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Ischemic preconditioning reduces ischemic brain injury by suppressing nuclear factor kappa B expression and neuronal apoptosis☆

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Abstract

Ischemic stroke induces a series of complex pathophysiological events including blood-brain barrier disruption, inflammatory response and neuronal apoptosis. Previous studies demonstrate that ischemic preconditioning attenuates ischemic brain damage *via* inhibiting blood-brain barrier disruption and the inflammatory response. Rats underwent transient (15 minutes) occlusion of the bilateral common carotid artery with 48 hours of reperfusion, and were subjected to permanent middle cerebral artery occlusion. This study explored whether ischemic preconditioning could reduce ischemic brain injury and relevant molecular mechanisms by inhibiting neuronal apoptosis. Results found that at 72 hours following cerebral ischemia, myeloperoxidase activity was enhanced, malondialdehyde levels increased, and neurological function was obviously damaged. Simultaneously, neuronal apoptosis increased, and nuclear factor-κB and cleaved caspase-3 expression was significantly increased in ischemic brain tissues. Ischemic preconditioning reduced the cerebral ischemia-induced inflammatory response, lipid peroxidation, and neurological function injury. In addition, ischemic preconditioning decreased nuclear factor-κB p65 and cleaved caspase-3 expression. These results suggested that ischemic preconditioning plays a protective effect against ischemic brain injury by suppressing the inflammatory response, reducing lipid peroxidation, and neuronal apoptosis *via* inhibition of nuclear factor-κB and cleaved caspase-3 expression.

Key Words

neural regeneration; brain injury; ischemic preconditioning; neural cells; apoptosis; nuclear factor kappa-B; cleaved caspase-3; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) Ischemic preconditioning reduced lipid peroxidation levels and neurological deficits following ischemic brain injury in the rat.
(2) Ischemic preconditioning attenuated neuronal apoptosis following rat brain injury.
(3) Ischemic preconditioning suppressed nuclear factor kappa B and cleaved caspase-3 expression in the rat brain.

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INTRODUCTION

Accumulating evidence shows that sublethal ischemic preconditioning induces a neuroprotective effect on subsequent lethal ischemic stroke[1-2]. The potential mechanisms underlying the neuroprotective effect of ischemic preconditioning have been extensively explored throughout the past decade[2-3], however, the effect of ischemic preconditioning on neuronal apoptosis in rats following permanent ischemic stroke and its possible mechanisms remain unknown.

Post-ischemic inflammation and apoptosis contribute to neuronal death and cerebral infarction, resulting in the eventual expansion of ischemic brain injury[3-4]. Our previous studies demonstrated that the pathophysiological events produced by permanent focal cerebral ischemia are characterized by an inflammatory peak at 24 hours and an apoptotic peak at 72 hours after the onset of ischemia[5]. Bowen *et al* [6] found that ischemic preconditioning provides neuroprotection against focal cerebral ischemia *via* preventing the inflammatory response and the expression of inflammatory genes. The present study first explored the neuroprotective effect of ischemic preconditioning in a rat model of permanent focal cerebral ischemia, and further investigated the effect of ischemic preconditioning on neuronal apoptosis and the possible molecular mechanisms.

RESULTS

Quantitative analysis of experimental animals

Forty-two rats were equally divided into three groups as follows: (1) sham surgery group, which underwent sham operation; (2) middle cerebral artery occlusion group, which was subjected to permanent middle cerebral artery occlusion; (3) ischemic preconditioning + middle cerebral artery occlusion group, which was subjected to ischemic preconditioning and permanent middle cerebral artery occlusion. Six rats died, and middle cerebral artery occlusion failed in four rats. We used 24 rats (8 rats in each group) for the final analysis.

Ischemic preconditioning reduced nerve cell damage in rats after middle cerebral artery occlusion

As shown in Figure 1, there was no neuronal damage in the sham surgery group (Figure 1A). Middle cerebral artery occlusion caused an increase in neuronal damage, characterized by a shrunken cytoplasm and nuclei in the ischemic brain (Figure 1B), which was attenuated by ischemic preconditioning (Figure 1C). Experimental data suggested that neuronal survival was significantly enhanced by ischemic preconditioning ($P < 0.01$; Figure 1D).

