

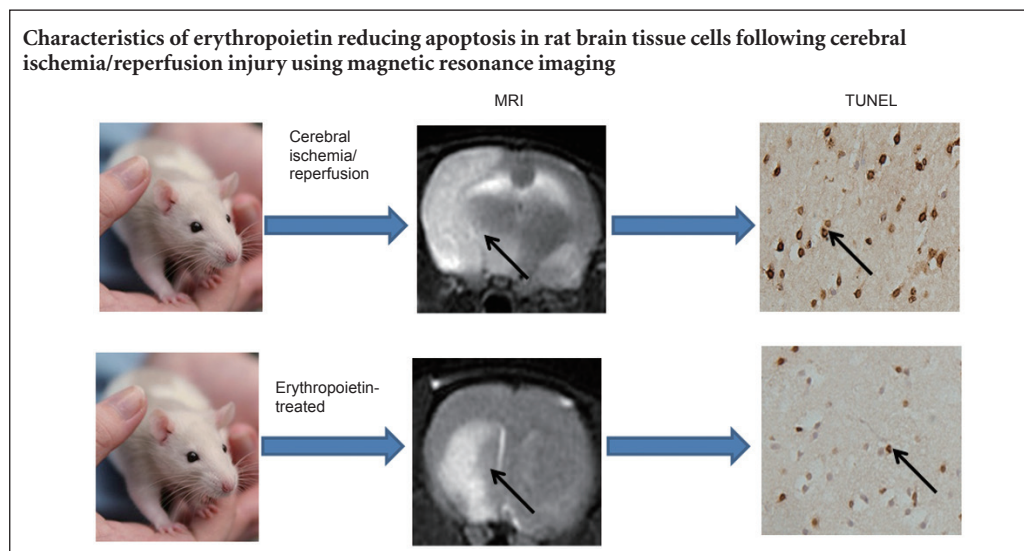
Erythropoietin reduces apoptosis of brain tissue cells in rats after cerebral ischemia/reperfusion injury: a characteristic analysis using magnetic resonance imaging

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Graphical Abstract



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Abstract

Some *in vitro* experiments have shown that erythropoietin (EPO) increases resistance to apoptosis and facilitates neuronal survival following cerebral ischemia. However, results from *in vivo* studies are rarely reported. Perfusion-weighted imaging (PWI) and diffusion-weighted imaging (DWI) have been applied successfully to distinguish acute cerebral ischemic necrosis and penumbra in living animals; therefore, we hypothesized that PWI and DWI could be used to provide imaging evidence *in vivo* for the conclusion that EPO could reduce apoptosis in brain areas injured by cerebral ischemia/reperfusion. To validate this hypothesis, we established a rat model of focal cerebral ischemia/reperfusion injury, and treated with intra-cerebroventricular injection of EPO (5,000 U/kg) 20 minutes before injury. Brain tissue in the ischemic injury zone was sampled using MRI-guided localization. The relative area of abnormal tissue, changes in PWI and DWI in the ischemic injury zone, and the number of apoptotic cells based on TdT-mediated dUTP-biotin nick end-labeling (TUNEL) were assessed. Our findings demonstrate that EPO reduces the relative area of abnormally high signal in PWI and DWI, increases cerebral blood volume, and decreases the number of apoptotic cells positive for TUNEL in the area injured by cerebral ischemia/reperfusion. The experiment provides imaging evidence *in vivo* for EPO treating cerebral ischemia/reperfusion injury.

Key Words: nerve regeneration; nerve protection; cerebral ischemia/reperfusion; erythropoietin; magnetic resonance imaging; diffusion-weighted imaging; apparent diffusion coefficient; perfusion-weighted imaging; cerebral blood volume; mean transit time; apoptosis; neural regeneration

Introduction

The effects of erythropoietin (EPO) on the brain and other organs are of great interest in clinical and scientific research (Shen et al., 2010). EPO plays a neuroprotective role in experimental models of ischemia/reperfusion, hypoxia-ischemia, subarachnoid hemorrhage, and cerebral infarction

