Effect of erythropoietin on Fas/FasL expression in brain tissues of neonatal rats with hypoxic-ischemic brain damage

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Hypoxic-ischemic brain damage (HIBD) occurs due to intrauterine hypoxia ischemia influencing the energy supply for fetal brain cells, which affects the metabolism of the brain to make the brain suffer a severe damage. Erythropoietin (EPO), which regulates hemacytopenia, is a kind of cytokine. EPO is sensitive to hypoxia ischemia. In this study, we aimed to investigate the effect of EPO on the expression of Fas/FasL in brain tissues of neonatal rats with HIBD. Neonatal rats were assigned randomly to sham, HIBD, and EPO groups. Five time points for observation were 6, 12, 24, 48, and 72 h after the HIBD rat model had been established, respectively. In the HIBD group, Fas/FasL expression began to rise at 6 h, reached the peak at 12–24 h, and dropped from 24 h. In the EPO group, the expression of Fas/FasL was lower than those in HIBD group at 12, 24, and 48 h (\(P < 0.05\)). Our findings suggest that EPO may reduce cell apoptosis after hypoxic-ischemic brain damage through reduction of the expression of Fas and FasL, and that optimal therapeutic time window is 6–24 h after HIBD. NeuroReport 30:262–268 Copyright © 2019 The Author(s). Published by Wolters Kluwer Health, Inc.

Keywords: erythropoietin, Fas, FasL, hypoxic-ischemic brain damage

Introduction

Hypoxic-ischemic brain damage (HIBD) refers to hypoxia ischemia that is caused by perinatal asphyxia, with reduction or suspension of the cerebral blood flow and serious damage of brain tissues [1]. It was reported that the incidence of neonatal HIBD is about 3–5%, and the mortality rates of children with HIBD is as high as 60%. Even survivors, nearly 25%, have been reported to be accompanied by lifelong defect of the nerve function, including epilepsy, cerebral palsy, mental retardation [2]. In recent years, although some new treatment methods, such as calcium channel antagonist, hyperbaric oxygen, mild hypothermia, and so on, have achieved more effective treatment effect compared with before, they have not been generalized extensively in clinical practice due to their various limitations [3,4]. Erythropoietin (EPO) is a glycoprotein excreted by the kidneys. Its main function is to stimulate the proliferation and differentiation of reticulocytes [5]. Further studies proved that EPO could also promote cell regeneration and vessel formation; resist inflammation, oxidation, and apoptosis; and accelerate vessel formation, cell proliferation, and cell protection. The levels of EPO and EPO receptor (EPO-R) in the normal brain tissue are very low. When the brain tissue is damaged by hypoxia ischemia, the levels of EPO and EPO-R will rise sharply [6]. Therefore, we hypothesize that EPO might have some positive effects on HIBD.

The Fas-singling pathway is a common way to induce apoptosis in cells. Its physiological ligand, FasL, is a member of the corresponding tumor necrosis factor (TNF) cytokine family. Fas and FasL play critical roles in the immune system, particularly in the killing of pathogen-infected target cells and the death of no-longer-needed, potentially deleterious and autoreactive, lymphocytes [7]. Various drugs and substances can induce neuronal apoptosis through FasL–Fas signaling. The expression of Fas and FasL increases significantly when HIBD occurs [8]. The pathophysiological process of HIBD mainly behaves as early neuronal necrosis and apoptosis after cerebral hypoxia ischemia, which trigger delayed neuronal death. This study attempts to evaluate the effect of EPO on Fas/FasL-mediated apoptosis-signaling pathways in brain tissues with hypoxic-ischemic damage by animal model of HIBD.

Materials and methods

Experimental animals and grouping

We chose 7-day-old Sprague-Dawley (SD) neonatal rats (male and female unlimited). In total, 120 SD neonatal rats were provided by experimental animal center of Hebei Medical University. All these neonatal rats were divided into three groups randomly: the sham group, the HIBD group, and the EPO group. Each group was further divided...
into five time points: 6, 12, 24, 48, and 72 h after HIBD model was established. Overall, 120 SD rats were randomly divided into three groups of 40 animals in each. Forty SD rats were randomly divided in five time points of eight animals in each. In the EPO group, each rat with HIBD was treated by intraperitoneal injection of EPO (3000 U/kg) for 3 days. Neonatal rats in the HIBD group and the sham group were treated by intraperitoneal injection of the same dose of normal saline at the same time.