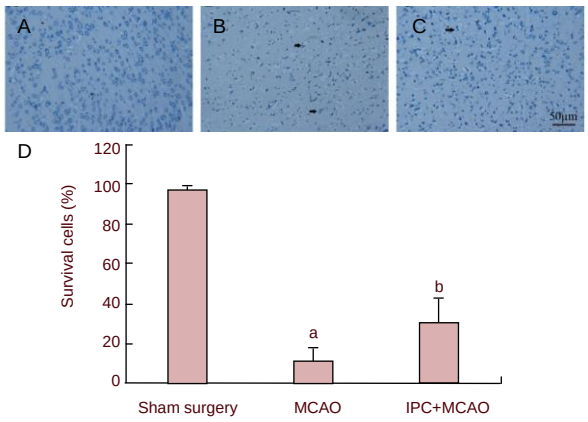

Figure 1 Effect of ischemic preconditioning on neuronal survival in the ischemic brain in permanent focal cerebral ischemia rats (Nissl staining).

(A–C) Representative images of neuronal damage in the ischemic hemisphere from sham surgery, MCAO and IPC + MCAO groups. Neuronal damage is characterized by a shrunken cytoplasm and nuclei (arrows). Scale bar: 50 μm.

(D) Number of intact neural cells in the ischemic hemisphere after 72 hours of MCAO. The total number of cells and damaged cells were counted in each section. The surviving cell ratio was calculated according to the following formulation: (total cells – damaged cells)/total cells ×100%. The data are expressed as mean ± SD from four rats in each group, $^{a}P < 0.01$, *vs*. sham surgery group; $^{b}P < 0.01$, *vs*. MCAO group (one-way analysis of variance followed by least significant difference test).

MCAO: Middle cerebral artery occlusion; IPC: ischemic preconditioning.

Ischemic preconditioning suppressed neuronal apoptosis in rats after middle cerebral artery occlusion

Middle cerebral artery occlusion caused a marked increase in terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL)-positive cells in the ischemic brain ($P < 0.01$), which was significantly decreased by ischemic preconditioning ($P < 0.05$; Figure 2). The experimental results suggested that ischemic preconditioning reduced neuronal apoptosis in the rat brain following cerebral ischemia.

Ischemic preconditioning inhibited nuclear factor kappa B p65 expression

As shown in Figure 3, few nuclear factor kappa B p65-positive cells were observed in sham-operated rats (Figure 3A), but numbers markedly increased after 72 hours of middle cerebral artery occlusion (Figure 3B). The number of nuclear factor kappa B p65-positive cells

significantly reduced in the ischemic preconditioning group ($P < 0.05$; Figures 3C, D). Western blot analysis showed increased expression of nuclear factor kappa B p65 in the ischemic brain in middle cerebral artery occlusion rats when compared with the sham surgery group ($P < 0.01$), which was significantly inhibited by ischemic preconditioning ($P < 0.05$; Figure 4).

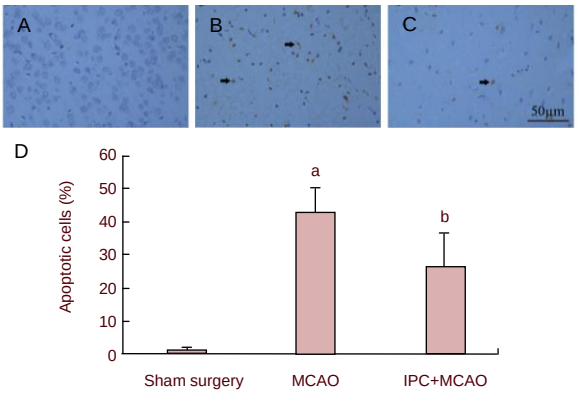

Figure 2 Effect of ischemic preconditioning on cell apoptosis in the ischemic brain in permanent focal cerebral ischemia rats (TUNEL staining).