(Wolfgang, 2007; Park et al., 2011; Xiong et al., 2011). Cerebral ischemia/reperfusion injury is an important pathophysiological process underlying cerebrovascular disease, and neuronal apoptosis following ischemia/reperfusion is a critical mechanism. Apoptotic cells can recover to normal cells if they are given proper treatment in time (Ferrer and Plans,

2003); therefore, the studies of possible drug-protective effects and mechanisms in apoptotic cells are more significant and valuable. Our former studies (Qian et al., 2014) showed that fludarabine exhibited neuroprotective effects through decreasing the number of apoptotic cells. In previous studies, many scholars have discussed the neuroprotective effects of EPO. Researchers (Kretz et al., 2005; Velly et al., 2010; Ryou et al., 2012; Zhao et al., 2015) demonstrated that EPO promoted central nervous system regeneration and facilitated cell survival after ischemia. Signore et al. (2006) reported that EPO could inhibit apoptosis through phosphorylating and activating Akt.

With the advances of fMRI, perfusion-weighted imaging (PWI) and diffusion-weighted imaging (DWI) have been applied successfully to distinguish acute cerebral ischemic necrosis and IP in living animals (Yang et al., 2010). Kidwell et al. (2003, 2013) put forward a new pattern of dividing the acute cerebral ischemic injury zone: the diffusion abnormality represents core, irreversibly injured tissue, and the outer rim of the visualized perfusion abnormality defines the periphery of the penumbra. The region with perfusion abnormality but no diffusion lesion (the mismatch region) identifies tissue that is hypoperfused but that has not yet experienced advanced bioenergetic failure and represents the penumbra.

However, there are few reports on the effects of EPO on MRI imaging at home and abroad. The purpose of the study was to evaluate the neuroprotective effects of EPO on the T2-weighted imaging-, DWI- and PWI-MRI and apoptosis of rat brains following ischemia/reperfusion injury. There are no reports using MRI-guided localization to determine the relative area of focal cerebral ischemia tissue, to analyze changes in DWI and PWI in the ischemic injury zone, to sample ischemic brain tissue, or to assess the number of apoptotic cells following acute cerebral ischemia/reperfusion with or without EPO treatment.

Materials and Methods

Animals

Male Sprague-Dawley (SD) rats ($n = 120$), aged 6 weeks and weighing 200–330 g, were provided by the Animal Center, Xuzhou Medical College, Jiangsu Province, China (License No. 2100133). The protocol described here received prior approval by the Committee on Animal Experimental Guidelines of the Affiliated Hospital of Xuzhou Medical College, China.

Administration and animal model preparation

Rats were randomly divided into four groups ($n = 30$ per group). The rats in the sham group were not given any treatment; those in the cerebral ischemia/reperfusion group were subjected to 2-hour ischemia and 24-hour reperfusion; those in the saline-treated group were subjected to 2-hour ischemia and 24-hour reperfusion, and received an injection of saline (10 μ L); and those in the EPO-treated group were subjected to 2-hour ischemia and 24-hour reperfusion, EPO was dissolved in 0.9 % normal saline, and the intra-cerebroventricular injection of EPO was conducted 20 minutes before the ischemia

(EPO for injection provided by 3SBio, Shenyang, China; 5,000 U/kg). After undergoing MRI, all rats in each group were sacrificed, and the brains were removed for further analysis.

A SD rat model of unilateral middle cerebral artery occlusion (MCAO) was established using a modified version of Longa's method (Kisseleva et al., 2002). Rats were anesthetized with 10% chloral hydrate (30 mg/kg). The left common carotid, internal carotid, and external carotid arteries were exposed with an incision in the neck and separated under a surgical microscope. The internal carotid artery was occluded, and the external carotid artery was ligated 0.8–1.0 cm from the common carotid artery. A treated thread was gently inserted through a small incision at the bifurcation into the internal carotid artery. When the thread was extended 17.5–18.5 mm from the common carotid artery bifurcation and met slight resistance, it suggested that the thread had been inserted into the origin of the MCA at the circle of Willis, occluding the blood flow in the MCA trunk. The thread was ligated with a slipknot in the internal carotid artery. The incision was then sutured, and rats were returned to their home cages with food and water. Body temperature was maintained near 37 °C using light bulbs. The thread was withdrawn 2 hours later, followed by 24 hours of reperfusion. For the sham group, the thread was inserted 5 mm deep and the other steps were the same as those for the cerebral ischemia/reperfusion surgery.

