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

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,
in 0.9% normal saline, and the intra-cerebroventricular in 2-hour ischemia and 24-hour reperfusion, EPO was dissolved (10 μL); and those in the EPO-treated group were subjected to and 24-hour reperfusion, and received an injection of saline in the saline-treated group were subjected to 2-hour ischemia.

Rats in the cerebral ischemia/reperfusion group were ment; those in the sham group were not given any treat. The rats in the sham group were not given any treat.

Rats were randomly divided into four groups (n = 30 per group). The rats in the sham group were not given any treat.

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. 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.

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 treat; 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 μL); 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.
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 1 MRI parameters

<table>
<thead>
<tr>
<th>Sequence</th>
<th>TR/TE (ms)</th>
<th>FOV (cm²)</th>
<th>Matrix</th>
<th>NEX</th>
<th>Slice thickness/spacing (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2WI</td>
<td>2,400/128</td>
<td>8 × 6</td>
<td>256 × 128</td>
<td>8</td>
<td>2.4/0.2</td>
</tr>
<tr>
<td>DWI</td>
<td>2,775/100</td>
<td>8 × 6</td>
<td>96 × 96</td>
<td>8</td>
<td>2.4/0.2</td>
</tr>
<tr>
<td>PWI</td>
<td>1,125/43</td>
<td>8 × 6</td>
<td>128 × 64</td>
<td>2</td>
<td>2.4/0.2</td>
</tr>
</tbody>
</table>

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.

Table 2 MRI parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Median neuroscore (score)</th>
<th>Infarct area (mm²)</th>
<th>TUNEL⁺ cells (× 10⁶/mm²)</th>
<th>NEX</th>
<th>Slice thickness/spacing (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0</td>
<td>0</td>
<td>30±18</td>
<td>8</td>
<td>2.4/0.2</td>
</tr>
<tr>
<td>Ischemia-reperfusion</td>
<td>2.68*</td>
<td>287±22</td>
<td>423±23</td>
<td>8</td>
<td>2.4/0.2</td>
</tr>
<tr>
<td>Saline-treated</td>
<td>2.59*</td>
<td>291±27</td>
<td>412±35</td>
<td>8</td>
<td>2.4/0.2</td>
</tr>
<tr>
<td>Erythropoietin-treated</td>
<td>1.43</td>
<td>151±24</td>
<td>239±29</td>
<td>2</td>
<td>2.4/0.2</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Ischemia/reperfusion</th>
<th>Saline-treated</th>
<th>Erythropoietin-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2WI (%)</td>
<td>0.01±0.10</td>
<td>25.5±7.7</td>
<td>30.5±8.7</td>
<td>16.6±6.9</td>
</tr>
<tr>
<td>DWI (%)</td>
<td>0</td>
<td>29.1±8.0</td>
<td>32.3±7.3</td>
<td>20.9±7.8</td>
</tr>
<tr>
<td>PWI (%)</td>
<td>0</td>
<td>33.9±6.8</td>
<td>52.0±7.2</td>
<td>26.3±8.9</td>
</tr>
<tr>
<td>rADC (%)</td>
<td>100.3±0.4</td>
<td>50.2±7.2</td>
<td>56.5±6.1</td>
<td>80.9±8.2</td>
</tr>
<tr>
<td>rCBV (%)</td>
<td>180.3±11.3</td>
<td>90.2±15.0</td>
<td>100.6±10.4</td>
<td>150.0±11.5</td>
</tr>
<tr>
<td>rMTT (%)</td>
<td>85.5±7.4</td>
<td>133.0±6.2</td>
<td>128.3±5.8</td>
<td>100.2±8.9</td>
</tr>
<tr>
<td>Ipsilateral ADC (× 10⁻⁶ mm²/s)</td>
<td>6.5±0.5</td>
<td>3.3±0.4</td>
<td>3.5±0.4</td>
<td>5.3±0.4</td>
</tr>
<tr>
<td>Contralateral ADC (× 10⁻⁶ mm²/s)</td>
<td>6.5±0.6</td>
<td>6.4³±0.5</td>
<td>6.5±0.4</td>
<td>6.6±0.6</td>
</tr>
</tbody>
</table>

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.

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

**Conflicts of interest:** None declared.

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**References**


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