Preparation of hypoxic-ischemic brain damage model
Neonatal rats were anesthetized with ether. The left common carotid artery of the rats was isolated and ligated with two 3-0 sterile thread. The pups were placed in glass chambers containing special standard gases (8% O₂ and 92% N₂), which were pumped into the glass chamber 2 h at a speed of 1.5–2.5 l/min. After modeling of 30 min, brain damage symptoms including disequilibrium, polynnea, dystonia, accidie, and drowsiness were found in experimental animals. That modeling was considered as successful. The sham group and HIBD group used the same ligation method. But the sham group was not included occluding the vessel and undergoing hypoxia.

Hematoxylin and eosin staining
After being treated by deparaffinage and hydration, the paraffin section was stained by hematoxylin for 5 min, washed by distilled water for 2 min, and separated by 1% hydrochloric acid alcohol for 3 s. It was washed to return to blue for 30 min and counterstained by 0.5% eosin solution for 3 min, then was washed by distilled water again for 30 s. Then the paraffin section was dehydrated by ethanol and treated by xylene. At last it was sealed by Permount TM Mounting Medium and observed under optical microscope.

Fas/FasL immunohistochemical staining
The paraffin section was conventionally dewaxed to water. The ganglia were cut 10 µm thick at a cryostat. The sections were incubated in 3% H₂O₂ for 10 min to block the endogenous peroxidase. Following incubation with normal goat serum for 20 min, the sections were incubated with rabbit anti Fas or rabbit anti FasL (1:200 diluted in PBS; Chemicon International Inc., Billerica, USA) for overnight at 4°C. After three rinses in PBS, the sections were then incubated with biotinylated goat antirabbit secondary antibody for 1 h at room temperature and streptavidin-horseradish peroxidase (Beijing Zhongshan Biotech Co., Beijing, China) for 30 min. The color was developed in DAB substrate. The section was counterstained by hematoxylin and treated by dehydration, clearing, and mounting. Image-Pro Plus 6.0 image (Media Cybernetics, Maryland, USA) analysis software was used to analyze the integrated optical density (IOD) of Fas and FasL.

RNA extraction and real-time PCR
The total RNA was isolated from the neonatal rat brain tissue by using the Trizol method according to the manufacturer’s instructions (Invitrogen Corporation, Carlsbad, California, USA). Briefly, a piece of tissue was homogenized in 1 ml of TRIzol reagent to extract the total RNA. It was then reverse-transcribed into complementary DNA (cDNA) with Revert Aid First Strand cDNA Synthesis Kit (TaKaRa, RNA-M-MLV kit). The cDNA was then stored at −20°C before further processing.

For real-time PCR, primer pairs are shown in Table 1. An equal amount of template DNA was used for the simplex assays, and water was added to make a final reaction volume of 25 pl. The amplification conditions of the assay were set as follows: activation of TaqMan at 95°C for 10 min, then 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 56°C for 30 s.

Table 1  Primer pairs used in the real-time PCR assay

| Name  | Primer sequence (5′–3′) | Product length (bp) |
|-------|------------------------|---------------------|
| Fas   | Forward: CTCCTGCTACCTCGTACG | 117 |
|       | Reverse: CTGTTGCGTGAGAGCTACGA | 182 |
| FasL  | Forward: TCACCTGTAACGCGCTTCCC | 194 |
| β-actin | Forward: CTTGTTAAAATGGGCCAATTC | 263 |
|       | Reverse: AGCCTGTGTGGCGTACAG | 194 |

Statistical analysis
All the experimental data were analyzed using the SPSS version 21 statistical analysis package (SPSS Inc., Chicago, Illinois, USA). Examined data were assessed using the variance analysis and χ²-test. In each test, the data were expressed as the mean±SD, and P less than 0.05 was accepted as statistically significant.

