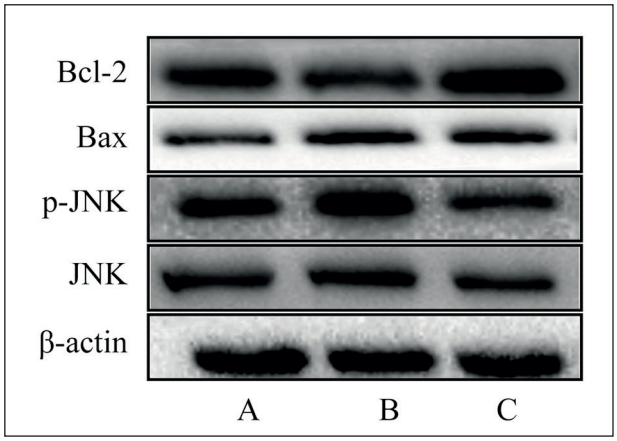

Figure 2. Protein expressions of JNK, p-JNK, Bax and Bcl-2 in brain tissues in each group. **A,** sham group. **B,** Model group. **C,** Sevoflurane group.

Table V. Comparisons of protein expressions of JNK, p-JNK, Bax, and Bcl-2 in brain tissues in each group ($\bar{x} \pm s$).

Group	JNK/β-actin	p-JNK/β-actin	Bax/β-actin	Bcl-2/β-actin
Sham group	0.924 ± 0.106	0.492 ± 0.025	0.047 ± 0.011	0.153 ±0.003
Model group	1.458 ± 0.064*	1.026 ± 0.013**	0.158 ± 0.009**	0.049 ± 0.002**
Sevoflurane group	1.051 ± 0.092$^{\#}$	0.523 ± 0.046$^{\#\#}$	0.072 ± 0.009$^{\#\#}$	0.095 ± 0.007$^{\#}$

**p<0.01 *vs*. sham group, *p<0.05 vs. sham group, $^{\#}p$<0.05 *vs*. model group, $^{\#\#}p$<0.05 *vs*. model group.

tosis. In the Bcl-2 family, it is the expressions of two proteins Bcl-2 and Bax that determine the presence or absence of apoptosis. The expressions of Bcl-2 and Bax, a pair of antagonistic factors, show the opposite trend. When the expression of Bcl-2 declines, that of Bax will rise, thus promoting apoptosis. On the contrary, apoptosis will be inhibited[20,21]. In this study, it was found that compared with those in the sham group, severe edema occurred in brain tissues, the apoptosis rate of brain cells was increased, and the neurological deficit and cerebral infarction became worse in the model group. At the same time, the protein expressions of JNK and p-JNK were significantly increased in the model group, indicating the activation of the JNK signaling pathway. Besides, model group had markedly higher gene and protein expressions of Bax, and markedly lower gene and protein expressions of Bcl-2.

Table VI. Comparisons of gene expressions of Bax and Bcl-2 in brain tissues in each group ($\bar{x} \pm s$).

Group	Bax	Bcl-2
Sham group	1.000 ± 0.321	1.000 ± 0.246
Model group	2.572 ± 0.230**	0.325 ± 0.097**
Sevoflurane group	1.460 ± 0.248$^{\#\#}$	0.726 ± 0.081$^{\#}$

**p<0.01 *vs*. sham group, $^{\#}p$<0.05 *vs*. model group, $^{\#\#}p$<0.05 *vs*. model group.

Sevoflurane is mainly applied in anesthesia in clinical practice currently, and studies have demonstrated that sevoflurane has a protective effect against cerebral ischemic injury, and it can protect the brain in CIRI, showing a delayed effect[22]. At present, there are two application methods of sevoflurane in cerebral ischemic injury, namely preconditioning and post-conditioning. Sevoflurane preconditioning can induce the cerebral ischemic tolerance in advance, reduce the concentration of pro-inflammatory factors in cerebral ischemic injury, inhibit lipid peroxidation, and alleviate the symptoms of brain injury after CIR. Sevoflurane post-conditioning can suppress the excessive oxidative stress and inflammatory response in brain tissues, and reduce the severity of CIRI. Besides, sevoflurane, by regulating the PI3K-Akt-mTOR and ERK1/2 signaling pathways, can reduce neuronal death during CIR, inhibit autophagy, and relieve focal CIRI, thereby improving the long-term prognosis[23]. In this study, sevoflurane preconditioning could mark-

edly reduce apoptosis in CIRI, lower the degree of brain tissue edema, reduce cerebral infarction, and improve neurological deficit. Compared with the model group, there were evidently decreased protein expressions of JNK and p-JNK, as well as gene and protein expressions of Bax, and evidently increased gene and protein expressions of Bcl-2 in the sevoflurane group.

Conclusions

In summary, sevoflurane can regulate the protein and gene expressions of Bax and Bcl-2, reduce apoptosis in CIRI, and alleviate cerebral edema and cerebral infarction by regulating the JNK signaling pathway, thereby exerting a protective effect on brain tissues and improving the symptoms of neurological deficit.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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