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## Inhibitory effect of genistein on activation of STAT3 induced by brain ischemia/reperfusion in rat hippocampus<sup>1</sup>

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**KEY WORDS** signal transduction; transcription factors; genistein; brain ischemia; tyrosine; phosphorylation; hippocampus

### ABSTRACT

**AIM:** To investigate the activation of signal transducer and activator of transcription-3 (STAT3) after brain ischemia/reperfusion (I/R) in rat hippocampus and effect of genistein on tyrosine phosphorylation and DNA binding activity of STAT3. **METHODS:** Four-vessel occlusion (4-VO) ischemia model of Sprague-Dawley (SD) rats was used in this study. The expression and activation of STAT3 were assessed by immunoblotting. Electrophoretic mobility shift assay (EMSA) was used to analyze DNA binding activity of STAT3. **RESULTS:** The phosphorylation level of STAT3 (p-STAT3) in cytoplasm increased after I/R 3 h and reached peak levels at 3 h and 72 h of reperfusion (1.7- and 2.5-fold *vs* sham), respectively. The protein level of STAT3 enhanced after 24 h of reperfusion and reached its peak at I/R 72 h (1.7-fold *vs* sham). The p-STAT3 in nucleus increased after ischemia and had also two peak levels at 3 h and 72 h of reperfusion (2.6- and 3.1-fold *vs* sham), respectively. Changes of STAT3 DNA binding activity showed a similar fashion to that of p-STAT3 in nuclear extracts (3.1-fold at I/R 3 h and 4.4-fold at I/R 72 h *vs* sham, respectively). Genistein prevented the increases of p-STAT3 and DNA binding activity at 72 h of reperfusion in a dose-dependent manner, but didn't change the expression of STAT3. **CONCLUSION:** I/R induces tyrosine phosphorylation and DNA binding activity of STAT3. Protein tyrosine kinase plays a crucial role in regulating the activation of STAT3 after I/R.

### INTRODUCTION

STAT3 (signal transducer and activator of transcription-3) was first identified as an acute-phase response factor (APRF) through its IL-6 signal pathway<sup>[1]</sup>, including three different nature isoforms, STAT3 $\alpha$  (92 kDa), STAT3 $\beta$  (83 kDa), STAT3 $\gamma$  (73 kDa). Upon

stimulation, STAT3 protein is phosphorylated on a specific tyrosine residue (Tyr705) by Janus kinase (JAK) or other tyrosine kinase. Activated STAT3 may associate as homodimers or heterodimers and translocate to the nucleus, then bind to special promoters, including the immediate early genes *c-fos* and *jun-B* promoter<sup>[2,3]</sup> in the regulation of gene expression involved in immune and stress response.

In the central nervous system, STAT3 is continuously expressed in neuron<sup>[4]</sup> and can be activated by a variety of pathologic stimuli, including oxidative stress<sup>[5]</sup>, ischemic insults<sup>[6,7]</sup>, and cytokines, *etc.* At present, little is known about changes in activation of STAT3 and DNA binding activity in rat hippocampus after brain

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ischemia, despite the finding that hippocampus is one of the most vulnerable brain regions to ischemic damages.

In this study, we examined the tyrosine phosphorylation and DNA binding activity of STAT3 at different time points of reperfusion after ischemia (I/R) in rat hippocampus of SD rats. To delineate the role of protein tyrosine kinase (PTK) in I/R-induced STAT3 activation *in vivo*, we attempt to determine whether genistein (a PTK inhibitor) could modulate the activation and DNA binding activity of STAT3 by immunoblotting (IB) and electrophoretic mobility shift assay (EMSA).

## MATERIALS AND METHODS

**Materials** Rabbit polyclonal STAT3 antibody and STAT3 probe were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal p-STAT3 antibody was from Cell Signalling Technology (New England Biolabs, England). Alkaline phosphatase conjugated goat anti-rabbit IgG and genistein was from Sigma (St Louis, MO, USA). Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and T4 polynucleotide kinase were from Promega (Madison, WI, USA). [ $\gamma$ - $^{32}$ P] ATP was from Yuhui Biological and Medical Engineering Co, Beijing.

