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Original Article

Activation of STAT3 is involved in neuronal apoptosis in focal cerebral ischemia/reperfusion rats via Bcl 2/Fas pathway

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Abstract: It is well-known that signal transducer and activator of transcription 3 (STAT3) plays an essential role in cell survival and proliferation. Therefore, we investigated the effects and mechanism of STAT3 is involved neuronal apoptosis in focal cerebral ischemia/reperfusion rats. Rat MCAO model was used to produce transient focal ischemia. The volume of cerebral infarction was measured by TCC stain. The localizations of STAT3, phosphorylated STAT3 Bcl-2 and Fas were immunohistochemically examined in rats after 0.5 h to 7 d of reperfusion following 3 h of middle cerebral artery occlusion (MCAO), and the neuronal apoptosis were detected by TUNEL assay. Our results showed that the amount of apoptosis cells reaches the peak at 3 h after ischemia/reperfusion. Low expressed STAT3, p-Tyr705-STAT3, Bcl-2 and Fas were detected in control group and sham operated group. In ischemia/reperfusion (I/R) group, the STAT3 firstly detected after 0.5 h reperfusion, and peaked at 24 hours in ischemic zone, including ischemic penumbra and ischemic core zone. The p-Tyr705-STAT3 positive cells peaked at 24 hours after reperfusion in the ischemic penumbra, while peaked at 3 hours after reperfusion in the ischemic core zone. The Bcl 2 positive cells peaked at 12 hours after reperfusion in the ischemic penumbra, while reached peak at 6 hours after reperfusion in the ischemic core zone. The Fas positive cells reached peak at 24 hours after reperfusion in the ischemic penumbra, while reached peak at 12 hours after reperfusion in the ischemic core zone. Correlative analysis showed that positive correlation with each other STAT3, p-Tyr705-STAT3, Bcl-2 and Fas in the ischemic zone ($P<0.01$). Moreover, p-Tyr705-STAT3, Bcl-2 and Fas were positive correlation with TUNEL positive cells ($P<0.01$), except STAT3. In conclusion, activation of STAT3 induces neuronal apoptosis in focal cerebral ischemia/reperfusion rats via Bcl 2/Fas pathway.

Keywords: Ischemia/reperfusion, neuronal apoptosis, STAT3 phosphorylation

Introduction

Stroke is one of the leading causes of death and the principal cause of disability in the world. However, there is currently no effective treatment [1]. Ischemic stroke is a major cause of death and disability in adults worldwide, and the clinical prognosis of acute cerebral ischemia is poor [2, 3]. The early reperfusion after cerebral ischemia is essential for the viability and functional recovery of the brain; however, the arrival of blood oxygen to the ischemic tissue causes ischemia/reperfusion injury (IRI) that eventually leads to neuronal death [4].

Signal transducer and activator of transcription factors (STATs) are a family of transcription factors that regulate cell growth, survival, differentiation, and motility [5]. Structural studies identified that STAT proteins consist of an N-terminal domain, a coiled-coil domain, a DNA-binding domain, a Src homology 2 (SH2) domain and a transactivation domain, of which the DNA-binding domain is required for the recognition of specific binding sequences. Until now, STAT family have identified seven members, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. STAT3 protein exists in the cytoplasm as an inactive form until phosphorylation by receptor-associated kinases. Activated JAK kinases or other tyrosine kinases phosphorylate STAT3 through binding of the SH2 domain to a phosphorylated tyrosine residue, by which the C-terminus of p-STAT3 triggers its release

from receptor, and form a homo- or heterodimerization of p-STAT3 [6, 7]. Tyrosine phosphorylation is necessary for STAT3 activity. STAT3 is primarily activated by tyrosine (Y705) phosphorylation, which traffic to the nucleus, bind DNA and activate transcription of key target genes involved in cellular proliferation, survival and migration. Evidence indicates that STAT3 signaling pathway transduces stress-activating extracellular chemical signals into cellular responses for a number of pathophysiological processes, such as immunity, inflammation and apoptosis, and is involved in cerebral IRI [8-10]. For example, Satriotomo et al. and Justicia et al. showed that STAT3 activation contributes to neuronal damage following transient focal cerebral ischemia [9, 11]. However, the mechanism of STAT3 in neuronal apoptosis in focal cerebral ischemia/reperfusion rats remains unclear. In the present study we elucidate the chronological, topographical and cellular alteration of STAT3 protein, Tyr705 phosphorylation of STAT3 protein, and apoptosis-associated protein (Bcl 2 and Fas) using immunohistochemical techniques, reveal the correlation between activation of STAT3 and neuronal apoptosis in focal cerebral ischemia rat.