(A–C) Representative images of TUNEL staining in the ischemic hemisphere from sham surgery, MCAO and IPC + MCAO groups. Arrows show TUNEL-positive cells. Scale bar: 50 μm.

(D) Number of TUNEL-positive cells in the ischemic hemisphere after 72 hours of MCAO. The total number of cells and TUNEL-positive cells were counted in each section. The TUNEL-positive cell ratio was calculated according to the following formulation: TUNEL-positive cells/total cells × 100%. The data are expressed as mean ± SD from four rats in each group, $^aP < 0.01$, *vs*. sham surgery group; $^bP < 0.05$, *vs*. MCAO group (one-way analysis of variance followed by least significant difference test).

MCAO: Middle cerebral artery occlusion; IPC: ischemic preconditioning; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay.

Ischemic preconditioning suppressed cleaved caspase-3 expression in rats after middle cerebral artery occlusion

Expression of cleaved caspase-3 in the ischemic brain was significantly elevated after 72 hours of middle cerebral artery occlusion ($P < 0.01$), demonstrating the presence of apoptosis, which was significantly down-regulated by ischemic preconditioning ($P < 0.05$; Figure 4).

Ischemic preconditioning suppressed the inflammatory response and lipid peroxidation in rats after middle cerebral artery occlusion

Results showed that middle cerebral artery occlusion caused an increase in myeloperoxidase activity in the ischemic brain when compared with sham-operated rats, which was significantly decreased by ischemic preconditioning ($P < 0.05$; Figure 5A). Middle cerebral artery occlusion caused an increase in malondialdehyde levels in the ischemic rat brain when compared with sham-operated rats, which was significantly decreased by ischemic preconditioning ($P < 0.05$; Figure 5B).

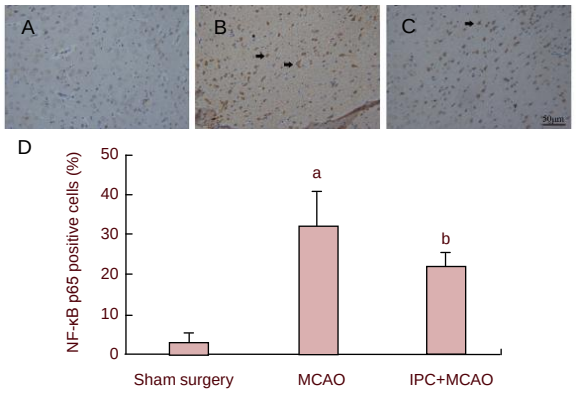

Figure 3 Effect of ischemic preconditioning on the expression of nuclear factor (NF)-κB p65 in the ischemic brain in permanent focal cerebral ischemia rats (immunohistochemical staining).

(A–C) Representative images of NF-κB p65 immunoreactivity (arrows) in the ischemic hemisphere from sham surgery, MCAO and IPC + MCAO groups. Scale bar: 50 μm.

(D) NF-κB p65 immunopositive cells in the ischemic hemisphere after 72 hours of MCAO. The total number of cells and immunopositive cells were counted in each section. The NF-κB p65 immunopositive cell ratio was calculated according to the following formula: immunopositive cells/total cells × 100%. The data are expressed as mean ± SD from four rats in each group. $^aP < 0.01$, *vs*. sham surgery group; $^bP < 0.05$, *vs*. MCAO group (one-way analysis of variance followed by least significant difference test).

MCAO: Middle cerebral artery occlusion; IPC: ischemic preconditioning.

Ischemic preconditioning reduced neurological deficits in rats following middle cerebral artery occlusion

Neurological deficit scores markedly increased at 72 hours after middle cerebral artery occlusion ($P < 0.01$), which were significantly reduced by ischemic preconditioning ($P < 0.01$; Figure 6).