The establishment of animal models was carried out in batches under the same conditions. After regaining consciousness, the ischemic animals were observed for symptoms of neurologic deficit. Hara's 5-point scale (Hata et al., 1997) was used: 0, no observable neurologic deficit; 1, failure to extend contralateral paw fully; 2, circling to the contralateral side; 3, falling to the contralateral side; and 4, inability to walk spontaneously and loss of consciousness. A score of 1 to 4 indicated the success of an ischemic animal model.

MRI parameters and analysis

Animals in each group underwent Signa HD 3.0-T MRI (GE Healthcare, Wauwatosa, WI, USA) with an animal experiment coil after 2 hours of cerebral ischemia and 24 hours of reperfusion. The rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate. The heads were then fixed in the middle of the coil (Shanghai Chenguang Medical Technologies Co., Ltd., Shanghai, China) in a prone position. A triplanar scout scan was performed first, followed by T2WI, DWI, and PWI. All sequences were coronal imaging. DWI and PWI used an echo planar imaging (EPI) sequence. MRI parameters are listed in **Table 1**. Gadolinium-DTPA (Gd-DTPA) (Bayer, Leverkusen, Germany) was injected in 2–3 seconds at a dose of 1.5 mL/kg to serve as a contrast agent.

After MRI scanning, DWI data were transmitted to the workstation for post-processing to obtain apparent diffusion coefficient (ADC) profiles. Slices with the largest ischemic areas were selected, and ADC values of the region of interest (ROI) were measured, as well as the corresponding regions in the contralateral hemisphere. The relative ADC (rADC)