Materials and methods

Animals

Adult male Wistar rats (weighing 280-300 g; 7 to 8 weeks of age) supplied by Experimental Animal Center, Central South University, Changsha, China, were housed in a temperature-controlled room (22-25°C) on a 12-h light/dark cycle with free access to food and water. All animal procedures were approved by the ethical committee for animal experiments, Central South University. All possible measures were taken to reduce animal suffering and numbers of animals in this study.

Model of focal cerebral ischemia

Middle cerebral artery (MCA) occlusion (MCAO) was carried out as previously described [12]. The animals were anesthetized by an intraperitoneal injection of 300 mg/kg chloral hydrate. Heating lamps were used to maintain rectal temperature at 37-37.5°C. The right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed through a midline neck incision, and the ECA was ligated close to its origin with a 3-0 silk suture. A 0.26-mm monofilament nylon suture with a blunt tip (Beijing Shandong Industrial Corp., Beijing, China) was inserted into the ICA, and advanced 18-20 mm until mild resistance was felt, effectively occluding the MCA. After 180 min of MCAO, the monofilament nylon suture was removed and ICA perfusion was restored. After surgery, animals were then placed into a cage to recover from anesthesia at room temperature and were allowed food and drink.

Experimental protocol

In total, 45 rats were randomly assigned to 9 groups (n=15 in each group): the control group, the sham-operated, I/R 0.5 h group, I/R 3 h group, I/R 6 h group, I/R 12 h group, I/R 24 h group, I/R 3 d group and I/R 7 d group (**Figure 1**). The I/R group underwent MCAO by occlusion of the right MCA for 180 min, followed by 0.5 h of cerebral reperfusion (I/R 0.5 h group), 3 h of cerebral reperfusion (I/R 3 h group), 6 h of cerebral reperfusion (I/R 6 h group), 12 h of cerebral reperfusion (I/R 12 h group), 24 h of cerebral reperfusion (I/R 24 h group), 3 d of cerebral reperfusion (I/R 3 d group), 7 d of cerebral reperfusion (I/R 7 d group). The sham-operated group underwent the same procedure as the I/R rats, but without occlusion of the right MCA.

Neurological evaluation

The animals were returned to their cages after the procedures were finished and again allowed free access to food and water. The neurological deficit score (NDS), as previously described by Longa *et al.* [13], was measured to assess neurological evaluation as follows: 0, no deficit; 1, failure to extend left forepaw fully; 2, circling to the left; 3, falling to the left; and 4, no spontaneous walking with a depressed level of consciousness.

Measurement of infarct size

After reperfusion, seven rats from each group were chosen randomly by staffs that were blinded to these rats, killed by an overdose of pentobarbital i.p. and their brains were rapidly removed. Infarct sizes were measured by stain-

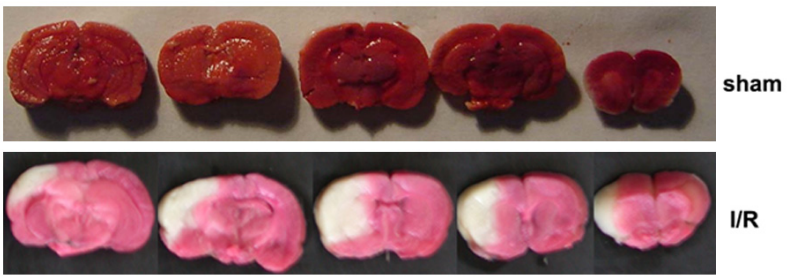

Figure 1. Ischemia reperfusion injury (IRI) induced cerebral infarction volume determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining (n=5); normal brain tissue is red, infarcted brain tissue is white. The sham-operated (sham) group underwent the operation without right middle cerebral artery (MCA) occlusion. The ischemia/reperfusion (I/R) group underwent MCA occlusion (MCAO) by occlusion of the right MCA for 3 h, followed by 12 h of reperfusion.

ing with 2,3,5-triphenyl-2H-tetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO, USA). Brains were cut into 2-mm-thick coronal sections in a cutting block and 6 slices were stained with 1% TTC solution for 30 min at 37°C followed by overnight immersion in 4% paraformaldehyde. The percentage of brain infarct was measured by normalizing to the entire brain tissue from the animals, as described previously [14].