DISCUSSION

Accumulating evidences shows that sublethal ischemic preconditioning exerts a neuroprotective effect against subsequent lethal ischemic stroke[6-7], however, the potential neuroprotective mechanisms remain unknown. Bowen *et al* [6] demonstrated that ischemic

preconditioning prevents ischemia-induced cerebral infarct volume including cortical infarction and striatal infarction in rats subjected to 1-hour transient middle cerebral artery occlusion and 24-hour reperfusion.

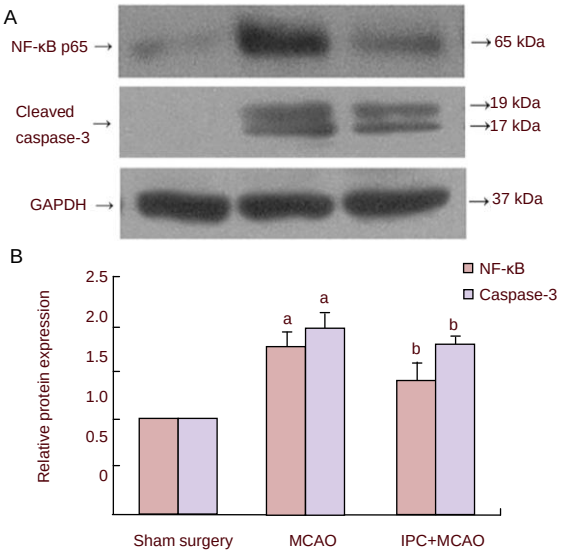

Figure 4 Effect of ischemic preconditioning on the expression of nuclear factor (NF)-κB p65 and cleaved caspase-3 in the ischemic brain in permanent focal cerebral ischemia rats.

(A) Representative bands of NF-κB p65 and cleaved caspase-3 in the ischemic hemisphere from the sham surgery, MCAO and IPC + MCAO groups.

(B) Quantitative analysis of NF-κB p65 and cleaved caspase-3 expression (absorbance ratio to GAPDH normorlized to sham surgery group). The data are expressed as mean ± SD from four rats in each group; $^{a}P < 0.01$, *vs*. sham surgery group; $^{b}P < 0.05$, *vs*. MCAO group (one-way analysis of variance followed by least significant difference test).

MCAO: Middle cerebral artery occlusion; IPC: ischemic preconditioning; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

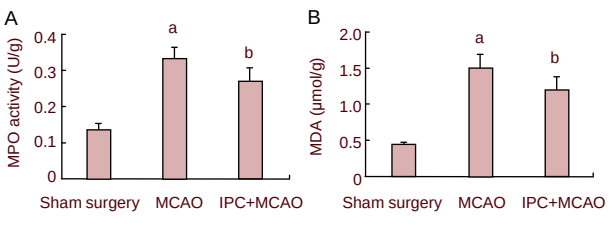

Figure 5 Effect of ischemic preconditioning on myeloperoxidase (MPO) activity (A) and malondialdehyde (MDA) content (B) in the ischemic brain in permanent focal cerebral ischemia rats.

The data are expressed as mean ± SD from four rats in each group. $^{a}P < 0.01$, *vs*. sham surgery group; $^{b}P < 0.05$, *vs*. MCAO group (one-way analysis of variance followed by least significant difference test).

MCAO: Middle cerebral artery occlusion; IPC: ischemic preconditioning.

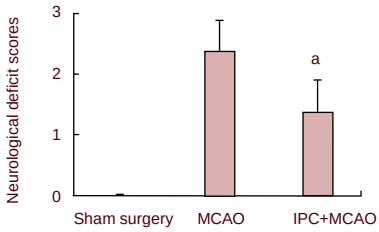

Figure 6 Effect of ischemic preconditioning on neurological deficits.

The data are expressed as mean ± SD from four rats in each group. $^{a}P < 0.01$, *vs*. MCAO group (one-way analysis of variance followed by least significant difference test). High neurological deficit scores indicate severe neurological dysfunction.

