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Effects of sevoflurane post-conditioning in cerebral ischemia-reperfusion injury via TLR4/NF-κB pathway in rats

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Abstract. – OBJECTIVE: The aim of the study was to investigate the anti-inflammatory effect of sevoflurane post-conditioning on cerebral ischemia-reperfusion injury in rats.

MATERIALS AND METHODS: Thirty Sprague Dawley (SD) rats were randomly divided into 3 groups: sham operation group (Sham), ischemia/reperfusion injury (I/R) group and sevoflurane post-conditioning group (Se). Hematoxylin-eosin (HE) staining was used to observe the inflammatory response in the brain tissue. The levels of TNF-α, IL-1β, IL-6 in serum were measured by ELISA. The mRNA and protein expression of TLR4 and NF-κB p65 were detected by RT-PCR and Western blot in the brain tissue.

RESULTS: The post-conditioning of sevoflurane decreased the level of inflammatory reaction in ischemic-reperfusion rat cerebral infarction area and reduced the levels of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 in rats with ischemia-reperfusion injury. In addition, after treatment with sevoflurane, the mRNA and protein expression of TLR4 and NF-κBp65 in TLR4/NF-κB pathway was inhibited.

CONCLUSIONS: Sevoflurane post-conditioning can decrease the inflammatory reaction in cerebral infarct area induced by cerebral ischemia-reperfusion injury in rats. The neuroprotective effect mechanism of sevoflurane may be related to TLR4/NF-κB signaling pathway.

Key Words
Sevoflurane post-conditioning, TLR4/NF-κB pathway, Cerebral ischemia-reperfusion injury.

Abbreviations

TLRs, Toll-like receptors; NF-κB, Nuclear factor-kappa B; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta; NO, nitric oxide; NOS, nitric oxide synthase; RT-PCR, real-time polymerase chain reaction; IL-6, interleukin-6; ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative polymerase chain reaction; SD, standard deviation; MCAO, middle cerebral artery occlusion.

Introduction

Insufficient blood flow to the brain to meet metabolic demand is called cerebral ischemia. This condition can lead to the death of brain tissue due to poor oxygen supply, or cerebral hypoxia. An episode of prolonged ischemic insult can result in fatal consequences for neurons that extend beyond the failure of cellular energy resources. The cellular changes include nuclear fragmentation, chromatin condensation and cell body shrinkage[1,2]. Reperfusion is the restoration of blood flow to the ischemic tissue. When the blood supply returns to the tissue after a period of a lack of oxygen or ischemia, this is a type of tissue damage called reperfusion injury. This type of damage can eventually lead to mortality and morbidity over a large range of pathologies including but not limited to myocardial infarction, acute kidney injury, ischemic stroke, circulatory arrest and trauma. Toll-like receptors (TLRs) are starting to be known as the primary non antigen-specific innate defense immune mechanism. They represent a family of receptors that, upon binding of their ligands, service to recognize molecular patterns associated with pathogens. They also induce activation of several kinases and nuclear factor-kappa B (NF-κB). Toll-Like Receptor 4 (TLR4) mediates the inflammatory response to Gram-negative bacteriaX[3]. NF-κB, as a central regulator of inflammatory response, also affect-

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ed the signal pathway TLR4 and induced many pro-inflammatory mediators involved in innate immunity[4]. In previous study, TLR4/NF-κB pathway was closely related to the myocardial ischemia reperfusion injury by releasing of inflammatory cytokines[5]. In recent years, the role of sevoflurane in ischemia-reperfusion injury attracts more and more people's attention. Studies have shown that sevoflurane post-conditioning may enhance immunity by decreasing serum tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), nitric oxide (NO), nitric oxide synthase (NOS)[6]. This reduced TLR2 and TLR4 expression as well as inflammatory markers in human endothelial cells[7]. But few studies have demonstrated the mechanism of the effects of sevoflurane post-conditioning in cerebral ischemia-reperfusion injury.

The present study is to investigate the effects of sevoflurane post-conditioning in cerebral ischemia-reperfusion injury via TLR4/NF-κB pathway in rats. An enhanced understanding of the underlying mechanisms of sevoflurane post-conditioning will help to improve the cerebral ischemia-reperfusion injury and identify novel diagnostic and therapeutic strategies.