Assessment of neuronal survival

The coronal brain sections were examined using a standard hematoxylin and eosin (H&E) staining protocol to examine cellular morphology by observing the number and shape of the neurons. The brain sections were stained with toluidine blue for Nissl bodies. Neuronal survival was assessed by observing the Nissl bodies in the neurons. Images of the ischemic penumbras of the 5 rats were captured, and quantitative analyses of the cells were performed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Neuronal apoptosis

According to the instruction of TUNEL apoptosis detection kit (Wuhan Boster Biotech. Co. Ltd., China), four paraffin sections form each group were conventionally de-waxed and hydrated to dispose with 3% H_2O_2 for 10 min at room temperature, and then digested by proteinase K (1:200) for 10 min at 37°C. Mixing TdT (1 μl) and Dig-dUTP (1 μl) into labeling buffer (18 μl) and add on each paraffin slice to react for 2 h at 37°C. Add biotin anti-digoxin antibody (1:100) and react for 30 min at 37°C, then add SABC (1:100) to react for 30 min at 37°C and colored with HRP-DAB reagent kit (Beijing Tiangen Biotech. Co. Ltd.). The TUNEL positive cells showed orange-brown nuclei under light microscope (Olympus CK2, Japan). Under high magnification (400 times), four non-overlapping visual fields in hippocampal area were randomly selected from each section to calculate the neuronal apoptosis index (NAI). NAI = the number of TUNEL-positive cells/the number of total cells in the field. Mean ± SD.

Immunohistochemical assay

The rabbit anti-rat STAT3 antibody, p-Tyr705-STAT3 antibody, Bcl-2 antibody and Fas antibody and immunohistochemical SP kit (Wuhan Boster Biotech. Co. Ltd.) were used to detect the expression in ischemic zone including ischemic penumbra and ischemic core zone. Referring to the instructions of kit, four sections form each group were de-waxed and hydrated to dispose 3% H_2O_2 for 10 min at room temperature. Added antibody to react for 2 h at 37°C and SP (1:100) to incubate at 37°C for 30 min, and colored with HRPDAB. The STAT3, p-Tyr705-STAT3, Bcl-2 and Fas positive cells appeared brown cytoplasm and measured by UV spectrophotometer (Beckmann USA) in four non-overlapping visual fields in each section. The protein expression intensity was presented by relative absorbance (A) index (RAI): the A value of positive cells minus the background A value, Mean ± SD.

Statistical analysis

Data analyses were performed using SPSS software 13.0 (SPSS Inc., Chicago, IL, USA). NDS values are expressed as the median (range) and were compared using Kruskal-Wallis tests. Other data are presented as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Student's t-tests were applied to determine differences between groups. A value of $P<0.05$ was

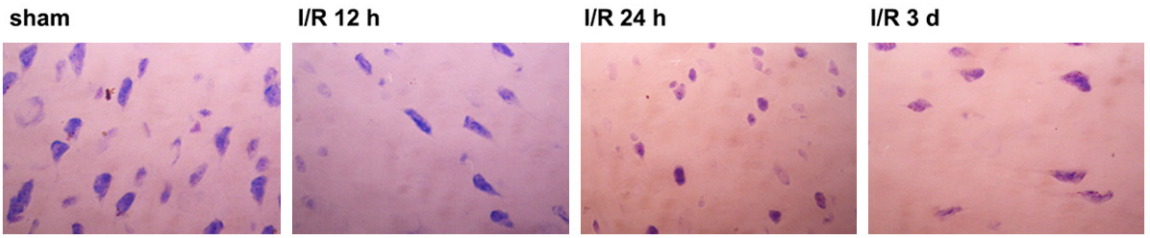

Figure 2. Nissl bodies in the ischemic zone in the 4 groups including sham group, I/R 12 h group, I/R 24 h group, I/R 3 d group (n=5). Nissl bodies are shown at ×400 magnification. In the rat cortex, Nissl bodies appear as large granular basophilic bodies in the neuronal cytoplasm and are composed of a rough endoplasmic reticulum. The damaged neurons were identified by the loss of Nissl substance.

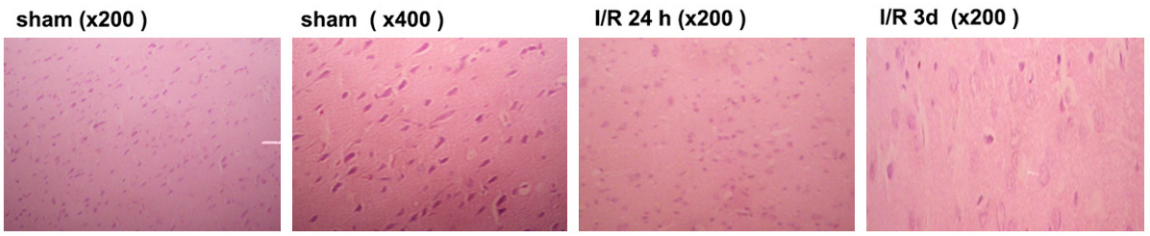

Figure 3. The cortex morphology assessed by hematoxylin and eosin (H&E) staining in the 4 groups including sham group, I/R 12 h group, I/R 24 h group, I/R 3 group (n=5). The neuronal morphology of the sham-operated (sham) group cortex was normal. The cellular morphology of the ischemic tissue in the I/R group was markedly worsen compared with sham group. There were a few neurons in the ischemic areas of the I/R groups.

considered to indicate a statistically significant difference.

