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Correlations of neuronal apoptosis with expressions of c-Fos and c-Jun in rats with post-ischemic reconditioning damage

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Abstract. – OBJECTIVE: Transcription factors (c-Fos and c-Jun) have been considered to play roles in the initiation of programmed nerve cell death. However, the roles of c-Fos and c-Jun protein expressions in neuronal apoptosis of rats with post-ischemic reconditioning damage were not clarified. Therefore, the aim of this study was to investigate the correlations of protein expressions of c-Fos and c-Jun with neuronal apoptosis of rats with post-ischemic reconditioning damage.

MATERIALS AND METHODS: Rat models of post-ischemic reconditioning were established firstly. Then, apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay, and the gene expression levels of apoptosis-related proteins [cytochrome c (Cyt c), B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax)] were detected by reverse transcription-polymerase chain reaction (RT-PCR). Lastly, Western blotting was used to determine the protein expression levels of c-Fos and c-Jun, and the expressions of c-Fos and c-Jun in brain tissues of models were measured by immunohistochemistry.

RESULTS: Treatment group had significantly increased malonaldehyde (MDA) level and significantly decreased superoxide dismutase (SOD) activity in rat cortex compared with those in control group ($p<0.05$). The number of TUNEL positive cells in the right cortex of rats in the treatment group was clearly higher than that in control group. Among them, post-ischemic reperfusion group had reduced level of Bax in the cytoplasm, but increased Bax level in the mitochondrion, and lowered expression level of Bcl-2 in both mitochondrion and cytoplasm in comparison with control group. Dynamic detection results of c-Jun were in synchronization with those of apoptosis proteins, and maximum expression occurred at 24 h after treatment.

CONCLUSIONS: c-Jun may play a role in the initiation of apoptotic cell death in these neurons.

Key Words:
Post-ischemic reconditioning, Neuronal apoptosis, c-Fos, c-Jun.

Introduction

Stroke is one of the major causes of death and disability. Advances in endovascular technology and thrombolytic agents have reduced functional deficits of stroke patients within the optimal time window. However, reperfusion itself leads to reperfusion injury[1]. Although many researches on the treatment of reperfusion injury have been conducted in the past few decades, there are few neuroprotective drugs from the successful basic researches applied in clinical practice.

Apoptosis is the main approach leading to cell death after cerebral ischemia-reperfusion[2]. After cerebral ischemia-reperfusion, mitochondria are oxidatively loaded, thereby causing permeabilization of the outer membrane[3] and eventually resulting in the release of Bcl-2-associated X protein (Bax) and cytochrome c (Cyt

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c) from the cytoplasm to the mitochondrial membrane space[4,5]. The translocation of such apoptotic proteins is controlled by the B-cell lymphoma 2 (Bcl-2) protein family[6].

A large number of *in vivo* and *in vitro* studies have indicated that transcription factors (c-Fos and c-Jun) may play roles in the regulation of programmed neuronal cell death. For example, it has been found that c-Fos expression is increased in several tissues of mouse mutant weavers before the death of normally developed programmed cells and with the death of apoptotic neuronal cells in cerebella[7]. In an *in vitro* model of apoptotic death of sympathetic neurons induced by withdrawal on nerve growth factors, c-Jun messenger ribonucleic acid (mRNA) expression is increased, and neurons are protected from death by intracellular microinjection of neutralizing antibodies of c-Jun[8]. In a similar model, other researchers discovered that nerve growth factors stop inducing c-Jun phosphorylation and increase c-Jun protein level[9]. In addition, it is showed that neurons are protected by the dominant-negative mutant transfected with the expression vector for c-Jun from apoptotic death. Bruckner et al[10] found that deprivation of nerve growth factors leads to increased c-Jun N-terminal kinase (JNK) activity in primary rat sympathetic neurons and rescues neurons by inhibiting this activity after early development. However, they also discovered that the occurrence of c-Jun expression and its timing of phosphorylation are not associated with death commitment. They also found that c-Jun expression is not restricted to apoptotic nuclei, so they thought that c-Jun expression may be necessary.