Materials and Methods

Animals

We selected 30 healthy Sprague Dawley rats (Shanghai Laboratory Animal Center, Chinese Academy of Sciences) with weight 250-270 g. The rats were feed in SPF animal rooms, room temperature 22-24°C, relative humidity 50-60%, animal laboratories in adaptive feeding week, free drinking water and feeding. The approval was given by the Medical Ethics Committee of Animal Care and Use Committee of the China Medical University.

The Establishment of the Rat MACAO Model

Cerebral ischemia/reperfusion model (MCAO) was prepared by clamping bilateral common carotid artery and systemic hypotension. Rats were anesthetized with 10% chloral hydrate (0.3 mL/kg, Bolait Chemical Co., Ltd., Wuhan, China) after intragastric administration for 8 h. The rats were anesthetized with endotracheal intubation and mechanical ventilation (20 mL/kg, frequency 60 times, R540 Reward Life Technology Co. Ltd., Shenzhen China). A midline incision was made to expose the left common carotid artery in the neck region. A 3-0 monofilament nylon suture (4.0 cm in length; Ethicon, Somerville, NJ, USA) was inserted into the external carotid artery lumen through a small nick to block the middle cerebral artery. Two hours after the induction of ischemia, the filament was slowly withdrawn and the animals were then returned to their cages for a period of 22h of reperfusion.

Experimental Group

In the experiment, 30 rats were randomly divided into 3 groups (n = 10). Sham group (Sham) was only exposed and isolated bilateral common carotid artery, no ligation; Ischemia/reperfusion injury group (I/R) was the bilateral common carotid artery was occluded by non-invasive arterial clamp for 20 min and then reperfusion 24 h. The sevoflurane post-conditioning group (Se) was infused with 2% sevoflurane (Maruishi Pharmaceutical Co., Ltd, Osaka, Japan) for 15 min before reperfusion.

Specimen Collection

The rats in each group were anesthetized with 10% chloral hydrate (Bolait Chemical Co., Ltd., Wuhan, China), and the brain was quickly decapitated and the left hemisphere (the infarct side) was isolated for the experiment. The left side of the brain tissue was soaked in 4% paraformaldehyde solution (Solaibao Biotechnology Companies, Beijing, China) at 4°C for HE staining. The rat serum was collected at 1100 bpm and centrifuged for 5 min. The obtained supernatant was transferred into a sterilized Eppendorf (EP: Hamburg, Germany) tube (Jinuotai Technology Development Co. Ltd., Beijing, China) and stored at -80 °C. Some of the left brain tissues were subjected to Real-time polymerase chain reaction (RT-PCR) and Western blot.

HE Staining

The brain tissues were immersed in 4% paraformaldehyde solution at 4°C. After 24 h, the water in the brain tissue was gradually removed with ethanol solution, followed by xylene transparent and paraffin embedded (Solaibao Biotechnology Companies, Beijing, China). The embedded wax blocks were sectioned into 5 μm continuous coronal brain tissue (Labsun CUT6062, Shanghai, China), dried and dewaxed, and then distilled water was used to enter the water. At last, they were stained with HE (Solarbio Co., Ltd, Beijing, China). Morphological characteristics of each brain tissue section were observed with an optical microscope (400×, Leica DM1000, Leica Microsystems, Wetzlar, Germany). Four slices were used in each group, and five visual fields were randomly selected for observation.

ELISA

The concentrations of TNF-α, IL-1β and interleukin-6 (IL-6) in serum were measured by enzyme-linked immunosorbent assay (ELISA) according to ELISA kit (R&D Systems, Minneapolis, MN, USA) instructions.

RT-PCR Analysis

Total RNA was extracted using TRIzol Kit (Cat. no. 74104, Qiagen, Germany) following the manufacturer's instructions strictly. The quality and quantity of RNA were detected. Then, 500 ng of total RNA were obtained to generate cDNA by a reverse transcription kit (TaKaRa, Dalian, China). Quantitative polymerase chain reaction (qPCR) was carried out to measure the mRNA levels. The relative levels of target mRNA were standardized through β-actin gene as reference. Primers for RT-PCR in this study were as follows:

TLR4: 5'-TCAGAGCCGTTGGTGTATCTT-3' (Forward) and 5'-TGTCCTCCCATTCCAGGTAG-3' (Reverse); NF-κB p65: 5'-GAGGCGTGTATTAGGGGCTA-3' (Forward) and 5'-ACGCTCAGGTCCATCTCCTT-3' (Reverse); β-actin: 5'-GTGGGGATAATGAACTTGCAG-3' (Forward) and 5'-GGAACCCCTGGTAGAACAGT-3'(Reverse).