Results
Brain tissues of the neonatal rat by hematoxylin and eosin staining
At each time point, the morphology and structures of the cerebral cortex tissue was basically normal with complete structure, distinct layers, and orderly arrangement of neurons in the sham operation group. The morphology and structures of neurons in the HIBD group were as follows: (a) neurons began to show mild edema at 6 h; (b) at 12 h, cerebral cortex sections showed edema and hemorrhage in different degrees, and the edema of neurons was deeper than that at 6 h; (c) from 12 to 24 h, the cerebral cortex tissue began to atrophy in varying degrees; neurons began to necrosis, and there was cell disintegration, cytoplasm light staining, nuclear pyknosis, as well as some nucleoli disappeared and showed vacuolar change; (d) at 72 h, there was cerebral cortex tissue atrophy and liquefaction necrosis; neurons necrosis aggrivated and neuron cells decreased significantly. The morphology of EPO group was relieved at each time point and compared with the HIBD group (Fig. 1).
The expressions of Fas and FasL protein by immunohistochemical staining in the hypoxic-ischemic brain damage

The expressions of Fas proteins by immunohistochemical staining in neonatal rat cerebral cortex neurons

The Fas-positive cells can be rarely seen in the neonatal rat cerebral cortex of the sham group. The results of Fas expression in HIBD and EPO groups are as follows: at 6 h, a small number of Fas-positive cells were seen in the neonatal rat cerebral cortex; at 12 h, the Fas-positive cells in the neonatal rat cerebral cortex increased gradually; at 24 h, the Fas expression in the neonatal rat cerebral cortex reached its highest level (Fig. 2). At each time point, the IOD values of Fas protein in the sham group were lower than those in the HIBD group ($P<0.05$). After being treated with EPO in the HIBD rats, the IOD values of Fas protein were lower than those in HIBD rats.
at each time point \( (P < 0.05) \). The IOD values of Fas protein in the EPO group were higher than those in the sham group at five time points \( (P < 0.05, \text{Table 2}) \).

**The expressions of FasL protein by immunohistochemical staining in neonatal rat cerebral cortex neurons**

The FasL-positive cells could be rarely seen in the neonatal rat cerebral cortex of the sham group. The results of FasL expression in HIBD and EPO groups were as follows: at 6 h, a small number of FasL-positive cells could be seen in the neonatal rat cerebral cortex; at 12 h, the FasL-positive cells in the neonatal rat cerebral cortex increased gradually; at 24 h, the Fas expression in the neonatal rat cerebral cortex reached its highest level (Fig. 3). At each time point, the IOD values of FasL protein in the sham group were lower than those in the HIBD group \( (P < 0.05) \). After being treated with EPO in the HIBD rats, the IOD values of FasL protein were lower than those in HIBD rats at each time point.

![Immunohistochemistry](image-url)
Table 2  The integrated optical density of Fas and FasL in neonatal rat cerebral cortex neurons in each group at different time points by immunohistochemistry

| Groups  | 6 h     | 12 h    | 24 h    | 48 h    | 72 h    |
|---------|---------|---------|---------|---------|---------|
| Fas     |         |         |         |         |         |
| Sham group | 1.76 ± 0.25 | 1.83 ± 0.22 | 1.67 ± 0.31 | 1.58 ± 0.28 | 1.81 ± 0.32 |
| HIBD group | 3.73 ± 0.42<sup>a</sup> | 4.25 ± 0.51<sup>a</sup> | 5.42 ± 0.28<sup>a</sup> | 5.26 ± 0.48<sup>a</sup> | 4.49 ± 0.64<sup>a</sup> |
| EPO group | 2.86 ± 0.38<sup>ab</sup> | 3.76 ± 0.43<sup>ab</sup> | 4.43 ± 0.47<sup>ab</sup> | 4.31 ± 0.55<sup>ab</sup> | 3.36 ± 0.29<sup>ab</sup> |
| Fas L    |         |         |         |         |         |
| Sham group | 1.45 ± 0.31 | 1.53 ± 0.25 | 1.61 ± 0.22 | 1.37 ± 0.16 | 1.48 ± 0.41 |
| HIBD group | 3.41 ± 0.72<sup>c</sup> | 3.79 ± 0.38<sup>c</sup> | 4.72 ± 0.31<sup>c</sup> | 4.19 ± 0.42<sup>c</sup> | 3.83 ± 0.44<sup>c</sup> |
| EPO group | 2.65 ± 0.35<sup>bc</sup> | 3.02 ± 0.50<sup>bc</sup> | 3.89 ± 0.67<sup>bc</sup> | 3.34 ± 0.32<sup>bc</sup> | 2.87 ± 0.26<sup>bc</sup> |