Results

Cerebral ischemia/reperfusion induces cerebral infarct volume

The cerebral infarct areas determined by TTC staining are illustrated in **Figure 1**. There were no conspicuous cerebral infarcts area in the sham-operated group rats, while the cerebral infarct volumes of the I/R rats was 26.7±2.1. The cerebral infarct volumes were significantly increased in the IR group compared with the sham group ($P<0.05$).

IRI-induced neuronal loss

Nissl bodies were used as a morphological indicator of neuronal survival. The number of Nissl-stained neurons in the ischemic zone in I/R groups (24 h and 3 d) were significantly reduced compared with the sham-operated group ($P<0.05$); however, this decrease was less significant in the I/R (12 h) group compared with the marked decrease observed in the I/R group (24 h and 3 d) (**Figure 2**). The Nissl bodies in the ischemic core areas in the I/R groups were wiped out in vast numbers.

IRI induces neuronal morphological changes

Neuronal morphology was assessed by H&E staining (**Figure 3**). The number of neurons in the ischemic zone of the I/R group was reduced. The obvious characteristics of the neurons were: decreased cell size, nuclear pyknosis, interstitial edema, cell disorder and chromatin condensation.

IRI induces neuronal apoptosis

Apoptosis was determined by TUNEL staining (**Figure 4**), which revealed that there were few apoptotic neurons in the sham-operated group. In the I/R group, there were large numbers of apoptotic neurons in the ischemic area, and the amount of apoptosis cells reaches the peak at 3~6 h after ischemia/reperfusion. As shown in **Figure 5**, *neuronal apoptosis* was evidently increased in both the ischemic core and the ischemic penumbra region after 0.5 h of reperfusion, reached peak at 3 h reperfusion and thereafter decreased slightly.

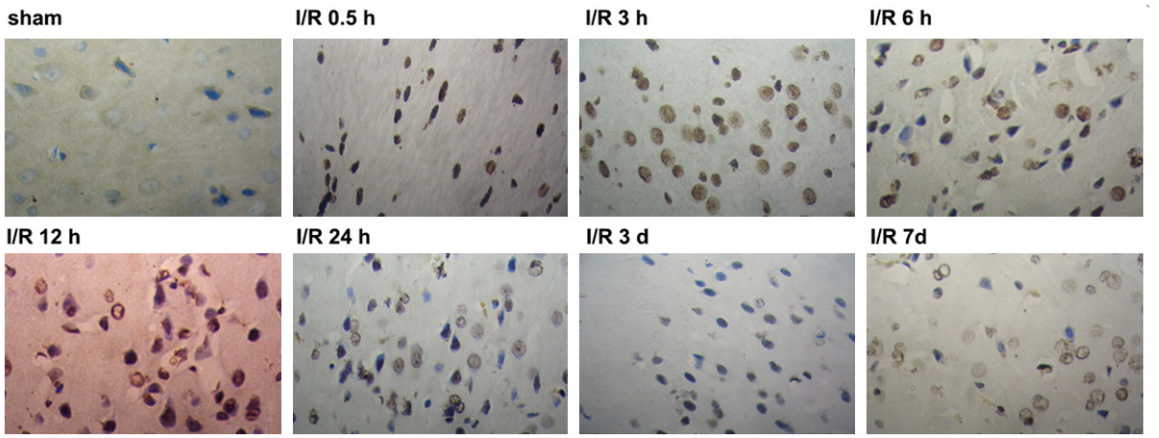

Figure 4. Ischemia reperfusion injury (IRI) induces neuronal apoptosis determined by TUNEL staining (n=5).