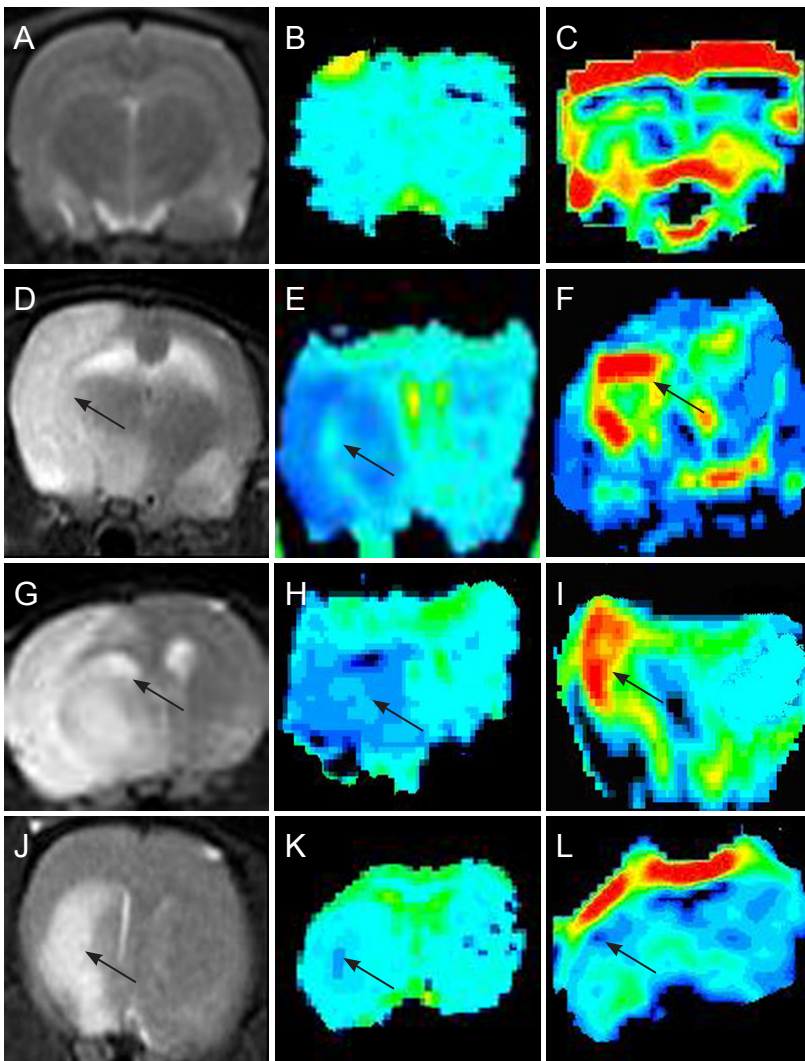


Figure 1 T2WI-, DWI- and PWI-MRI findings in each group.

(A–C) T2WI, ADC, and PWI in the sham group revealed similar signals in both cerebral hemispheres. (D–F) T2WI, ADC, and PWI in the cerebral ischemia/reperfusion group; T2WI imaging revealed a large, ischemic hyperintense area (arrow) in the left hemisphere; ADC pseudo-color showed an abnormal blue signal (arrow); and PWI revealed a large area with hypoperfusion (arrow). (G–I) T2WI, ADC, and PWI in the saline-treated group; T2WI revealed a large, ischemic hyperintense area (arrow) in the left hemisphere; ADC pseudo-color image showed a large, blue area indicative of abnormal signal (arrow); and PWI revealed a large hypoperfused area (arrow). (J–L) T2WI, ADC, and PWI in the erythropoietin-treated group; T2WI showed a few ischemic hyperintense patches in the subcortex of the left hemisphere (arrow); ADC pseudo-color image revealed a few small, blue, abnormal signal regions (arrow); and PWI showed a few hypoperfused patches (arrow). T2WI: T2-weighted imaging; DWI: perfusion-weighted imaging; MRI: magnetic resonance imaging; ADC: apparent diffusion coefficient.

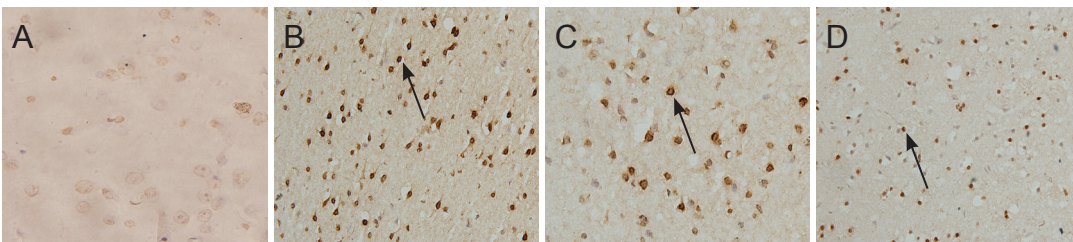


Figure 2 Effects of EPO on apoptotic cells in rat brain tissue after ischemia/reperfusion injury (TUNEL staining, light microscope, × 400)

(A) The sham group imaging revealed sporadic apoptotic cells characterized by brown staining. (B, C) The cerebral ischemia/reperfusion and saline-treated groups imaging revealed much larger numbers of apoptotic cells (arrow) characterized by brown staining, irregular and punctate shapes, and abnormal nuclei. (D) The distribution of apoptotic cells (arrow) in the EPO-treated group was similar to that of the cerebral ischemia/reperfusion and saline-treated groups, but the number was significantly reduced. TUNEL: TdT-mediated dUTP-biotin nick end-labeling; EPO: erythropoietin.

value was calculated as follows: rADC value = ADC value of ROI/contralateral ADC value × 100%. The percentage of abnormal DWI signal region on the selected slice with the largest ischemic area in the whole brain was also calculated. Negative enhancement integral (NEI), mean time to enhance (MTE), and time-signal intensity curve were obtained from the original PWI images by post-processing at the workstation. NEI was the cerebral blood volume (CBV), and MTE was the mean transit time (MTT). A slice matching the DWI

was selected, and the percentage of the PWI perfusion defect region on the same slice was calculated, and the relative CBV (rCBV) and relative MTT (rMTT) values of region showing defective PWI perfusion were determined in comparison with the contralateral side.

