Diffusion-weighted magnetic resonance imaging reflects activation of signal transducer and activator of transcription 3 during focal cerebral ischemia/reperfusion
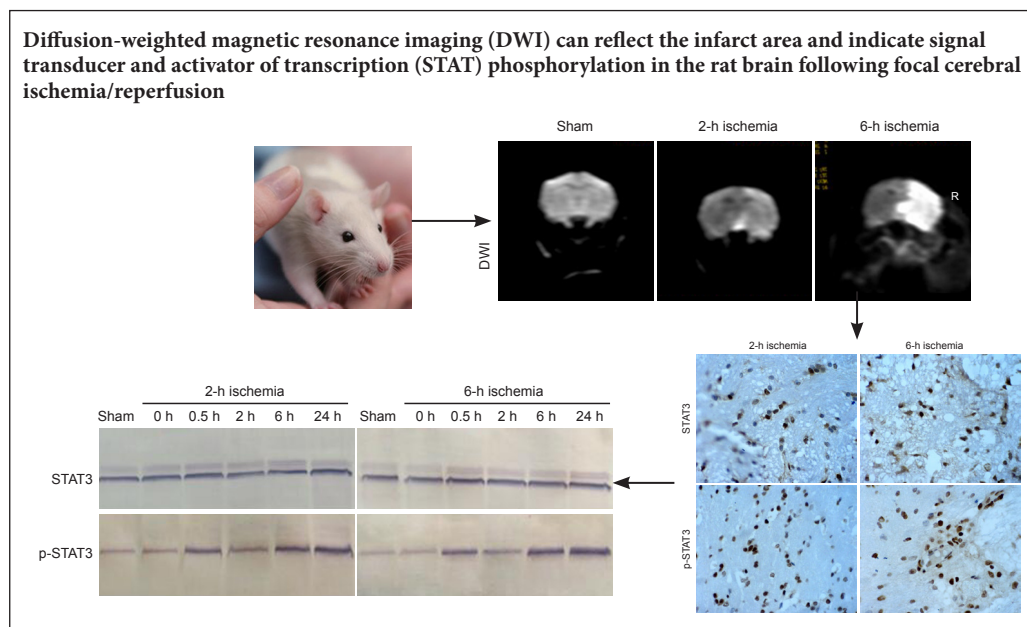
Wen-juan Wu[1,2], Chun-juan Jiang[1], Zhui-yang Zhang[1], Kai Xu[2], Wei Li[1,*]

1 Department of Radiology, Nanjing Medical Unversity Affiliated Wuxi Second People's Hospital, Wuxi, Jiangsu Province, China
2 Department of Radiology, Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu Province, China

Graphical Abstract

Diffusion-weighted magnetic resonance imaging (DWI) can reflect the infarct area and indicate signal transducer and activator of transcription (STAT) phosphorylation in the rat brain following focal cerebral ischemia/reperfusion

Correspondence to:
Wei Li, 409631600@qq.com.

orcid:
0000-0002-3808-4399
(Wei Li)

Abstract

Signal transducer and activator of transcription (STAT) is a unique protein family that binds to DNA, coupled with tyrosine phosphorylation signaling pathways, acting as a transcriptional regulator to mediate a variety of biological effects. Cerebral ischemia and reperfusion can activate STATs signaling pathway, but no studies have confirmed whether STAT activation can be verified by diffusion-weighted magnetic resonance imaging (DWI) in rats after cerebral ischemia/reperfusion. Here, we established a rat model of focal cerebral ischemia injury using the modified Longa method. DWI revealed hyperintensity in parts of the left hemisphere before reperfusion and a low apparent diffusion coefficient. STAT3 protein expression showed no significant change after reperfusion, but phosphorylated STAT3 expression began to increase after 30 minutes of reperfusion and peaked at 24 hours. Pearson correlation analysis showed that STAT3 activation was correlated positively with the relative apparent diffusion coefficient and negatively with the DWI abnormal signal area. These results indicate that DWI is a reliable representation of the infarct area and reflects STAT phosphorylation in rat brain following focal cerebral ischemia/reperfusion.