EPO, erythropoietin; HIBD, hypoxic-ischemic brain damage.
<sup>a</sup>Compared with sham group, P < 0.05.
<sup>b</sup>Compared with HIBD group, P < 0.05; each group with five rats (n = 5).

Fig. 3

Fas L immunohistochemistry to neonatal rat cerebral cortex neurons in each group at different time points. (a) Sham group, (b) HIBD group at 6 h, (c) HIBD group at 12 h, (d) HIBD group at 24 h, (e) HIBD group at 48 h, (f) HIBD group at 72 h, (g) EPO group at 6 h, (h) EPO group at 12 h, (i) EPO group at 24 h, (j) EPO group at 48 h, (k) EPO group at 72 h, all scale bars = 100 μm, each group with five rats (n = 5). EPO, erythropoietin; HIBD, hypoxic-ischemic brain damage.
(P < 0.05). The IOD values of FasL protein in the EPO group were higher than those in the sham group at five time points (P < 0.05, Table 2).

**The expressions of Fas and FasL mRNA by real-time PCR in the hypoxic-ischemic brain damage**

The expression of Fas and FasL mRNA in the neonatal rat cerebral cortex in the sham group remained at a low level at different time points. The expression of Fas mRNA began to increase in the neonatal rat cerebral cortex in the HIBD group 6 h later, reached the maximum at 12 h, then began to decrease gradually, and dropped to near the basic level after 72 h in the EPO group. The expression of Fas mRNA in the EPO group was significantly lower than that in the HIBD group at 12, 24, and 48 h (P < 0.05), but it was significantly higher than that in the sham group at each time point (P < 0.05). FasL mRNA expression in the neonatal rat cerebral cortex of the HIBD group began to increase and decrease rapidly (6–24 h) in a short time, and maintained a high level at 12 and 24 h. After 24 h, FasL mRNA expression began to decrease, and dropped to near the basic level. At 6, 12, 24, and 48 h, FasL mRNA expression in the EPO group was significantly lower than that in the HIBD group, but it was significantly higher than that in sham group at each time point (P < 0.05, Table 3).

**Discussion**

HIBD to the developing brain remains a major cause of significant long-term morbidity and mortality. Currently, despite the advances offered by therapeutic hypothermia in terms of neuroprotection, its effect is limited and has to be initiated in a very short time window [9]. Perinatal asphyxia remains the major cause of permanent neurological disability in survived children [2]. Therefore, one of the most important goals while approaching patients with HIBD remains actually determining the exact period in which the effects of potential damaging factors occur [10,11].

EPO is a 34-kDa glycoprotein hormone. It is mainly used in the treatment of anemia in clinical settings at present. EPO and EPO-R are upregulated following hypoxic-ischemic injury and EPO has an antioxidant as well as anti-inflammatory effect. In recent researches, its neuroprotective role has been recognized and evaluated in various animal models of cerebral ischemia [12]. In particular, EPO reduced the damage of apoptosis to cells [13,14]. A Phase I trial demonstrated that a moderately high dose of 1000 U/kg achieved levels based on animal studies that would protective maximal neuroprotection and minimize risks of excessive Epo [15]. In addition, EPO has been shown to protect cultured hippocampal and cortical neurons against glutamate toxicity, hypoxia, and glucose deprivation and serum deprivation-induced apoptosis in-vitro experiments [16,17]. The effects of EPO on SVZ cell fate and number play a significant role in the long-term histological and functional improvement previously reported with prolonged EPO treatment [18]. As our results showed, the cellular structures and levels of the rats in the sham group are intact and clear; the cellular structures of the rats in the HIBD group are damaged obviously, and the damage becomes worse gradually over time. The morphology of the EPO group was relieved at each time point and compared with the HIBD group. These findings suggest that EPO may improve the cell structures of the HIBD rat brain.