**Animals and induction of ischemia** Adult male Sprague-Dawley (SD) rats (purchased from Sippr-BK Experimental Animal Ltd Co, Shanghai, Grade II, Certificate No D52) weighing 250-300 g were used. Transient brain ischemia was induced by four-vessel occlusion (4-VO) as described before<sup>[8]</sup>. Briefly, rats were anesthetized (chloral hydrate, 350 mg/kg, ip) and common carotid arteries were exposed and bilateral vertebral arteries were occluded permanently by electrocautery. On the next day, both carotid arteries were occluded with aneurysm clips for 15 min. During ischemia, animals were required to meet the following criteria: completely flat EEG, maintenance of dilated pupils, absence of a cornea reflex when exposed to strong light stimulation, and maintenance of rectal temperature at 37.0-37.5 °C. Animals not meeting these criteria were excluded. Sham control animals received the same surgical procedures except those carotid arteries were not occluded.

To study the time course of I/R-induced STAT3 activation, SD rats were occluded 15 min and sacrificed at 0, 3, 6, 12, 24, 48, 72, and 120 h of reperfusion,

respectively. To evaluate the effect of PTK on I/R-induced activation of STAT3, animals were given genistein (5, 10, 15, or 20 mg/kg, ip) by abdominal injection 20 min before ischemia and then subjected to 72 h of reperfusion following 15 min of ischemia. Control rats were ip injected dissolvent (Me<sub>2</sub>SO).

### Tissue preparation and nuclear extracts

Nuclear extracts of hippocampus were carried out and a modification of a previously described procedure<sup>[9]</sup>. Briefly, tissue samples were homogenized in ice cold Buffer A (pH 7.9, HEPES 10, NaF 50, DTT 1, Na<sub>3</sub>VO<sub>4</sub> 1 mmol/L and enzyme inhibitors: PMSF 0.5 mmol/L, aprotinin 10, leupeptin 10, and pepstatin A 10 mg/L). The homogenates were allowed to swell on ice-water for 10 min. Then the last concentration of 0.6 % solution of NP-40 was added, the tubes were vigorously vortexed for 30 s and centrifuged at 800×g, at 4 °C for 10 min. The supernatants containing the cytoplasmic fraction were collected and stored at -80 °C until use. The nuclei-containing pellets were washed three times with buffer A, then resuspended in ice-cold buffer B (pH 7.9, HEPES 20, NaCl 400, DTT 1, Na<sub>3</sub>VO<sub>4</sub> 1 mmol/L, 20 % glycerine, and enzyme inhibitors above). The tubes were vigorously rocked at 4 °C for 30 min on a shaking platform. After a centrifugation at 12000×g, at 4 °C for 15 min, the nuclear extracts were aliquoted and frozen in liquid nitrogen, then stored at -80 °C until use. Protein concentration was determined using Lowry method.

**Immunoblotting (IB)** IB was performed as described previously<sup>[8]</sup>. Briefly, samples were mixed with loading buffer and boiled for 5 min. Proteins (100 μg) were fractionated by 7.5 % SDS-PAGE and then electrotransferred onto nitrocellulose membrane. The membrane was probed with anti-STAT3 antibody (1:200) or polyclonal anti-p-STAT3 antibody (1:1000) and visualized using the image analyzer (LabWorks Software, UVP upland, CA).

### Electrophoretic mobility shift assays (EMSA)

A double-stranded 24-mer DNA probe: 5'-GAT CCT TCT GGG AAT TCC TAG ATC-3' containing the sis-inducible element (SIE) consensus sequence (in boldface) and the reverse complement 3'-CTA GGA AGA CCC TTA AGG ATC TAG-5' were labeled with [ $\gamma$ - $^{32}$ P] ATP by T4 polynucleotide kinase. For binding assays, 10 μg of proteins from hippocampal extracts and 1 μg of [poly (dI·dC)(dI·dC)] (Amersham Pharmacia) were incubated for 10 min prior to addition of labeled probe. Reaction was allowed to take place at