Key Words: nerve regeneration; cerebral ischemia/reperfusion; magnetic resonance imaging; diffusion weighted imaging; signal transducer and activator of transcription 3; phosphorylated signal transducer and activator of transcription 3; apparent diffusion coefficient; relative apparent diffusion coefficient; immunohistochemistry; western blot assay; neural regeneration

Introduction

Cerebral ischemia/reperfusion injury is an important pathophysiological process that underlies cerebrovascular disease. Magnetic resonance imaging (MRI) can reveal ischemic brain tissue. In hyperacute cerebral infarction (< 6 hours), the infarcted area can be seen in diffusion-weighted MRI (DWI), and magnetic resonance perfusion imaging can show the location and extent of the ischemic zone at around 10 minutes (Beck et al., 2014).

Cerebral ischemia/reperfusion can activate signal transducers and activators of transcription (STATs) (Li et al., 2015b). The Janus kinase (JAK)-STAT pathway is activated after cerebral ischemia. Membrane receptor signaling by various ligands induces activation of JAK kinases, which then leads to tyrosine phosphorylation of various STAT transcription factors (Kim et al., 2017). STAT1 and STAT3 are members of the STAT family, and phosphorylated (p-) STAT3 is the activated form of STAT3 (Jia et al., 2017). These proteins play an important role in neuronal survival and antiapoptosis. p-STAT3 is a mediator of growth factors, hormones and cytokines, and exerts its protective and regenerative effects in cerebral ischemia/reperfusion partly through transcriptional upregulation of neuroprotective and neurotrophic genes (Jiang et al., 2012). In the present study, we analyzed the changes in DWI, STAT3 and p-STAT3 in the ischemic injury zone in a rat model of focal cerebral ischemia/reperfusion injury.

Materials and Methods

Animals

A total of 110 healthy male Sprague-Dawley rats, 45–60 days old and weighing 290–330 g, were provided by the Animal Center of Xuzhou Medical University, Jiangsu Province, China. The rats were randomized into three groups: sham (n = 10), 2-hour ischemia (n = 50), and 6-hour ischemia (n = 50). Rats in the ischemia groups underwent 2- or 6-hour ischemia followed by reperfusion for 0, 0.5, 2, 6, or 24 hours (n = 10 rats per time point). All rats were housed under diurnal lighting and had free access to food and water before the experiments. The protocols were approval by the Committee on Animal Experimental Guidelines of the Affiliated Hospital of Xuzhou Medical University (XZMU-A201204-057R).

Focal cerebral ischemia injury modeling

A rat model of unilateral middle cerebral artery occlusion was established using the modified Longa method (Longa et al., 1989). Rats were anesthetized intraperitoneally with 10% chloral hydrate (3 mL/kg). The left common, external, and internal carotid arteries were exposed *via* an incision in the neck and separated under a surgical microscope. The external carotid artery was ligated 0.8–1.0 cm from the common carotid artery, and the internal carotid artery was occluded. A 3-0 surgical monofilament nylon suture, blunted at the end, was gently inserted into the internal carotid artery through a small incision at the bifurcation. When the thread was extended 17–19 mm from the bifurcation and a slight resistance was felt, this indicated that the thread had been inserted into the origin of the middle cerebral artery at the circle of Willis, blocking blood flow in the middle cerebral artery trunk. The thread was ligated with a slipknot in the internal carotid artery, and the incision was sutured. The rats were returned to their cages with food and water and allowed to recover. Body temperature was maintained near 37°C using a heat pad. After 2 or 6 hours, the thread was withdrawn by approximately 10 mm to begin reperfusion. For the sham group, the procedure was identical except the thread was only inserted to a depth of 5 mm.

MRI scan

3.0 T MRI examination (Signa HD 3.0, GE Healthcare, Chicago, IL, USA) was performed at various time points after ischemia/perfusion. A rat coil (Chenguang Medical Technology Co., Ltd., Shanghai, China) was used as the receiver coil. The rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate. Their heads were then placed in the center of the coil in the prone position. Echo planar imaging was used with the following parameters: repetition time, 6,800 ms; echo time, 93 ms; field of view, 8 cm × 6 cm; matrix, 64 × 64; number of excitations, 2; thickness, 2.4 mm; slice gap, 0.2 mm. After the scan, DWI data were transmitted to the workstation for postprocessing to obtain apparent diffusion coefficient (ADC) profiles. The relative ADC (rADC) for the abnormal signal area on a slice with marked ischemia in the region of interest (ROI) was calculated as follows: $rADC = ADC_{ROI} / ADC_{contralateral} \times 100\%$. The ratio of the DWI abnormal signal area (rS-DWI) on the selected slice (with marked ischemia) to that in the whole-brain slice area was calculated. Brains were removed after scanning.