In an *in vivo* model of hypoxic-ischemic injury, Dragunow et al[11-13] found that c-Jun is expressed in neurons that experienced delayed death, which may be caused by programmed cell death. Wang et al[14] also observed that c-Jun is expressed in an *in vivo* study of cerebellar neurons that undergo apoptosis upon exposure to ionizing radiation. These researchers also found that c-Jun is expressed during apoptosis in the death process of naturally developed cells.

Therefore, the primary purpose of this study was to investigate the roles of c-Fos and c-Jun in the initial signaling phase of neuronal apoptosis in rats with cerebral ischemia-reperfusion injury, and the correlations of c-Fos and c-Jun expressions with apoptosis-related protein expressions.

Materials and Methods

Experimental Materials

An *in situ* cell death assay kit (Roche, Basel, Switzerland), a laser Doppler flowmeter (Periflux System 5000, Perimed, Stockholm, Sweden), avidin-biotin horseradish peroxidase complex (ABC, Vector Laboratories, Burlingame, CA, USA), 2,2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, MO, USA), Image J software (NIH Image, version 1.61), c-Jun (Ab-1, Oncogene Science, Uniondale, NY, USA), c-Fos (Ab-5, Oncogene Science, Uniondale, NY, USA) and diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA).

Methods

Experimental Animals and Rats With Post-Ischemic Reconditioning Damage

Adult male Sprague-Dawley (SD) rats weighing 250-280 g were selected according to the *Guide for the Care and Use of Laboratory Animals* and anesthetized *via* chloral hydrate anesthesia at a dosage of 350 mg/kg. Then, a monocular nylon suture was used to obstruct the middle cerebral artery. After 60 min of the occlusion of the middle cerebral artery, the suture was removed to restore blood flow (reperfusion was confirmed by the laser Doppler) (n=15). Control group did not receive occlusion of the middle cerebral artery (n=15). As previously described, local cerebral blood flow was monitored using a flexible probe on the skull *via* the laser Doppler flow meter, and measured before ischemia, during the occlusion of the middle cerebral artery and during reperfusion. After that, animals in the experimental group that had a reduction of at least 70% in local cerebral blood flow and animals that died after the induction of ischemia were excluded. Core temperature was monitored with a rectal probe and maintained at 37°C throughout the procedure. Mean arterial pressure (left femoral artery), pH, and arterial blood gas and glucose levels were measured before, during and after ischemia. All surgical procedures were performed under a stereoscopic microscope. This study was approved by the Animal Ethics Committee of Shenzhen Second People's Hospital.

Measurement of Infarct Volume

After 24 h and 72 h of reperfusion, rats were decollated to quickly take out brains. Brains (n=6, each

group) were cut into coronal sections with 2 mm in thickness, stained with 2,2,3,5-triphenyltetrazolium chloride at 37°C for 30 min and then immersed in 10% formalin overnight. Then, the infarct area was divided and analyzed by Image J software (Rawak Software, Inc., Hamburg, Germany). The infarct areas of all sections were added to calculate the total infarct area, and then the total infarct area was multiplied by the thickness of brain sections to obtain the infarct volume. To compensate for the effects of cerebral edema, the corrected infarct volume was calculated as follows: Corrected infarct area = Measured infarct area× {1 − [(ipsilateral hemisphere region − contralateral hemisphere region) / contralateral hemisphere area]}.