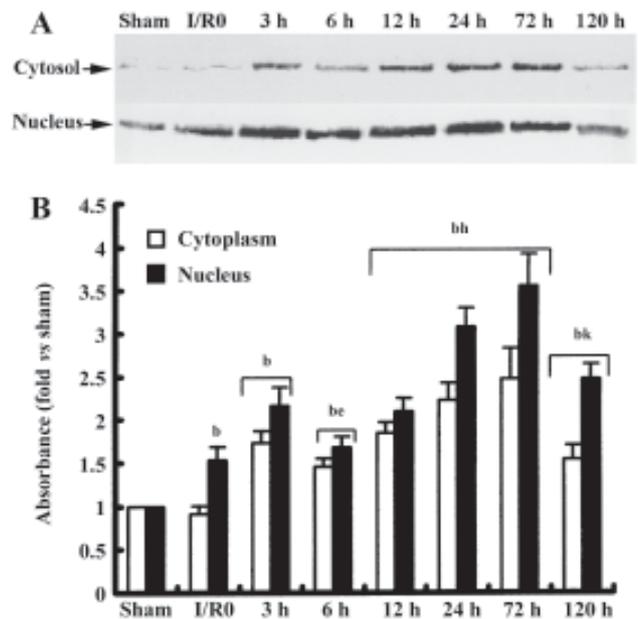
room temperature for 20 min. Samples were mixed with loading buffer and loaded on 4 % nondenaturing polyacrylamide gel in 0.5×Tris/borate/ethylene diamine tetraacetic acid (EDTA). Autoradiograms were developed by exposing vacuum-dried gels to x-ray film at -80 °C with intensifying screens for 20-48 h. Competition experiment was carried out using 100-fold excess non-radioactive STAT3 probe. Specificity control was carried out using 100-fold excess non-radioactively irrelevant probe that was specific for SP-1. Supershift of STAT3 was incubated with anti-STAT3 antibody.

**Statistical analysis** Values were expressed as mean±SD from three independent animals. Statistical software package (Stata, version 4.0) was used for data analysis. Statistical analysis of the results was carried out by one-way analysis of variance (ANOVA) followed by the Duncan's new multiple range method or Newman-Keuls test and  $P < 0.05$  was considered significant.

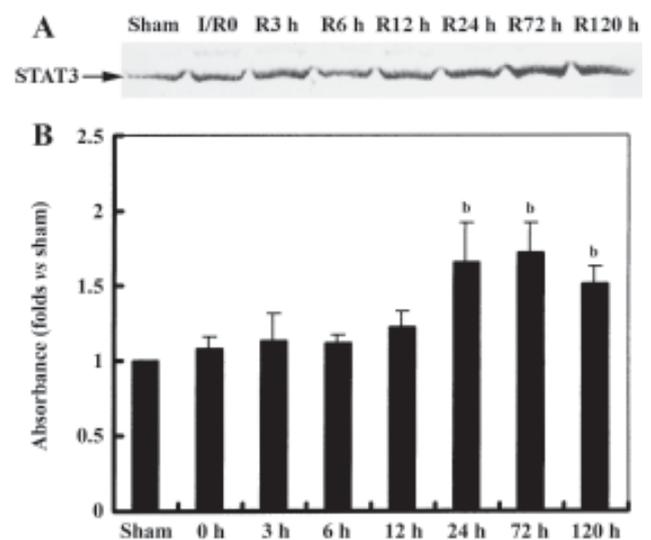
## RESULTS

**Time course of expression and tyrosine phosphorylation of STAT3 in hippocampus after I/R** Homogenates were prepared from sham and I/R rats that had 0, 3, 6, 12, 24, 72, and 120 h of reperfusion after ischemia. IB was performed with anti-STAT3 and anti-p-STAT3 antibodies to assess the protein and phosphorylation levels of STAT3. Phosphorylation levels of STAT3 in cytoplasm increased continuously after 3 h of reperfusion and reached two peaks at I/R 3 h (1.7-fold vs sham) and 72 h (2.5-fold vs sham), respectively (Fig 1). The content of STAT3 protein increased evidently at 24 h and peaked at 72 h of reperfusion (1.7-fold vs sham, Fig 2). Fig 1 also shows the time course of p-STAT3 in nuclear extracts, which increased significantly after 15 min of ischemia and had a similar rule to that of p-STAT3 in cytoplasm. It was shown 2.6-(I/R 3 h) and 3.6-(I/R 72 h) fold compared with the case in control rats, respectively.