Immunohistochemistry

After MRI, the whole brain from five rats in each group was removed immediately en bloc and postfixed for 24 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS). The tissue was then frozen, and 12-μm consecutive coronal sections were prepared. Microwave antigen retrieval was performed in citrate buffer (pH 6.0), and the sections were then incubated in 3% hydrogen peroxide at 37°C to block endogenous peroxidase activity. Normal goat serum was added dropwise to block nonspecific binding, and STAT3 (1:100) and p-STAT3 (1:100) rabbit polyclonal antibodies (both from Cell Signaling Technology, Danvers, MA, USA) were added and incubated at 4°C overnight. Biotin-labeled goat anti-rabbit IgG (1:30; Yu Bo Biological Technology, Shanghai, China) was then added dropwise at 37°C for incubation for 25 minutes, followed by dropwise addition of horseradish peroxidase-labeled streptavidin working solution. Diaminobenzidine was added for color development, and hematoxylin was applied as a counterstain. PBS (0.01 M) was added instead of primary antibody for the negative control sections. Cells with a brown-stained nucleus or cytoplasm under a light microscope (Olympus, Tokyo, Japan) were considered positive.
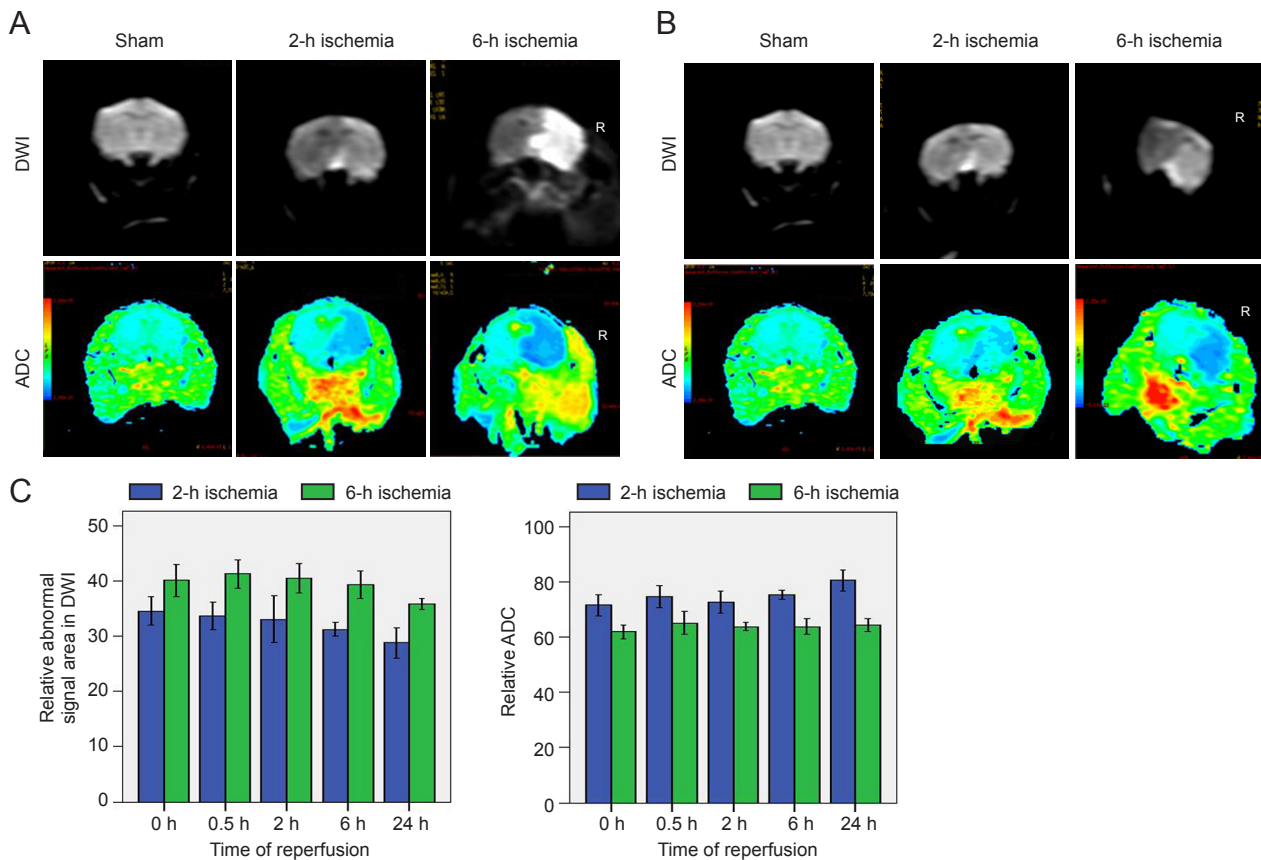
Figure 1 Brain diffusion-weighted magnetic resonance imaging (DWI) and apparent diffusion coefficient (ADC) in rat models of focal cerebral ischemia/reperfusion injury.