Histological Examination

After 24 h of reperfusion, rats were deeply anesthetized with chloral hydrate and perfused with heparin phosphate-buffered saline, followed by perfusion with 4% paraformaldehyde in phosphate-buffered saline. For c-Fos and c-Jun staining, 30-μm sections were alternately stored and collected in phosphate-buffered saline (PBS). Striatum was post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 1 week, then serially sectioned and stained with thionine to determine lesion location. While for c-Fos and c-Jun immunostaining, all sections were subjected to free-floating treatment, and washed with PBS, 0.5% bovine serum albumin (BSA)-PBS, 0.5% BSA-0.1% Triton-PBS solution at 4°C for 15 min successively. Then, additional washing in PBS was carried out, and sections and antiserum (1:1000) were incubated with anti-c-Fos (1:1) in PBS containing 0.5% BSA or c-Jun at 4°C for 48 h. After that, sections were washed in PBS containing 0.5% BSA and incubated with biotinylated protein A (1:100) prepared in our laboratory at room temperature for 60 min. Then, sections were washed in PBS containing 0.5% BSA, incubated with avidin-biotin horseradish peroxidase complex (1:600) at room temperature for 60 min and then incubated in diaminobenzidine under the presence of hydrogen peroxide to produce brown colored products. After that, sections were placed on gelatin and counterstained with thionine to identify cellular morphology and apoptotic chromatin clusters in the nucleus. Oncogene Ab-5 rabbit polyclonal serum was prepared for residues 4-17 of human fos, which did not cross-react with the jun protein of 39,000 kDa. A previous immunohistochemical study of rat brains proved that Ab-5 is specific for c-Fos protein[15].

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate-Biotin Nick End Labeling (TUNEL) Analysis

TUNEL analysis was used to assess apoptosis (n=6). TUNEL-positive cells showed brown staining in apoptotic nuclei. Deoxyribonucleic acid (DNA) breakage was quantitatively analyzed at high power magnification (400) without the researcher's knowledge and expressed as n/mm^2.

Determination of Oxidative Stress

Cortical samples (n=6 in each group) were weighed. The amount of lipid peroxides was measured by measuring the absorbance of malonaldehyde (MDA) at 532 nm *via* the thiobarbituric acid method as MDA production. Superoxide dismutase (SOD) activity was measured by spectrophotometrically detecting absorbance at 550 nm through xanthine oxidation method.

Western Blotting Analysis

The Western blotting analysis was performed to detect apoptosis-related proteins. Right cortical samples (n=6 in each group) were weighed and homogenized in a homogenizer to obtain whole cell lysate (n=6 in each group). Then, homogenate was further centrifuged at 7500 g and 4°C for 15 min to separate the sample into supernatant A and precipitate A. Supernatant A containing cytoplasmic/mitochondrial proteins was further centrifuged at 16000 g and 4°C for 30 min to separate supernatant B. Supernatant B was used as a cytoplasmic component, and precipitate B was reused as a mitochondrial component in buffer. After that, protein samples were separated on 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20-50 g/lane), and then, transferred to a membrane and followed by blocking and incubation with primary antibodies overnight. Primary antibodies and their dilutions are as follows: Bcl-2, Cyt c and Bax (1:200), actin β-actin and COX IV (1:5000). Lastly, the membrane was washed with Tris buffered saline with Tween®20 (TBS-T), incubated with secondary antibodies (1:2000) at room temperature for 1 h and then subjected to color development with an electrochemiluminescence system (ECL kit, Millipore, Billerica, MA, USA).

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA (2 μg) was extracted from the right cortex using TRIzol method (n=6 in each group)

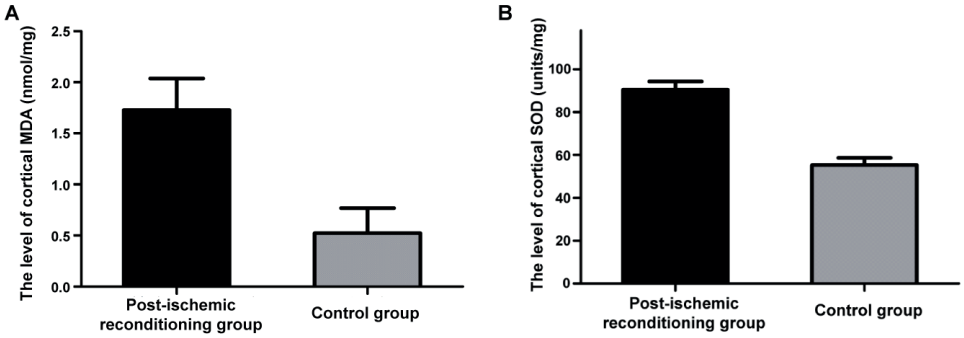

Figure 1. Cortical MDA level and SOD activity in rats with post-ischemic reconditioning injury.