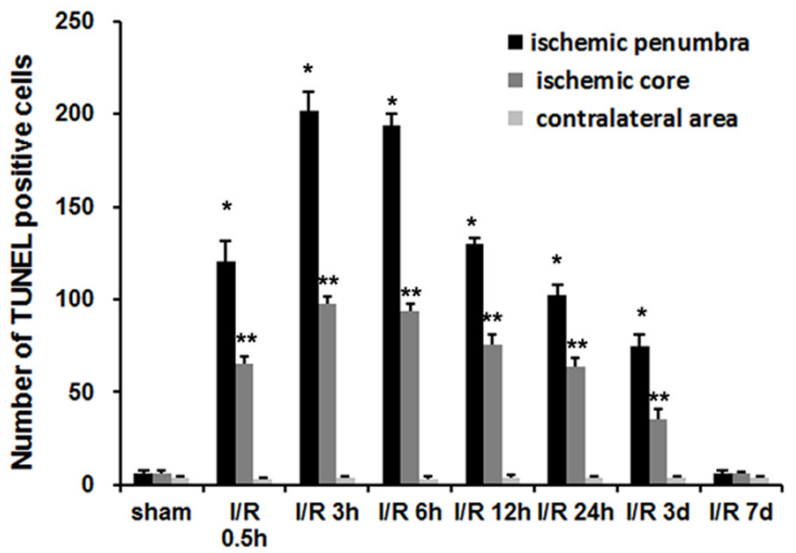

Figure 5. Ischemia reperfusion injury (IRI) induces neuronal apoptosis determined by TUNEL staining (n=5). The numbers of TUNEL positive cells were counted in ischemic core, the ischemic penumbra and contralateral region. *P<0.01 vs. sham group in ischemic penumbra. **P<0.01 vs. sham group in ischemic core.

STAT3 and p-STAT3 protein expression in the ischemic zone area

In the sham animals, no STAT3 was detected in any regions of the cerebral cortex (**Figure 6A**). STAT3 was noted in both the ischemic core and the penumbra region after 0.5 h of reperfusion, and peaked at 24 h reperfusion and thereafter decreased (**Figure 6C**). At each time point, the immunoreactivity tended to be more prominent in the cytoplasm than in the nucleus. In the contralateral cortex, a few of STAT3 was also detected in neurons during the reperfusion period.

Next, we detected the p-STAT3 expression (**Figure 6B**). In the sham animals, no phosphorylated STAT3 was detected in any regions of the cerebral cortex. Phosphorylated STAT3 was noted in both the ischemic core and the penumbra region after 0.5 h of reperfusion. The phosphorylated STAT3 expression reached peak in the ischemic penumbra region at 24 h reperfusion and thereafter decreased at 7 d reperfusion. At each time point, phosphorylated STAT3 was detected in both the cytoplasm and the nucleus. In the ischemic core, phosphorylated STAT3 expression in neurons peaked at 3 h and subsequently decreased (**Figure 6D**). In the contralateral cortex, phosphorylated STAT3 was also detected in neurons during the reperfusion period.

IRI-induced Bcl-2 and Fas expression in the ischemic zone

Consistent with the sham animals, a few Bcl-2 protein was detected in the cerebral cortex in I/R 0.5 h group (**Figure 7A**). Bcl-2 expression was subsequently increased and reached peak in both the ischemic core and the penumbra region after 6 h and 12 h of reperfusion respec-