(A) Before reperfusion. In sham-operated animals, DWI findings were as expected and ADC values were similar across both cerebral hemispheres. In animals that underwent ischemia for 2 or 6 h, DWI showed patchy hyperintensity in the right hemisphere, and ADC pseudocolor images showed an abnormal blue signal with blurred edges. (B) After 24 h of reperfusion, the relative abnormal signal area was smaller in the 2- and 6-h ischemia groups than before reperfusion. (C) DWI relative abnormal signal area (%). (D) Relative ADC in region of interest (%). Data are expressed as the mean ± SD. *$P < 0.05$, *vs*. 0 h (before reperfusion) (one-way analysis of variance). h: Hours.
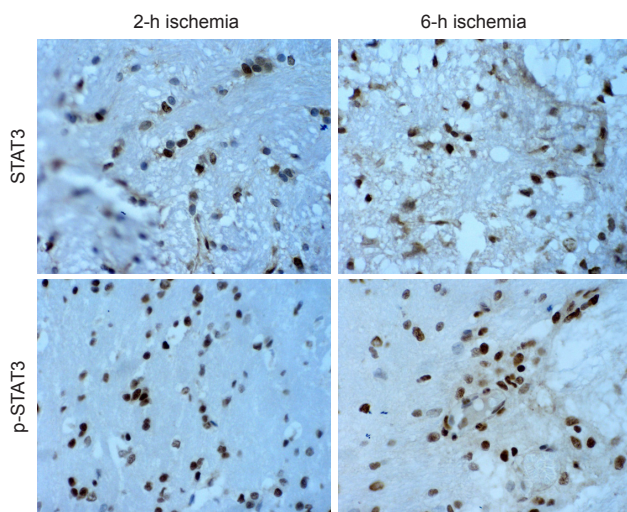

Figure 2 Immunohistochemical staining of STAT3 and p-STAT3 in the brain 24 h after reperfusion (× 400).

STAT3-positive cells were round or oval. The yellow-brown immunoreactive products were distributed in the cytoplasm. p-STAT3-positive cells were elliptical, and mainly astrocytes and neurons. The yellow-brown immunoreactive products were mainly distributed in the nucleus. h: Hours; STAT3: signal transducer and activator of transcription 3; p-STAT3: phosphorylated-STAT3.

Western blot assay

After MRI, five rats in each group were decapitated and their brains quickly removed. The ROI on the slice with the largest ischemic area revealed by MRI was rapidly separated. Nuclear proteins were extracted, and equal amounts of protein sample were separated by 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis, then wet-transferred onto nitrocellulose membranes. The membranes were then blocked with 3% bovine serum albumin, incubated overnight at 4°C with diluted primary antibody (rabbit anti-STAT3 or rabbit anti-p-STAT3, both 1:100; Cell Signaling Technology) with freshly prepared PBS containing 3% skim milk powder, and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:30; Yu Bo Biological Technology). Nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) was used for color development. Bands were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Relative optical density was calculated as: optical density of ischemic ROI/optical density of equivalent region in the sham group.