Determination of infarct area

The infarct area was expressed as a percentage (%) of the whole brain area. To measure the infarct area, brains were

Table 2 MRI parameters

Group	Median neuroscore (score)	Infarct area (mm ²)	TUNEL ⁺ cells (/mm ²)	NEX	Slice thickness/spacing (mm)
Sham	0	0	30±18	8	2.4/0.2
Ischemia-reperfusion	2.68*	287±22*	423±23*	8	2.4/0.2
Saline-treated	2.59*	291±27*	412±35*	8	2.4/0.2
Erythropoietin-treated	1.43	151±24	239±29	2	2.4/0.2

Data are expressed as the mean ± SD (n = 30; one-way analysis of variance and SNK test). *P < 0.05, vs. erythropoietin-treated group. TUNEL: TdT-mediated dUTP-biotin nick end-labeling; NEX: number of excitations.

Table 3 T2WI-, DWI- and PWI-MRI findings in each group

	Sham	Ischemia/reperfusion	Saline-treated	Erythropoietin-treated
T2WI (%)	0	25.5±7.7*	26.8±8.7*	16.6±6.9
DWI (%)	0	29.1±8.0*	30.5±9.2*	20.9±7.8
PWI (%)	0	33.9±6.8*	32.3±7.3*	26.5±8.9
rADC (%)	100.3±0.4	52.0±7.2*	56.5±6.1*	80.9±8.2
rCBV (%)	180.3±11.3	90.2±15.0*	100.6±10.4*	150.0±11.5
rMTT (%)	85.5±7.4	133.0±6.2*	128.3±5.8*	100.2±8.9
Ipsilateral ADC (× 10 ⁻¹⁰ mm ² /s)	6.5±0.5	3.3±0.4*	3.5±0.4*	5.3±0.4
Contralateral ADC (× 10 ⁻¹⁰ mm ² /s)	6.5±0.6	6.4±0.5*	6.5±0.4*	6.6±0.6

Data are expressed as the mean ± SD (n = 30; one-way analysis of variance and Student-Newman-Keuls tests). *P < 0.05, vs. erythropoietin-treated group. T2WI: T2-weighted imaging; PWI: perfusion-weighted imaging; rCBV: relative cerebral blood volume; rMTT: relative mean transit time; DWI: diffusion-weighted imaging; T2WI: T2-weighted imaging; ADC: apparent diffusion coefficient; rADC: relative ADC.

Table 1 MRI parameters

Sequence	TR/TE (ms)	FOV (cm ²)	Matrix	NEX	Slice thickness/spacing (mm)
T2WI	2,400/128	8 × 6	256 × 128	8	2.4/0.2
DWI	2,775/100	8 × 6	96 × 96	8	2.4/0.2
PWI	1,125/43	8 × 6	128 × 64	2	2.4/0.2

MRI: Magnetic resonance imaging; TR: repetition time; TE: echo time; FOV: field of view; NEX: number of excitations; T2WI: T2-weighted imaging; DWI: diffusion-weighted imaging; PWI: perfusion-weighted imaging.

removed after ischemia for 2 hours and reperfusion for 24 hours and evaluated using tetrazolium chloride (TTC)-stained 1-mm-thick slices. These slices were stained for 20 minutes in a 2% TTC and fixed in 10% buffered formalin solution. The stained slices were photographed using a digital camera. Infarction areas were quantitated using NIH image analysis software (Research Services Branch, NIH, USA). To compensate for brain edema, the infarction area was calculated (Hara et al., 1997).

Detection of apoptosis

After MRI scanning, the rat brains were removed. An *in situ* cell death detection kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) based on TdT-mediated dUTP-biotin nick end-labeling (TUNEL) was used according to the manufacturer’s instructions. The general procedure was as follows: rat brain tissue was incubated with proteinase K working solution after fixation; TUNEL reaction mixture was then added and reacted in a dark, humid chamber at

37°C for 1 hour; peroxidase was added for further reaction in a dark, humid chamber at 37°C for 30 minutes; and diaminobenzidine was added for color development and hematoxylin was applied as a counterstain. Four sections were used as specimens, and five fields (× 400) were randomly selected from each section. TUNEL-positive cells were counted using Image-Pro 9.2 software (Media Cybernetics, Inc., Rockville, MD, USA). The cell number was expressed as cells per mm².