MCAO: Middle cerebral artery occlusion; IPC: ischemic preconditioning.

Yin *et al* [7] demonstrated that ischemic preconditioning could suppress ischemia-induced inflammation in neonatal Sprague-Dawley rats subjected to hypoxic ischemia *via* activating the phosphatidylinositol-3-kinase/protein kinase B signaling pathway. The present study first demonstrated that sublethal ischemic preconditioning attenuates neurological deficit scores and exerts a neuroprotective effect against subsequent lethal ischemic stroke in a rat model of permanent middle cerebral artery occlusion. Our experiments further demonstrated that ischemic preconditioning could attenuate middle cerebral artery occlusion-induced neuronal damage in the rat brain. Neuronal damage characterized by a shrunken cytoplasm and nuclei was considered cell death[4]. Our study further explored the effect of ischemic preconditioning on neuronal apoptosis, and experimental results showed that ischemic preconditioning attenuated neuronal apoptosis following 72 hours of lethal middle cerebral artery occlusion. In the search for mechanisms involved in this effect, we first demonstrated an inhibitory effect of ischemic preconditioning on myeloperoxidase activity and malondialdehyde content in the ischemic rat brain. Previous studies showed that inflammation induces neuronal death following cerebral ischemia. Muralikrishna Adibhatla *et al* [8] showed that increased lipid peroxidation and over-production of reactive oxygen species may induce apoptotic neuronal injury, eventually contributing to ischemic brain damage. Accordingly, our results revealed that ischemic preconditioning attenuates neuronal apoptosis possibly because of its inhibitory effect on the inflammatory response and lipid peroxidation.

Experimental results showed that, after 72 hours of lethal middle cerebral artery occlusion, sublethal

ischemic preconditioning inhibited nuclear factor kappa B expression in the rat brain. Cerebral ischemia caused the overexpression and activation of nuclear factor kappa B[9], which induced the expression of pro-inflammatory, pro-oxidative and pro-apoptotic genes, which promote the inflammatory response, oxidation and neuronal apoptosis[10], eventually aggravating ischemic brain damage. Because nuclear factor kappa B is involved in cerebral ischemia-induced neuronal apoptosis[11], we can presume that ischemic preconditioning attenuates neuronal apoptosis *via* inhibiting the expression of nuclear factor kappa B.

Furthermore, this study demonstrated the effect of ischemic preconditioning on cleaved caspase-3 in rats following cerebral ischemia. Cleaved caspase-3, the active form of caspase-3, is an indicator of cellular apoptosis[5]. The elevated expression of cleaved caspase-3 in the rat brain after 72 hours of middle cerebral artery occlusion in this study indicated ischemia-induced neuronal apoptosis. The inhibitory effect of ischemic preconditioning on the expression of cleaved caspase-3 demonstrated that ischemic preconditioning attenuates neuronal apoptosis possibly *via* inhibiting the activation of caspase-3.

MATERIALS AND METHODS

Design

A randomized, controlled animal study.

Time and setting

Experiments were performed at the Fujian Medical University, Fujian Neurosurgical Institute from 2008 to 2011.

Materials

A total of 42 clean adult male Sprague-Dawley rats weighing 250–300 g were purchased from Shanghai Laboratory Animal Center (SCXK (Hu) 2003-0003).

Methods

Ischemic preconditioning and permanent middle cerebral artery occlusion establishment

Rats were intraperitoneally anesthetized with chloral hydrate (300 mg/kg). Ischemic preconditioning was induced by occluding the bilateral common carotid artery temporarily (15 minutes), followed by a period of reperfusion (48 hours). Subsequently, permanent middle cerebral artery occlusion was induced in the middle cerebral artery occlusion group and ischemic preconditioning + middle cerebral artery occlusion group using the intraluminal filament occlusion method as described in our previous report[5].