Statistical analysis

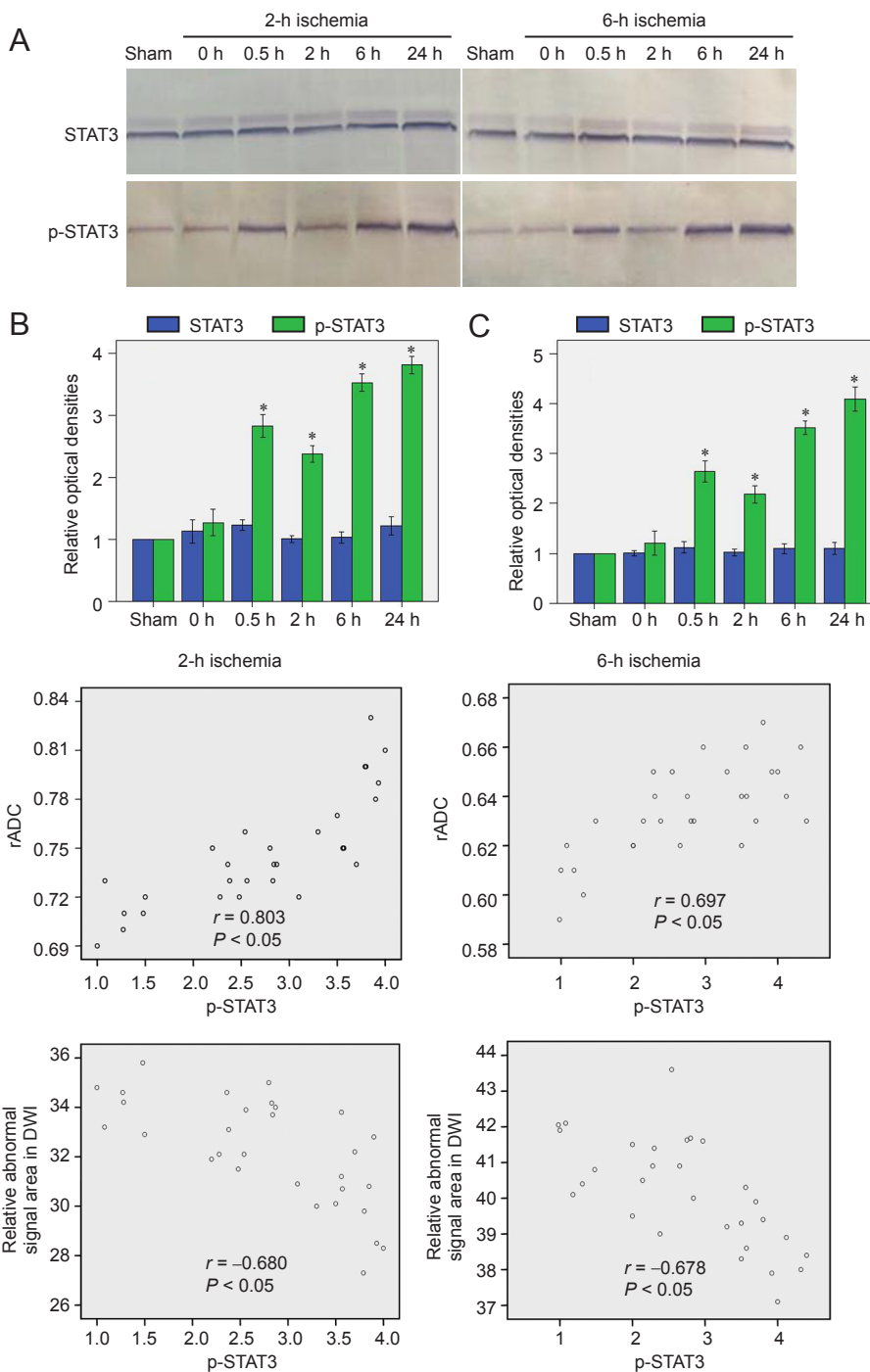SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA) was

Figure 3 Changes in STAT3 expression and activation in the brain after acute cerebral ischemia/reperfusion.
(A) Western blot assay of STAT3 and p-STAT3. (B, C) Relative optical densities of STAT3 and p-STAT3 in the 2-h (B) and 6-h (C) ischemia groups ($n = 10$). (C) Relative optical densities of STAT3 and p-STAT3 in the 6-h ischemia group ($n = 10$). Data are expressed as the mean ± SD. *$P < 0.05$, *vs.* 0 h (before reperfusion) (one-way analysis of variance). h: Hour(s); STAT3: signal transducer and activator of transcription 3; p-STAT3: phosphorylated-STAT3.