according to the instructions of the Revert Aid H Minus M-uLV RT kit (Thermo Fisher Scientific, Waltham, MA, USA). In all experiments, Actin was used as an internal standard for stable expression (housekeeping gene). PCR was performed using a Gene Cycler, and the band optical density of products was analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical product and service solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by a Post-Hoc test (Least Significant Difference). p-values < 0.05 were considered statistically significant

Results

Cortical MDA Level and SOD Activity in Rats With Post-Ischemic Reconditioning Injury

Compared with that in control group, the level of cortical MDA (lipid peroxidation index) of rats was significantly increased in the post-ischemic reconditioning group (Figure 1A). While the cortical SOD activity of rats in the post-ischemic reconditioning group was clearly lower than that in control group (p<0.05) (Figure 1B).

TUNEL Changes in the Right Cortex Of Rats With Post-Ischemic Reconditioning Injury

In TUNEL experiments, a large number of TUNEL-positive cells were observed in the right cortex of rats that undergone reconditioning after ischemia, while no TUNEL-positive cells were detected in the right cortex of rats receiving control operation (p<0.05) (Figure 2).

Expressions of Apoptosis-Related Proteins (Cyt c, Bax, and Bcl-2) After Post-Ischemic Reperfusion

Compared with control operation group, post-ischemic reperfusion group had overtly decreased Cyt c expression in mitochondria and clearly increased Cyt c expression in cytoplasm. In comparison with control operation group, the level of Bax was decreased in the cytoplasm and increased in mitochondria in post-ischemic reperfusion group. The expression of Bcl-2 in mitochondria was lowered in post-ischemic reperfusion group compared with that in control operation group (Figure 3).

Relative Expressions of c-Fos and c-Jun in Neurons of Rats in Post-Ischemic Reconditioning Group

According to Figure 4, the expression of c-Fos in the neurons of rats with post-ischemic

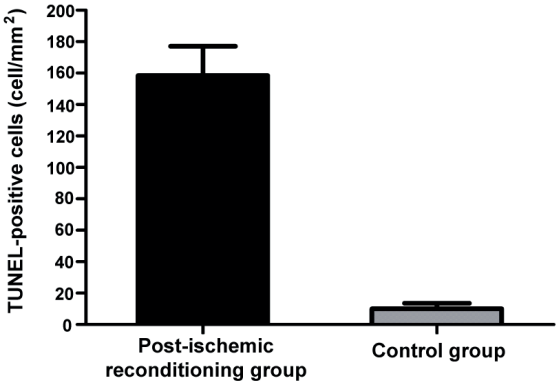

Figure 2. TUNEL changes in the right cortex of injured rats after 24 h of post-ischemic reconditioning (n=6).