Western Blot Analysis

The protein expression of TLR4 (76B357.1, Abcam, Shanghai, China) and NF-κB p65 (ab16502, Abcam, Shanghai, China) was detected by Western Blot. The total brain protein was extracted and the protein concentration was determined by butyleyanoacrylate (BCA, Pierce Biotechnology, Waltham, MA, USA) method. Then, proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into polyvinylidene difluoride (PVDF) membrane. After blocking with 5% fat-free dry milk for 2 h at room temperature, the membranes were incubated overnight at 4°C with polyclonal antibodies. Finally, specific antibody ECL luminescence were using for detection. Labwork 4.6 image analysis software was used to analyze the target protein band.

Statistical Analysis

All data were performed for variance analysis by SPSS 19.0 statistical software (SPSS Inc., Armonk, NY, USA). The *t*-test was used for comparison between groups. The analysis results were presented by mean ± standard deviation (SD). $p<0.05$ was considered statistically significant.

Results

Sevoflurane Post-conditioning Decreased the Level of Inflammatory Response in Ischemic-Reperfusion Rat Cerebral Infarct

In order to investigate the effect of sevoflurane on immunoinflammation, the inflammatory response of brain tissue slices of rats in each group was observed by optical microscope (400×). As shown in Figure 1, the cerebral cortex of the sham group was homogeneous, the neurons were clear, the number of cells was abundant, the cytoplasm was abundant, the nucleus was round and the necrotic degeneration and necrosis were not found. In I/R group, cytoplasm concentration, nucleus pyknosis, fragmentation or dissolution, disorder of cell arrangement, unclear level and interstitial edema were found. The pathological changes of ischemic cortex in the Se group were less than those in the I/R group, the numbers of nerve cells were more, the arrangement was still regular and the interstitial edema was alleviated. These results demonstrated that sevoflurane post-conditioning can decrease the level of inflammatory response in ischemic-reperfusion cerebral infarct in rats.

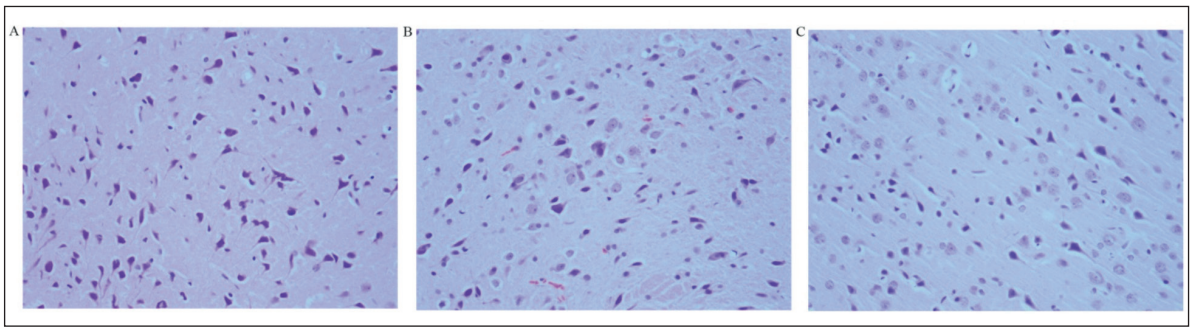

Figure 1. Effects of sevoflurane postconditioning on inflammatory response in rats with cerebral infarction (400 ×). (**A**) Sham, (**B**) I/R, (**C**) Se.

Sevoflurane Post-conditioning Decreased the Concentration of Proinflammatory Cytokines TNF-α, IL-1ß, and IL-6 in Serum of Rats with Ischemia-reperfusion

As shown in Figure 2, the concentrations of TNF-α, IL-1β and IL-6 in the sham group were low at 40.86 pg/mL, 31.20 pg/mL and 78.96 pg/mL, respectively. After the perfusion injury model was established, the secretion of the three pro-inflammatory cytokines in the serum was significantly increased to 210.45 pg/mL, 68.06 pg/mL and 276.50 pg/mL (p<0.05). The levels of three pro-inflammatory cytokines after treatment with sevoflurane were 108.34 pg/mL, 43.69 pg/mL and 183.59 pg/mL, respectively, which were significantly lower than those in the I/R group (p<0.05) and higher than those in the Sham group (p<0.05). These data demonstrated that sevoflurane can decrease the concentration of pro-inflammatory cytokines in serum of rats with ischemia-reperfusion injury.