Figure 4 Correlation of phosphorylated signal transducer and activator of transcription 3 (p-STAT3) with relative apparent diffusion coefficient (rADC) and relative abnormal signal area in diffusion-weighted magnetic resonance imaging at different reperfusion time points.
p-STAT3 expression (expressed as relative optical density) showed a positive correlation with rADC and a negative correlation with relative abnormal signal area in diffusion-weighted magnetic resonance imaging. h: Hours.

used for statistical analysis. Experimental data are expressed as the mean ± SD. One-way analysis of variance was used to compare DWI and ADC before and after cerebral ischemia/reperfusion, and STAT3 and p-STAT3 expression. The relationship between p-STAT3 expression and rADC or rS-DWI was analyzed by Pearson correlation analysis. $P < 0.05$ was considered statistically significant.

Results
DWI in rat brain before and after acute cerebral ischemia/reperfusion
In the sham group, DWI findings were as expected, and ADC values were similar across both cerebral hemispheres. In both ischemia groups, before reperfusion, DWI showed patchy hyperintensity in the right corpus striatum and frontoparietal cortex, and ADC pseudocolor images showed an abnormal blue signal with blurred edges. The 6-hour ischemia group had larger rS-DWI and smaller ADC values than the 2-hour ischemia group (**Figure 1**). In the 2-hour ischemia group, after 24 hours of reperfusion, the rS-DWI was significantly smaller than before reperfusion, and the ADC was partially restored. After 24 hours of reperfusion in the 6-hour group, the rS-DWI was slightly lower than before reperfusion, and the ADC value was slightly greater (**Figure 1**).

Changes in STAT3 expression and activation after acute cerebral ischemia/reperfusion

Immunohistochemistry showed that p-STAT3-positive cells were rarely expressed in brain tissue from sham-operated rats, but in rats with ischemia, expression in the ischemic area increased with reperfusion time. The positive cells were circular or oval, and mainly astrocytes, followed in number by neurons. After 24 hours of reperfusion, there was neural nuclear condensation, cell body shrinkage and deformation, and larger astrocytes with abundant cytoplasm (**Figure 2**).

In the western blot assay, a low level of p-STAT3 was detected in brain tissue from sham-operated rats. In rats with ischemia, expression increased significantly after 0.5 hours of reperfusion, decreased slightly after 2 hours, and peaked at 24 hours (**Figure 3**).

Correlation between p-STAT3 expression and DWI

Data from different time points after reperfusion showed that p-STAT3 expression was positively correlated with rADC (2-hour ischemia group: $r = 0.803$, $P < 0.05$; 6-hour ischemia group: $r = 0.697$, $P < 0.05$) and negatively correlated with the rS-DWI (2-hour ischemia group: $r = -0.680$, $P < 0.05$; 6-hour ischemia group: $r = -0.678$, $P < 0.05$) (**Figure 4**).

Discussion

The JAK-STAT pathway has been clearly described in recent years (Jiang et al., 2013). As a family of cytoplasmic proteins capable of binding to DNA in the regulatory region of target genes, STATs are important downstream targets of JAKs (Liu et al., 2014). After binding to the corresponding ligand, cytokine receptors on the cell membrane induce mutual phosphorylation of JAK in the cell body. Cytoplasmic STAT has an SH2 domain, by which STAT is translocated to the specific tyrosine phosphorylation site of the receptor complex and becomes activated. Activated STAT separates from the receptor to form homo- or heterodimers and translocates to the nucleus where it initiates gene transcription (Yang et al., 2010). STAT3 is in a class of DNA-binding proteins involved in regulating various physiological functions, such as cell growth, malignant transformation, and apoptosis. Hoffmann et al. (2015) reported that STAT3 nuclear translocation in astrocytes in the cerebral cortex induced a variety of changes in glial cells after ischemia. In the present study, immunohistochemistry confirmed that non-activated STAT3 was expressed in the cytoplasm. p-STAT3-positive cells were rarely expressed in sham-operated rat brain tissue, but increased with longer ischemia/reperfusion times. Most immunoreactive products were observed in the nuclei of astrocytes, followed in number by neurons. This finding is likely associated with the mechanism by which activated STAT3 mediates gene transcription by forming homo- or heterodimers and translocating to the nucleus. However, the reason for high STAT3 protein expression and activation after cerebral ischemia remains unclear. It may be related to the rapid synthesis and expression induction of interleukin-6 and its receptors by neuronal and glial cells during cerebral ischemia.