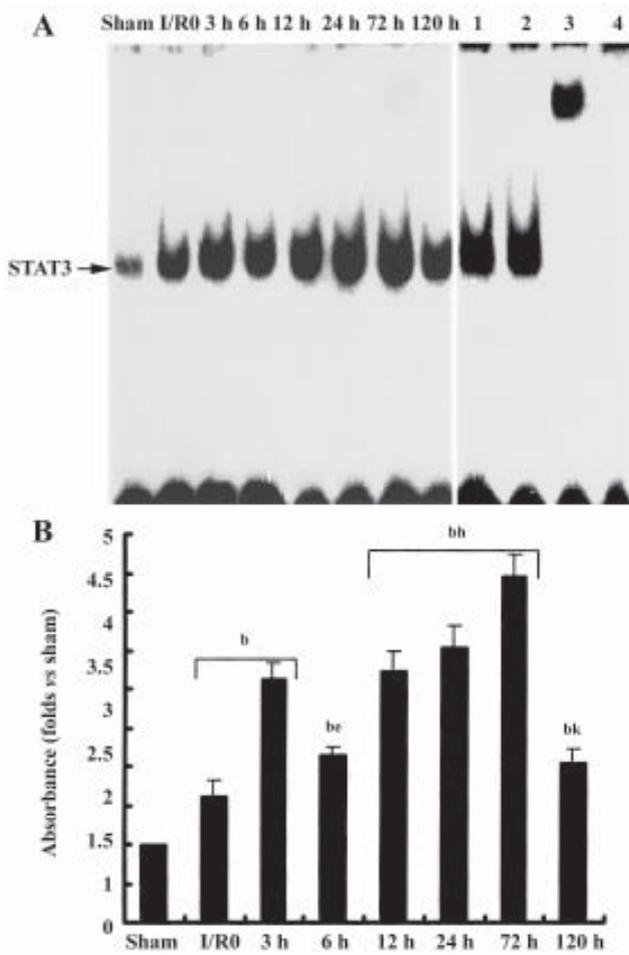
**Time course of DNA binding activity of STAT3 induced by I/R in rat hippocampus** EMSA was used to evaluate STAT3 DNA binding activity in nuclear extracts from rats subjected to various periods of reperfusion. The DNA binding activity of STAT3 increased from ischemia and reached two peaks at 3 h (3.1-fold vs sham) and 72 h (4.4-fold vs sham) of reperfusion, respectively (Fig 3).



**Fig 1.** Time course of alterations of STAT3 tyrosine phosphorylation in the cytoplasm and nuclear extracts after brain ischemia. (A): IB analysis of STAT3 phosphorylation level in cytoplasm and nucleus extracts with anti-p-STAT3 antibody. (B): Bands corresponding to p-STAT3 were scanned and the intensities were represented as fold vs sham control.  $n=3$  independent animals. Mean±SD. <sup>b</sup> $P < 0.05$  vs sham. <sup>c</sup> $P < 0.05$  vs I/R 3 h. <sup>e</sup> $P < 0.05$  vs I/R 6 h. <sup>k</sup> $P < 0.05$  vs I/R 72 h.

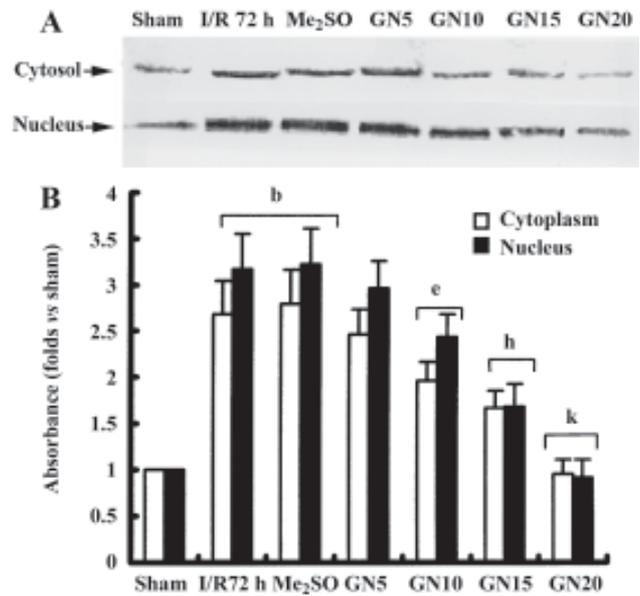


**Fig 2.** Time course of STAT3 expression in rat hippocampus after a 15-min ischemia. (A) IB analysis of cytoplasm protein level with anti-STAT3 antibody. (B) Bands corresponding to STAT3 were scanned and the intensities were represented as fold vs sham control.  $n=3$  independent animals. Mean±SD. <sup>b</sup> $P < 0.05$  vs sham.