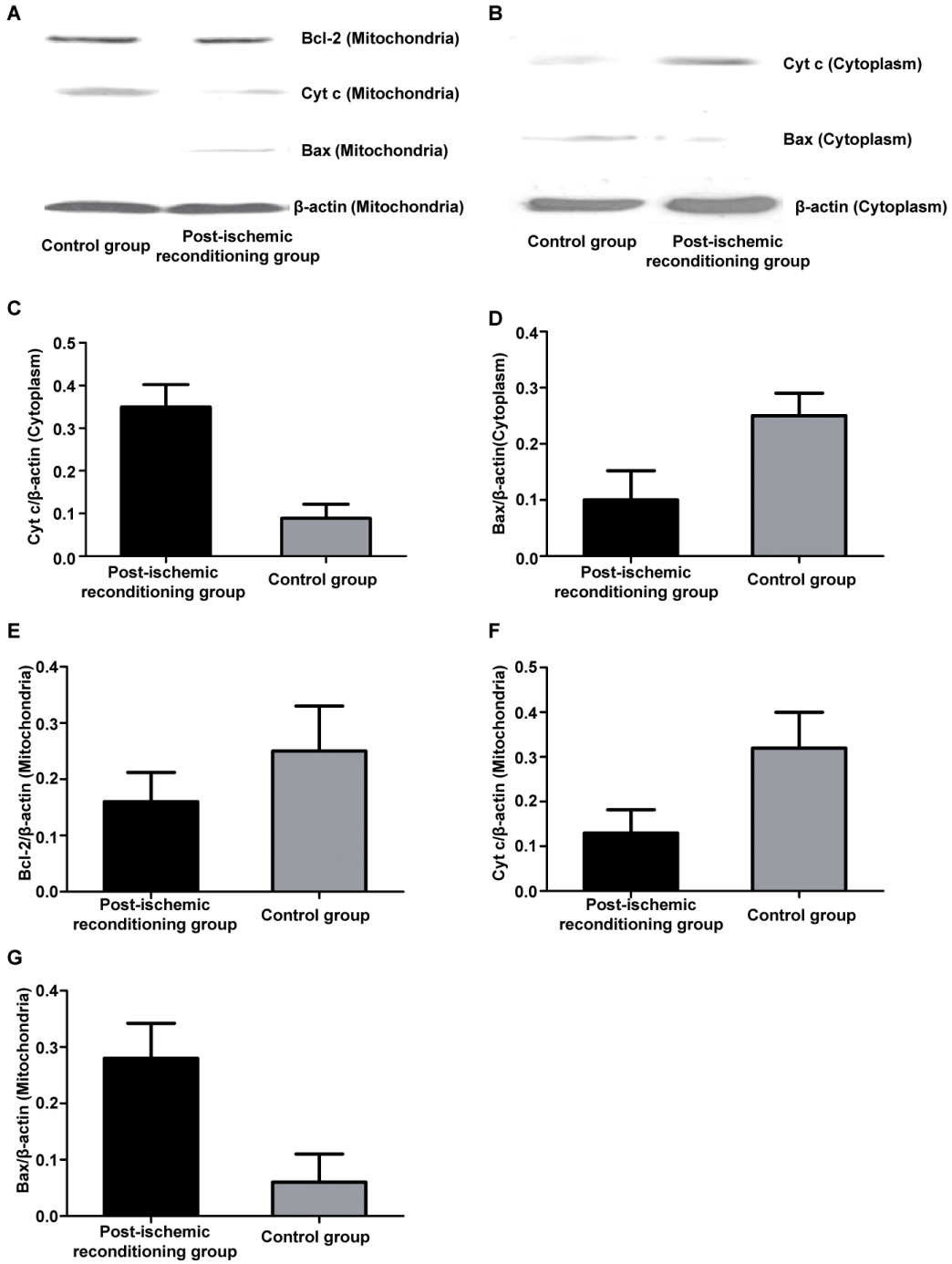

Figure 3. Cyt c, Bax and Bcl-2 expression levels at 24 h after post-ischemic reperfusion. ***A,*** Immunoblots showing differences in the expressions of Cyt c, Bax and Bcl-2 in mitochondria. ***B,*** Immunoblots showing differences in Cyt c and Bax expressions in cytoplasm. ***C,*** The relative band density of Cyt c and the average value of controls in cytoplasm. ***D,*** The relative band density of Bax and the average value of controls in cytoplasm. ***E,*** The relative band density of Bcl-2 and the average of controls in mitochondria. ***F,*** The relative band density of Cyt c and the average value of controls in mitochondria. ***G,*** The relative band density of Bax and the average value of controls in mitochondria. For comparisons between post-ischemic reconditioning group and control group, $p<0.05$ (n=6).

reconditioning injury was increased to the peak at 12 h after treatment. However, the relative expression of c-Jun was peaked at 24 h after treatment.