Sevoflurane Post-conditioning Inhibited the mRNA Expression of TLR4/NF-kB Pathway

As shown in Figure 3, the expression of TLR4 mRNA and NF-κB p65 mRNA increased to 3.08 eq and 2.11 eq in the I/R group compared with the sham group, 1.79 eq and 1.39 eq in the sevoflurane

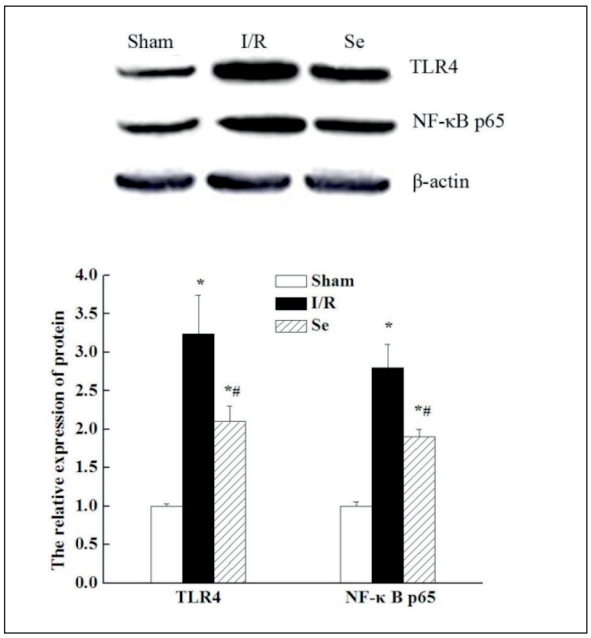

Figure 2. Effects of sevoflurane post-conditioning on serum pro-inflammatory cytokine concentrations. Note: Compared with sham group, *p<0.05; compared with I/R group, #p<0.05.

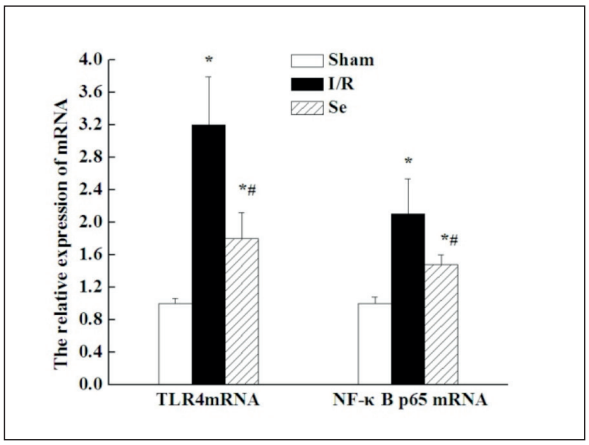

Figure 3. Effect of sevoflurane post-conditioning on TLR4/NF-κB pathway. Note: Compared with sham group, *p<0.05; compared with I/R group, #p< 0.05.

treatment group, the differences were statistically significant (p<0.05). The expression of TLR4 mRNA and NF-κB p65 mRNA was significantly decreased in the Se group compared with the I/R group (p<0.05). The above results demonstrated that sevoflurane post-conditioning could significantly inhibit the mRNA expression of TLR4/NF-κB pathway.

Sevoflurane Post-conditioning Inhibited the Protein Expression of TLR4/NF-kB Pathway

As shown in Figure 4, the protein expression of TLR4 and NF-κB p65 increased to 3.18 eq and 2.53 eq in I/R group compared with the sham group, respectively (p<0.05). Compared with I/R group, the expression of TLR4 and NF-κB p65 in Se group were significantly decreased (p<0.05). These results demonstrated that sevoflurane post-conditioning could significantly inhibit the protein expression of TLR4/NF-κB pathway.