**Fig 3.** Time course of changes of STAT3 DNA-binding activity in the nuclear extracts of hippocampus. (A): STAT3 DNA binding activity was assessed by EMSA analysis. Lane 1 shows normal STAT3 DNA binding reaction. Lane 2 is non-competitive reactions with nonrelated probe SP1. Lane 3 indicates supershift assay with anti-STAT3 antibody, and lane 4 specifies competitive reactions with unlabeled STAT3 probe. The retarded bands are indicated by an arrow. (B): Bands corresponding to STAT3 binding complex were scanned and the intensities were represented as fold vs sham control. *n*=3 independent animals. Mean±SD. <sup>b</sup>*P*<0.05 vs sham. <sup>c</sup>*P*<0.05 vs I/R 3h. <sup>b</sup>*P*<0.05 vs I/R 6 h. <sup>k</sup>*P*<0.05 vs I/R 72 h.

**Effects of genistein on tyrosine phosphorylation and DNA binding activity of STAT3 in hippocampus following I/R 72 h** To verify whether activation of STAT3 after I/R 72 h was associated with PTK, 4-VO rats were treated with the PTK inhibitor genistein (5, 10, 15, or 20 mg/kg, ip) or with an equal volume dissolvent (Me<sub>2</sub>SO). IB analysis of homogenates from cytoplasm and nuclear extracts in rat hippocampus was performed with p-STAT3 antibody. As shown in Fig 4, pretreatment with genistein inhibited the I/R-induced

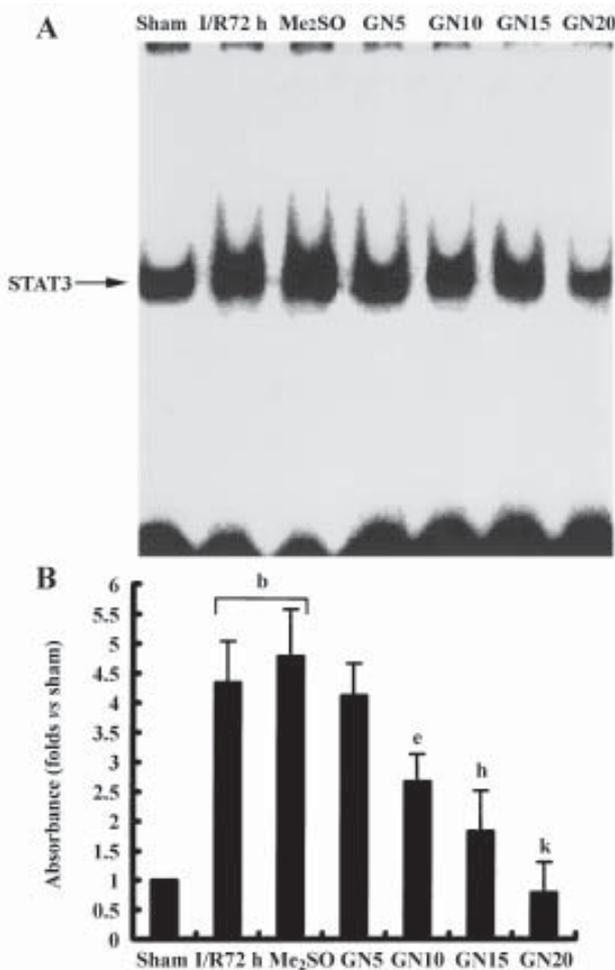


**Fig 4.** Inhibition of genistein on tyrosine phosphorylation of STAT3 in hippocampus after I/R 72 h. (A): IB analysis of p-STAT3 in cytoplasm and nuclear extracts with anti-p-STAT3 antibody. (B): Bands corresponding to p-STAT3 were scanned and the intensities were represented as fold vs sham control. *n*=3 independent animals. Mean±SD. <sup>b</sup>*P*<0.05 vs sham control. <sup>c</sup>*P*<0.05 vs I/R 72 h+Me<sub>2</sub>SO. <sup>h</sup>*P*<0.05 vs GN 5. <sup>k</sup>*P*<0.05 vs GN 10. GN 5, 10, 15, 20: genistein of 5, 10, 15, or 20 mg/kg was administered to the rats 20 min before ischemia and hippocampus was removed at 72 h of reperfusion. (GN: genistein)

increase of p-STAT3 in rat hippocampus after I/R 72 h in a dose-dependent manner, but it didn't change the content of STAT3 protein in cytoplasm at this time point (data not shown). Genistein also inhibited DNA binding activity of STAT3 in nucleus extracts (Fig 5). These data indicate that the observed activation of STAT3 *in vivo* may be regulated by PTK after brain I/R.