To sum up, the results showed that there was a significant change in rats with post-ischemic reconditioning injury at 24 h after treatment, which was mainly manifested by evident elevated level

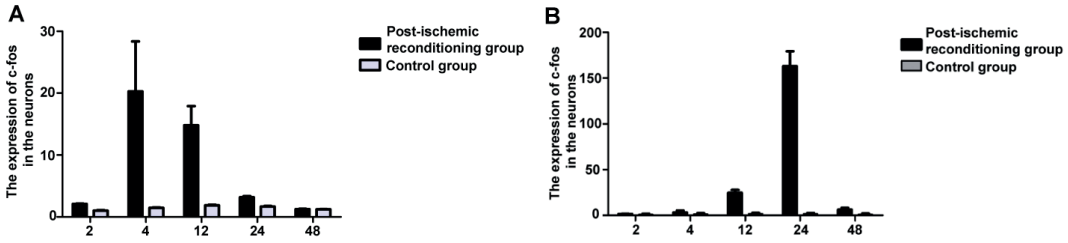

Figure 4. Relative expressions of c-Fos (***A,***) and c-Jun (***B,***) in neurons (n=6).

of MDA (lipid peroxidation index) in the cortex. The SOD activity in rat cortex was decreased significantly compared with that in control operation group. The number of TUNEL positive cells in the right cortex of rats in the treatment group was evidently higher than that in the control group. The expressions of apoptosis-related proteins (Cyt c, Bax, and Bcl-2) were overtly changed, in which Bcl-2 expressions in both mitochondria and cytoplasm were decreased in post-ischemic reperfusion group in comparison with control group. The dynamic detection results of c-Jun were synchronized with those of apoptosis proteins, and the maximum expression occurred at 24 h after treatment.

Discussion

Reperfusion plays an important role in the pathogenesis of ischemic injury in an early stage. Post-ischemic conditioning reduces brain damage. However, it remains unclear whether harmful mechanisms are impaired or whether beneficial mechanisms are triggered by post-ischemic conditioning. In addition, early studies are performed in models of permanent focal cerebral ischemia after partial reperfusion. Whether this protection phenomenon is applicable to other models of post-ischemic reperfusion remains to be elucidated. In this study, the control group was tested in rat models.

It is well-known that excessive reactive oxygen species (ROS) produced during reperfusion plays a major role in stroke-related brain injury[16]. Due to the low activity of antioxidase, the brain is easy to be damaged by ROS in post-ischemic reconditioning injury, which could lead to brain dysfunction and cell death[17-19]. In this study, post-ischemic conditioning lowered the level of MDA and increased the activity of SOD, which inhibited oxidant production and oxidant-mediated damage in reconditioning injury after myocardial ischemia. Those results were consistent with previous studies[18,20].

Recent studies have indicated that post-ischemic conditioning has an anti-apoptotic effect on the heart *in vivo* and *in vitro*. Our study showed that post-ischemic conditioning significantly inhibited the apoptosis of cortical neurons induced by post-ischemic reconditioning injury. Post-ischemic reperfusion increased the level of Bax protein in mitochondria, decreased the expression levels of anti-apoptotic Bcl-2 and Cyt c in mitochondria, reduced the expression level of Bax protein in the cytoplasm and increased the expression level of Cyt c in the cytoplasm. Those results suggested that mitochondrial pathway is an important target of post-ischemic conditioning.

Conclusions

This study clarified that post-ischemic conditioning increased c-Fos and c-Jun expressions in neuronal cells during reperfusion after cerebral ischemia. Among them, the expression level of c-Fos was peaked at 12 h after post-ischemic conditioning, while the expression level of c-Jun reached the peak at 24 h after post-ischemic conditioning. The expression of c-Jun was synchronized with the change of apoptosis-related protein Bcl-2 (anti-apoptotic)/Bax (pro-apoptotic) after post-ischemic conditioning.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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