Discussion

In the treatment of acute stroke, restoration of the blood supply could decrease more extensive brain tissue[8]. According to this mechanism, reperfusion after thrombolysis could improve clinical outcome acute stroke patients[9]. However, fatal edema or intracranial hemorrhage following thrombolysis happened in some patients with acute stroke[10]. In some animal stroke models, reperfusion could cause a larger infarct after a long ischemic period than that associated with permanent vessel occlusion[11,12]. In some patients,

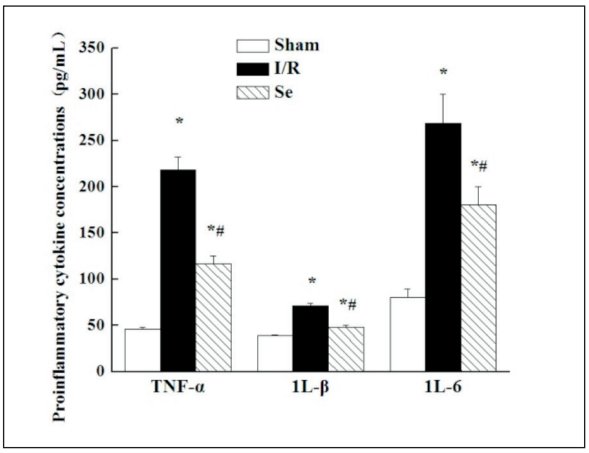

Figure 4. Effect of sevoflurane post-conditioning on TLR4/NF-κB pathway protein expression. Note: Compared with sham group, $*p<0.05$; compared with I/R group, $^{\#}p<0.05$.

reperfusion may improve clinical outcomes and reduce infarct size. However, it may also produce a "cerebral reperfusion" and make the brain injury worse[11,13,14]. Therefore, attention should be paid to reperfusion injury. Some studies demonstrated that sevoflurane post-conditioning may reduce cerebral ischemia-reperfusion induced oxidative injury in rats[15]. However, the underlying mechanism and signaling pathway were rarely studies clearly.

In the present study, we investigated the effects of sevoflurane post-conditioning in cerebral ischemia-reperfusion injury via TLR4/NF-κB pathway in rats. It was found that the pathological changes of ischemic cortex in the sevoflurane post-conditioning group were less than those in the ischemia-reperfusion group. These results demonstrated that sevoflurane post-conditioning can decrease the level of inflammatory response in ischemic-reperfused rat cerebral infarct.

Activation of NF-κB following MCAO-induced cerebral injury, it has been previously shown, was accompanied by the elevated expression of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6[16]. These cytokines serve as mediators of inflammation and are produced by diverse cell types. Our data also indicated that the post-conditioning of sevoflurane could decrease the levels of proinflammatory cytokines TNF-α, IL-1β, IL-6 in rats with ischemia-reperfusion injury[17]. Our findings are in essential agreement with previous reports that suggested, by inhibiting nuclear factor kappa B activation and subsequent alterations in inflammatory cytokines interleukin-1(IL-1), IL-6, TNF-α and IL10 release, sevoflurane could protect the liver from ischemia/reperfusion injury[18].

Our results also suggested that post-conditioning of sevoflurane could decrease the mRNA and the protein expression of TLR4 and NF-κBp65 in TLR4/NF-κB pathway. NF-κB is a transcription factor that targets genes involved in cell proliferation, apoptosis, and inflammation, and that controls the expression of target genes. It is also known to be highly activated in inflammatory disease states such as cerebral ischemia and traumatic brain injury[19,20]. These pro-inflammatory cytokines TNF-α, IL-6, and IL-1β could also stimulate NF-κB activation in inflammatory diseases[21]. The production of cytokine may possibly act as positive feedback to further activate the NF-κB. These findings are consistent with previous studies reporting that, the inhibition of sevoflurane post-conditioning, was against cerebral ischemia-reperfusion induced oxidative injury in rat[15,22]. Our results are in agreement with other studies that suggested the beneficial effects of sevoflurane post-conditioning of ischemia-reperfusion injury via TLR-4/NF-κB pathway in different organs, such as liver, lung and heart[18,23,24].

Conclusions

We demonstrated that sevoflurane post-conditioning could decrease the inflammatory reaction in cerebral infarct area and decrease the concentration of pro-inflammatory cytokines in the serum induced by cerebral ischemia-reperfusion injury in rats. The neuroprotective effect mechanism of sevoflurane may be related to TLR4-NF-κB signaling pathway. The results can provide new potential diagnostic and therapeutic strategies of cerebral ischemia-reperfusion injury and result in an improvement in ischemia-reperfusion outcome.

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Conflict of Interest

The authors declared that there was no conflict of interest regarding the publication of this article.

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