**DISCUSSION**

STAT3 is a unique protein possessing dual function in cytokine signaling. Much evidence has shown that STAT3 was activated when cytokines or growth factors bound to their cognate cell surface receptors. In the present study, we explore whether it was activated in rat hippocampus following I/R. The results indicate that brain I/R induced two-face activation of STAT3 during the process of reperfusion after ischemia. The first peak at 3 h of reperfusion suggests that ischemic brain injury induces a rapid activation of p-STAT3 protein in the ischemic area neurons and



**Fig 5.** Effects of genistein on I/R-induced increase of STAT3 DNA-binding activity in rat hippocampus. (A): EMSA analysis of STAT3 DNA binding activity in nuclear extracts from rats treated with genistein or vehicle. The retarded bands are indicated by an arrow. (B): Bands corresponding to STAT3 binding complex were scanned and the intensities were represented as fold vs sham control.  $n=3$  independent animals. Mean $\pm$ SD. <sup>b</sup> $P<0.05$  vs sham control. <sup>e</sup> $P<0.05$  vs I/R 72 h+Me<sub>2</sub>SO. <sup>h</sup> $P<0.05$  vs GN5. <sup>k</sup> $P<0.05$  vs GN10. GN 5, 10, 15, 20: genistein of 5, 10, 15, or 20 mg/kg was administered to the rats 20 min before ischemia and hippocampus was removed at 72 h of reperfusion. (GN: genistein)

p-STAT3 may be involved in the cytokine regulation of immediate early gene<sup>[3,6,10,11]</sup>. In sham group, a certain amount of p-STAT3 and DNA binding activity were found contributing to maintain the basal biological function<sup>[12]</sup>. Our findings also show that they all reached their second peak at 72 h of reperfusion, and in this time point the content of STAT3 protein increased significantly. These are consistent with previous reports that STAT3 is highly expressed in large reactive astrocytes and abundant reactive microglia macrophages in the areas undergoing neural cell death from 4-d

postischemia<sup>[6,13]</sup>. The increase of STAT3 expression may contribute to the enhancement of p-STAT3 and DNA binding activity. Whether the activation of STAT3 and increased DNA-binding activity in neurons and reactive astrocytes at distinct time points in process of I/R would play different roles in ischemia-induced injury will be studied in our later work.

When rapid changes in protein activity in response to stimuli are required, phosphorylation is an important fashion for regulating these activities. Previous reports had shown that genistein can inhibit tyrosine phosphorylation of *N*-methyl-*D*-aspartate (NMDA) receptor subunit 2B (NR2B)<sup>[8]</sup> and mitogen-activated protein (MAP)-2 kinase<sup>[14]</sup> in hippocampus following I/R. In this report, we determined its effect on activation of STAT3 following brain I/R 72 h. The findings show that genistein inhibited markedly the tyrosine phosphorylation and DNA-binding activity of STAT3 in a dose-dependent manner. These results could be explained that genistein prevents the upstream tyrosine kinase of STAT3, such as JAKs, Src or inhibits directly the tyrosine phosphorylation of STAT3<sup>[5]</sup>. The study suggests that genistein may serve as a new tool to study the precise role of STAT3 activation after brain I/R and it might be a novel method for therapy of ischemia-induced brain damage.

Recently, a multitude of studies about aberrant activation of STAT3 in the pathophysiological condition have been reported. However, the actual biological effect seems to depend on the particular factors considered and involved in diseases. Indeed, ischemic brain injury is a complicated pathological process. p-STAT3-positive showed severe signs of necrosis in the cerebral ischemic area by electron micrographs<sup>[10]</sup>. But the precise biological role of ischemia-induced STAT3 pathway activation remains unknown. It poses a new field to lead us to continue investigation.

Taken together, we demonstrated firstly that brain I/R induced the increase of p-STAT3 and DNA binding activity in rat hippocampus. PTK may participate in the regulating the activation of STAT3 after I/R.

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