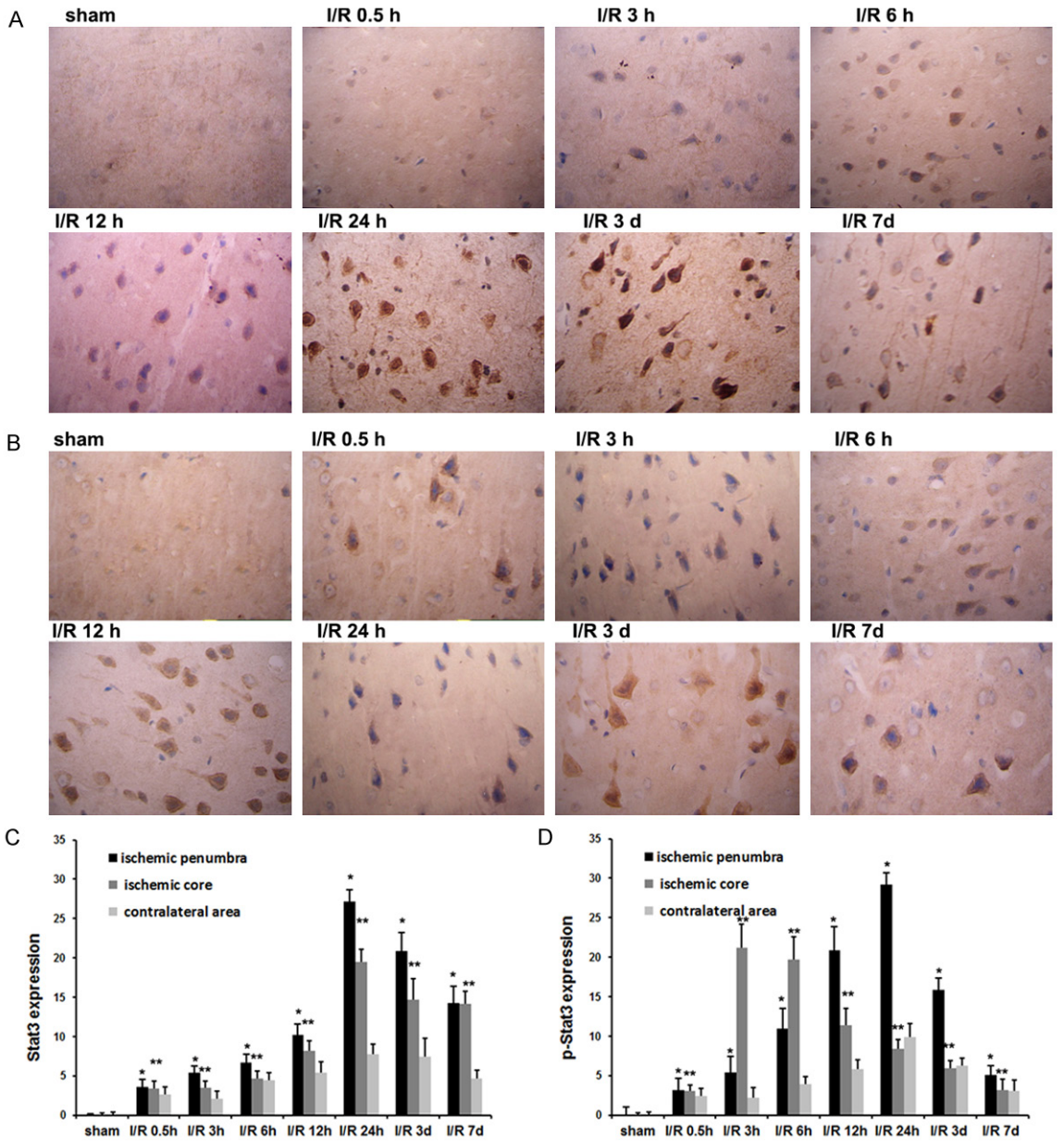

Figure 6. Temporal profiles showing the numbers of STAT3 and phospho-STAT3 immunopositive cells in three different regions of the cerebral cortex. (A) STAT3 and (B) p-STAT3 expression were detected by immunohistochemistry. The locations of the ischemic core, penumbra region, and the contralateral cortex are shown in (C and D). *P<0.01 vs. sham group in ischemic penumbra. **P<0.01 vs. sham group in ischemic core.

tively, and thereafter decreased. At each time point, the immunoreactivity tended to be more prominent in the cytoplasm than in the nucleus. In the contralateral cortex, a few of Bcl-2 was also detected in neurons during the reperfusion period.

Next, we detected the Fas expression (**Figure 7B**). In the sham animals, no Fas was detected in any regions of the cerebral cortex. Fas was noted in both the ischemic core and the penumbra region after 0.5 h of reperfusion. Fas expression reached peak in the ischemic penumbra region at 24 h reperfusion and thereafter decreased at 7 d reperfusion. At each time point, Fas tended to be more prominent in the cytoplasm than in the nucleus. In the ischemic core, Fas expression in neurons peaked at 12 h