Assessment of neurological deficits

Neurological deficit scores were assessed at 72 hours after middle cerebral artery occlusion as follows: 0, no observable deficits; 1, contralateral forelimb flexion; 2, decreased resistance to lateral push without circling; 3, circling to the contralateral side; 4, death.

Sample preparation

After 72 hours of middle cerebral artery occlusion, rats were sacrificed, perfused and the ischemic brain cortex was removed, fixed, and embedded in paraffin. Coronal sections (4 μm thick) were obtained and deparaffinized for Nissl staining, TUNEL staining and immunohistochemical analysis.

Nissl staining for nerve cell damage

To confirm the effect of ischemic preconditioning on focal cerebral ischemia-induced neuronal injury, Nissl staining (0.1% (w/v) toluidine blue; Amresco, Solon, OH, USA) was performed to observe the morphological characteristics of neurons in the ischemic brain. The sections were visualized with a light microscope (Olympus). The survival cell ratio was calculated.

TUNEL analysis for cell apoptosis

TUNEL staining was performed according to the manufacturer's instructions. The paraffin-embedded sections were deparaffinized and incubated in the TdT enzyme at 37°C for 60 minutes, followed by two washes in standard saline citrate to stop the reaction. Brain sections were incubated in streptavidin horseradish-peroxidase solution for 30 minutes at room temperature. Diaminobenzidine was used as a color substrate, and the sections were counterstained with hematoxylin. The TUNEL-positive cell ratio was calculated under light microscopy.

Immunohistochemical analysis for nuclear factor kappa B expression

The paraffin-embedded sections were deparaffinized, then incubated with a mouse anti-rat monoclonal antibody against nuclear factor kappa B p65 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Biotinylated goat anti-mouse IgG (1:500; Zhongshan Biotechnology, Beijing, China) was used as a secondary antibody at 37°C for 2 hours. Diaminobenzidine was used as the chromogen, and the sections were counterstained with hematoxylin. The

number of nuclear factor kappa B p65 immunopositive cells was calculated under light microscopy.

Western blot analysis for nuclear factor kappa B and cleaved caspase-3 expression

Total protein was extracted and separated on a 10% (w/v) sodium dodecyl sulphate polyacrylamide gel, transferred to nitrocellulose membrane, then incubated overnight with a mouse anti-rat monoclonal antibody against nuclear factor kappa B p65 (1:200; Santa Cruz Biotechnology), or a rabbit polyclonal antibody against cleaved caspase-3 (1:1 000; Cell Signaling, Boston, MA, USA) at 4°C overnight, followed by incubation with horseradish-peroxidase conjugated goat anti-rabbit/mouse IgG at 4°C for 2 hours (1:2 000; KPL Inc., Gaithersburg, MD, USA). Protein expression was detected with an enhanced chemiluminescence detection system (KPL Inc.). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The absorbance of nuclear factor kappa B p65, cleaved caspase-3 and GAPDH bands on the X-ray film were quantitatively analyzed with Quantity One software (Bio-Rad, Hercules, CA, USA). The results were expressed as an absorbance ratio of nuclear factor kappa B p65/GAPDH and cleaved caspase-3/GAPDH.

Biochemical analysis for myeloperoxidase activity and malondialdehyde content

Myeloperoxidase activity in the rat brain was measured according to the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China). The results were expressed as unit per gram of tissue (U/g tissue). Malondialdehyde content in the rat brain was detected according to the manufacturer's instructions (Jiancheng Bioengineering Institute). The results were expressed as μmol/g.

Statistical analysis

Experimental data were presented as mean ± SD. Statistical significance was assessed by one-way analysis of variance followed by Least Significant Difference test with SPSS 13.0 software (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Songsheng Shi was in charge of animal model establishment. Weizhong Yang was responsible for experimental design. Xiankun Tu obtained the funding and wrote the manuscript. Chunmei Chen and Chunhua Wang performed the experiments. All authors have read and agree to the manuscript as written.

Conflicts of interest: None declared.

Ethical approval: The surgical procedures were approved by Animal Ethics Committee of Fujian Medical University in China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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