Semi-quantitative western blot assay revealed that STAT3 protein expression did not change significantly with different reperfusion periods following ischemia for 2 or 6 hours. By contrast, p-STAT3 expression increased gradually, whereas it was barely detectable in the sham group. The increase in p-STAT3 levels after 0.5 hours of reperfusion may be related to the involvement of STAT3, c-Fos, and c-Jun in the transcriptional regulation of immediate early genes in neurons (Amantea et al., 2011). The significant increase in p-STAT3 levels after 24 hours of reperfusion may be related to ATP depletion during ischemia and significantly increased ATP levels after reperfusion; conversely, a marked proliferation of reactive glial cells and microglia was induced by ischemic brain damage, and increased cytokines and growth factors were released as reperfusion continued.

The basic pathology of the rS-DWI is that Na^+/K^+–ATP enzyme pump function is reduced due to ischemia and hypoxia, which leads to sodium retention and consequent cytotoxic edema, resulting in slowed molecular diffusion; this is demonstrated by the low ADC and DWI hyperintensity (Kim et al., 2006; Cereda et al., 2015; Lago et al., 2015; Song et al., 2015; Aoki et al., 2016; Freitag et al., 2016; Grams et al., 2016; Jiang et al., 2016; Kaseka et al., 2016; Kohno et al., 2016; Kvistad et al., 2016; Onofrj et al., 2016; Tamura et al., 2016; Xin and Han, 2016; Zhang et al., 2016; Zhou et al., 2016; Abdelgawad et al., 2017; Bekiesinska-Figatowska et al., 2017; Heiss and Zaro Weber, 2017). The decrease in ADC was highly consistent with the ATP-labeled defect area and decreased tissue pH area; and the decrease in ADC in ischemic brain damage was consistent with the level of cytotoxic edema caused by cell energy metabolism disorders (Anticoli et al., 2015; Baron et al., 2015; Brown et al., 2015; Eom et al., 2015; Gory et al., 2015; Kate et al., 2015; Landais, 2015; Li et al., 2015a; Makin et al., 2015; Mawet et al., 2015; Michałowska et al., 2015; Odland et al., 2015; Ostwaldt et al., 2015; Sasai et al., 2015; Yaghi et al., 2015). As infarction time increases (> 24 hours), there are corresponding increases in vasogenic edema, extracellular space water, diffusion speed, and ADC values (Maruyama et al., 2015).

As the JAK-STAT signaling pathway is activated during cerebral ischemia, JAK1 expression in cortical pyramidal neurons and striatal cells increases, and STAT3 nuclear translocation also increases, in rats with focal cerebral ischemia/reperfusion injury; this results in extensive proliferation of reactive microglia and macrophages (Li and Zhang, 2003; Zechariah et al., 2010; Jiang et al., 2013; Feng et al., 2015; Jung et al., 2015; Deng et al., 2016). Previous studies have demonstrated that the gp130-STAT signaling pathway could be activated by the nuclear translocation of STAT3, and that this activation in astrocytes correlates closely with gp130 expression (Jang et al., 2014; Song et al., 2014; Xu et al., 2015; Zhang et al., 2015; Guo et al., 2016). Selection of the samples used in the present experiment was based on MRI findings. STAT3 activation levels before and after ischemia/reperfusion correlated with the rS-DWI in the ischemic region and rADC. This finding indicated that with aggravation of cytotoxic and vasogenic edema, the

status of some membrane channels changed, which affected certain cell signaling pathways and STAT3 activation. This correlation helps to identify biochemical changes of JAK-STAT signaling in brain tissue after ischemia/reperfusion. Further research is needed to determine whether blocking STAT3 phosphorylation could prevent neuronal necrosis or apoptosis due to ischemia to achieve neuroprotective effects and minimize ischemia/reperfusion injury, and whether it could be reflected in MRI. This correlation also provides a theoretical and experimental basis for the clinical treatment of cerebral ischemia.

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