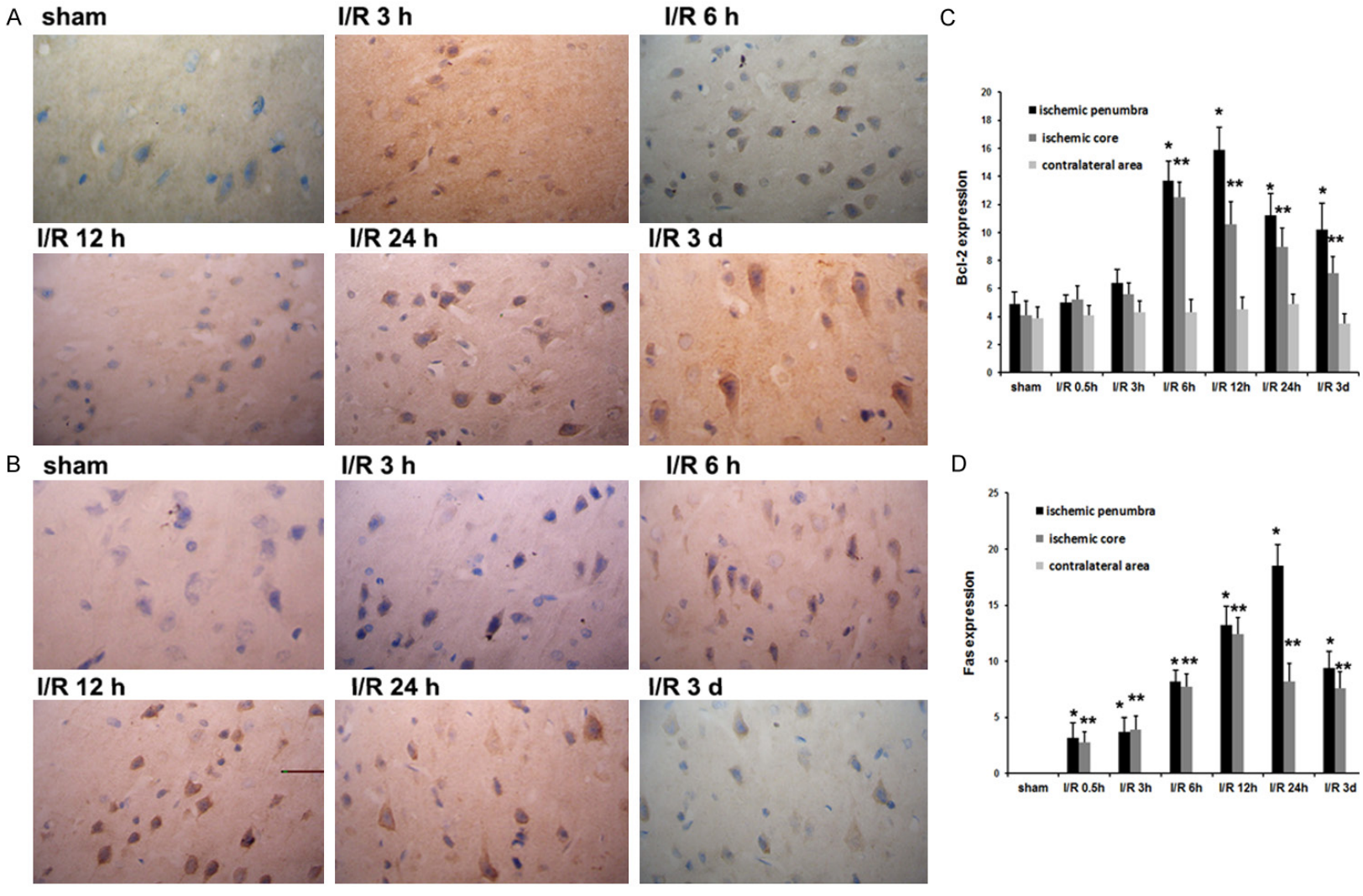

Figure 7. Temporal profiles showing the numbers of Bcl-2 and Fas immunopositive cells in three different regions of the cerebral cortex. (A) Bcl-2 and (B) Fas expression were detected by immunohistochemistry. The locations of the ischemic core, penumbra region, and the contralateral cortex are shown in (C and D). *P<0.01 vs. sham group in ischemic penumbra. **P<0.01 vs. sham group in ischemic core.

Table 1. Correlation between activation of STAT3 and neuronal apoptosis

	STAT3		p-STAT3		Bcl-2		Fas		Neuronal apoptosis	
r	IC	IP	IC	IP	IC	IP	IC	IP	IC	IP
STAT3									0.179	0.159
P									0.091	0.135
p-STAT3	0.445	0.385							0.445	0.886
P	0.000	0.000							0.000	0.000
Bcl-2	0.524	0.488	0.776	0.693					0.576	0.606
P	0.000	0.000	0.000	0.000					0.000	0.000
Fas	0.685	0.528	0.907	0.707	0.825	0.822			0.549	0.658
P	0.000	0.000	0.000	0.000	0.000	0.000			0.000	0.000

IC: ischemic core, IP: ischemic penumbra, r: Pearson correlation coefficient.

and subsequently decreased. In the contralateral cortex, no Fas was also detected in neurons during the reperfusion period.

Correlation between activation of STAT3 and neuronal apoptosis

As shown in **Table 1**, STAT3 is positive correlation with p-Tyr705-STAT3 in both ischemic core and ischemic penumbra, with the Pearson correlation coefficient 0.445 and 0.385 respectively. Moreover, STAT3 and p-Tyr705-STAT3 were positive correlation with Bcl-2 and Fas in both ischemic core and ischemic penumbra. However, p-Tyr705-STAT3, Bcl-2 and Fas were positive correlation with neuronal apoptosis, except STAT3. Overall, our finding indicates that the activation of STAT3 are positive associated with neuronal apoptosis in in focal cerebral ischemia/reperfusion rats.