Statistical analysis

Experimental data are expressed as the mean ± SD. SPSS 16.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. All data were tested for normality and homogeneity of variance. Comparisons among groups were made by one-way analysis of variance and Student-Newman-Keuls tests. A two-sided test was used to determine significance, and values of P < 0.05 were considered statistically significant.

Results

Behavioral symptoms in each group after cerebral ischemia for 2 hours and reperfusion for 24 hours

The sham group showed no behavior change while the cerebral ischemia/reperfusion, saline-treated and EPO-treated groups showed varying degrees of neurobehavioral impairment. According to Longa’s 5-point scale, the overall score of the EPO-treated group was lower than that of the cerebral ischemia/reperfusion and saline-treated groups. The EPO-treated group showed only mild neurologic deficits with a significantly lower median score compared with the cerebral ischemia/reperfusion and saline-treated groups (P < 0.05; **Table 2**). No

significant difference was found between the cerebral ischemia/reperfusion and saline-treated groups ($P > 0.05$).

T2WI-, ADC- and PWI-MRI findings

In the sham group, T2WI, ADC, and PWI showed similar signals in both cerebral hemispheres. In the cerebral ischemia/reperfusion, saline-treated and EPO-treated groups, T2WI revealed a hyperintense area with a clear border in the left cerebral hemisphere, ADC pseudo-color showed an abnormal blue signal, and PWI post-processing images were suggestive of perfusion defects in the region supplied by the left MCA. Groups of cerebral ischemia/reperfusion and saline-treated revealed large, abnormal signal areas in the cortex and subcortex of the left hemisphere, and the EPO-treated group showed a few abnormal patches either in the cortex or in the subcortex of the left hemisphere. Compared with the cerebral ischemia/reperfusion and saline-treated groups, the relative area of the abnormal signal region was significantly reduced in the EPO-treated group ($P < 0.05$; **Figure 1, Table 3**). No significant difference was found between the cerebral ischemia/reperfusion and saline-treated groups in the relative areas of the abnormal areas shown on DWI or in the PWI perfusion defect region ($P > 0.05$).

Infarct areas in each group after cerebral ischemia for 2 hours and reperfusion for 24 hours

In the sham group, TTC staining showed no ischemic region in both cerebral hemispheres. In the cerebral ischemia/reperfusion, saline-treated and erythropoietin-treated groups, TTC staining revealed infarcts in the left cerebral hemisphere supplied by the left MCA. The cerebral ischemia/reperfusion and saline-treated groups revealed a large infarct area in the cortex and striatum of the left hemisphere. Treating rats with EPO led to a significant decrease in the cortical and striatal infarct area compared with the cerebral ischemia/reperfusion and saline-treated groups ($P < 0.05$; **Table 2**). No significant difference was found between the cerebral ischemia/reperfusion and saline-treated groups ($P > 0.05$).

Effect of EPO on apoptosis

Sporadic apoptotic cells were observed in the sham group, but much greater numbers were noted in the cerebral ischemia/reperfusion and saline-treated groups. Apoptotic cells were characterized by brown staining, irregular and punctate shapes, and abnormal nuclei. The distribution of apoptotic cells in the EPO-treated group was similar to that in the cerebral ischemia/reperfusion and saline-treated groups, but the number was significantly reduced ($P < 0.05$) (**Figure 2, Table 2**).