Discussion

In this study, we used an in vivo model of MCAO-induced focal ischemia, which has been widely used to study the effects and mechanisms of focal cerebral ischemia. We used immunohistochemistry to detected expressions of STAT3, phosphorylation-tyrosine 705-STAT3, Bcl-2 and Fas, and TUNEL assay to detect the neuronal apoptosis, which elucidate the chronological, topographical and cellular alteration of STAT3 protein, Tyr705 p-STAT3 protein, and apoptosis-associated protein (Bcl 2 and Fas), reveal the correlation between activation of STAT3 and neuronal apoptosis in focal cerebral ischemia rat.

Phosphorylation of STAT3 at Tyr705 in response to gp130-stimulating cytokines leads to formation of STAT3 dimers, followed by translocation of these dimers to the nucleus, where they regulate transcription of target. In the present study, STAT3 was localized mainly in the cytoplasm neurons in the control animals. The wide distribution of STAT3 protein in normal rat neurons has been reported [10]. After 0.5 h reperfusion, STAT3 was detected in both the cytoplasm and the nuclei of most neurons, indicating that translocation of STAT3 occurred soon after cerebral ischemia as the initial step of activated signal transduction. STAT3 expression was slightly upregulated in both the ischemic core and the penumbra region and peaked at 24 h reperfusion and thereafter decreased. Recently, translocation of STAT3 into the nuclei of reactive microglia and astrocytes was reported to occur after transient cerebral ischemia, suggesting activation of the JAK/STAT pathway [9]. Phosphorylated STAT3 was noted in both the ischemic core and the penumbra region after 0.5 h of reperfusion. The phosphorylated STAT3 expression reached peak at 24 h and 3 h reperfusion in the ischemic penumbra region and ischemic core, respectively, and thereafter decreased at 7 d reperfusion. At each time point, phosphorylated STAT3 was detected in both the cytoplasm and the nucleus. These results indicated that activation of STAT3 was significant in neurons, with particular chronological features in each region.

Cerebral ischemia involves in a series complex of pathophysiological mechanisms, including excitotoxicity, oxygen free radical injury and inflammatory reaction [15]. These pathophysiological mechanisms are closely related to apoptosis. The purpose of apoptosis is to regulate organism development and keep homeostatic state [16, 17]. Growing evidence reveals that apoptosis is one of the major mechanisms that lead to the cell death and tissue injury after cerebral ischemia [18, 19]. In this study, apoptosis was determined by TUNEL staining, which revealed that there were few apoptotic neurons in the sham-operated group. Neuronal

apoptosis was evidently increased in both the ischemic core and the ischemic penumbra region after 0.5 h of reperfusion, reached peak at 3 h reperfusion. Bcl-2, an anti-apoptotic member from Bcl-2 family, is key regulators of apoptosis [20]. Recent studies of cerebral ischemia in rats reveal that dysregulation of the Bcl-2 family of proteins can exacerbate ischemic neuronal injury. Inhibition or deficiency of Bcl-2 family has been associated with reduced neurons to death induced by excitotoxic, metabolic and oxidative insults relevant to Alzheimer's disease, stroke and other disorders [21, 22]. In addition, it is well known that Fas is an important apoptosis-associated protein and apoptosis induced by Fas plays an important role in then triggered by its ligand or antibody crosslinking [23]. Recent studies showed that Fas involves neuronal apoptosis [24]. Liu et al. revealed that TFs from R. laevigata Michx fruit show good effects against H_2O_2-induced oxidative injury in PC12 cells by downregulating the expressions of Fas, FasL, caspase-3, caspase-9 and p53 [25]. In this study, we detected Bcl-2 and Fas expression in ischemic region. Bcl-2 expression was subsequently increased and reached peak in both the ischemic core and the penumbra region after 6 h and 12 h of reperfusion respectively, and thereafter decreased. In the sham animals, no Fas was detected in any regions of the cerebral cortex. Fas was noted in both the ischemic core and the penumbra region after 0.5 h of reperfusion. Fas expression reached peak at 24 h and 12 h reperfusion in the ischemic penumbra and core region respectively and thereafter decreased at 7 d reperfusion. Finally, correlative analysis showed that positive correlation with each other STAT3, p-Tyr705-STAT3, Bcl-2 and Fas in the ischemic zone ($P<0.01$). Moreover, p-Tyr705-STAT3, Bcl-2 and Fas were positive correlation with TUNEL positive cells ($P<0.01$), except STAT3.

In conclusion, these results suggest that activation of STAT3 is associated with neuronal apoptosis after cerebral ischemia and reperfusion by regulation of apoptosis-related protein Bcl-2 and Fas expression.

Disclosure of conflict of interest

None.

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