Discussion

Cerebral ischemia/reperfusion can activate signal transducers and activators of transcription (STATs) (Takagi et al., 2002), and these proteins play an important role in neuronal survival and anti-apoptosis. P-STAT1 subsequently activates downstream genes to initiate neuronal apoptosis following cerebral ischemia/reperfusion (Qian et al., 2014). P-STAT3, a mediator of growth factor, hormones and cytokines, exerts protective

and regenerative effects in cerebral ischemia/reperfusion in part through transcriptional up-regulation of neuroprotective and neurotrophic genes (Dziennis and Alkayed, 2008; Amantea et al., 2011; Wang et al., 2013). Our former studies showed that EPO exerts its protective and regenerative effects in cerebral ischemia/reperfusion targeted P-STAT3 (Chunjuan et al., 2013). After EPO intervention, P-STAT3 expression significantly increased further, the neurological deficit symptoms were palliative, and the infarct areas were significantly reduced. Neuronal death induced by cerebral ischemia can be divided into necrosis and apoptosis. A large number of studies (Kidwell et al., 2006, 2013) have reported that neuronal necrosis occurs mainly in the ischemic core area, and apoptotic cells in the region of ischemic penumbra. Ischemia-induced neuronal necrosis is difficult to reverse. However, apoptosis can be prevented by altering upstream signals. Treatments for cerebral ischemia are designed to target apoptotic cells at the edge of the necrotic region (Stephanou et al., 2001; Zechariah et al., 2010). With the advances of fMRI, PWI and DWI have been applied successfully to distinguish acute cerebral ischemic necrosis and ischemic penumbra in living animals. Our former studies showed that the expression of P-STAT3 was negatively associated with the abnormal signal area of ADC, which means that the P-STAT3 activation reduced the abnormal signal area of DWI and the infarct area (Wu et al., 2009). However, examinations of the effects of EPO using MRI were rarely reported in the international research literature. There are no reports about the neuroprotective effects of EPO based on DWI- and PWI-MRI techniques, which were used for "target" biochemical analysis to determine the location of ROIs of cerebral ischemic tissue. Therefore, we used a cerebral ischemia/reperfusion animal model to investigate changes in MRI and apoptosis with or without EPO treatment.

The experimental results revealed that after EPO treatment, the neurological deficit symptoms were palliative, the relative area of cerebral ischemia as assessed by MRI was significantly reduced, the rADC increased significantly, and the rCBV and rMTT significantly increased and decreased, respectively. Because the ADC decrease is linearly related to cytotoxic edema induced by cellular energy metabolism dysfunction (Brissaud et al., 2010) and CBV and MTT can inform about ischemic brain tissue perfusion (Meng et al., 2004; Chen et al., 2007), the above parameters in the EPO group reflect a reduction in brain tissue injury. TUNEL also demonstrated a significant decrease in the number of apoptotic cells. These findings suggest that EPO increases the expression of genes regulating neuronal survival, inhibits apoptosis, and promotes injury repair in the ischemic penumbra, thereby reducing the relative area of abnormal signal assessed by MRI, all of which contribute to the survival of central nervous system neurons. This is consistent with the findings of Xie et al. (2007) that decreases in the number of apoptotic neurons in the ischemic penumbra were consistent after cerebral ischemia/reperfusion injury. Our findings support the conclusion of Li et al. (2007) that intraperitoneal injection of large doses of EPO (5,000 U/kg) given 30 minutes before ischemia and 24 hours after ischemia could significantly reduce the infarct area and inhibit neuronal

apoptosis in a mouse model of persistent focal cerebral ischemia. However, the best time window for clinical administration of EPO, the optimal dosage, and whether there are adverse reactions need to be investigated in further experimental and clinical studies.

In summary, intraperitoneal injection of EPO decreases the cerebral ischemic area and the number of apoptotic cells in the ischemic penumbra in a rat model. These effects may be achieved *via* EPO-mediated protection of cells against apoptosis. Exogenously administered EPO is expected to provide novel ideas for the prognosis and treatment of nervous system diseases. It offers significant neuroprotection against animal models of Parkinson's disease and motor neuron disease (Xue et al., 2010).

Author contributions: CJJ designed this research; ZJW and YJZ performed this research; ZYZ and JJT analyzed data; JYM and CJJ wrote the paper. All authors approved the final version of this paper.

Conflicts of interest: None declared.

Plagiarism check: This paper was screened twice using Cross-Check to verify originality before publication.

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