So far, it is generally believed that Fas and FasL play an important role in cell apoptosis. In the current study, levels of Fas and FasL were evaluated as the extrinsic apoptosis signals. Fas/FasL-signaling pathway is involved in and mediates the apoptosis in HIBD [19]. Fas (Apo-1, or CD95) is a kind of type-I transmembrane protein, with a molecular weight of 45 kD. Fas belongs to the TNF superfamily and it is the receptor of TNF [20]. FasL is the ligand of Fas, and it is a kind of type II transmembrane protein, with a molecular weight of about 40 kD; that FasL combines with the Fas which exists at the surface of the cell adjacent can activate FADD, to form the protein composite trimer of caspase-10/FADD/pro-caspase-8 in the cytoplasm; this further activates other members in caspase family to cause caspase cascade, and transfer death signals to the cells, to trigger and lead to apoptosis [21,22]. In HIBD, apoptosis mainly occurs around the ischemic area, and it is the main form of the

**Table 3 The expressions of Fas/FasL mRNAs by real-time PCR in each group**

| Groups     | 6 h     | 12 h    | 24 h    | 48 h    | 72 h    |
|------------|---------|---------|---------|---------|---------|
| Fas        |         |         |         |         |         |
| Sham group | 1.75 ± 0.21 | 1.63 ± 0.28 | 1.67 ± 0.32 | 1.39 ± 0.26 | 1.58 ± 0.46 |
| HIBD group | 3.89 ± 0.42 | 12.04 ± 0.89 | 6.91 ± 0.64 | 4.31 ± 0.52 | 2.43 ± 0.26 |
| EPO group  | 3.72 ± 0.22* | 5.68 ± 0.54* | 3.95 ± 0.41** | 3.83 ± 0.27** | 1.79 ± 0.28* |
| P          | 0.39    | 0.01    | 0.00    | 0.04    | 0.24    |
| Fas L      |         |         |         |         |         |
| Sham group | 1.52 ± 0.20 | 1.73 ± 0.31 | 1.82 ± 0.35 | 1.42 ± 0.27 | 1.48 ± 0.34 |
| HIBD group | 4.69 ± 0.61 | 19.54 ± 1.67 | 22.61 ± 2.06 | 5.29 ± 0.73 | 2.92 ± 0.21 |
| EPO group  | 2.78 ± 0.17* | 6.94 ± 0.78** | 6.25 ± 0.52** | 3.12 ± 0.36* | 2.92 ± 0.23* |
| P          | 10.97   | 51.34   | 42.75   | 14.09   | 4.23    |

*Compared with HIBD group, P < 0.05.
*Compared with sham group, P < 0.05.
delayed neuronal death. Some studies have found that, in the rat model induced by hypoxia ischemia, the expression of Fas and Fasl in brain hippocampus increased, and this increase was positively correlated with the apoptosis rate of the neurons. Thus we can speculate that Fas/Fasl-signaling pathway is involved in the apoptosis of neonatal rats with HIBD [23]. The view that Fas/Fasl-signaling pathway is critical in inducing the HIBD has been accepted by many international and domestic scholars.

However, the ‘therapeutic window’, that is, the period in which damaged but still live cells can be rescued by neuroprotective strategies, is a main point of interest in hyperbaric oxygen therapeutic studies. Following hypoxia-ischemic injury, the apoptotic pathway is thought to be activated after 6–12 h, peak after 24–72 h, and continue for 7 days [24]. In this study, we observed that EPO protects neonatal rat brain against hypoxia-ischemic brain-damage-induced apoptosis at 6, 24, 48, and 72 h after the HIBD. By observing the changes of Fas and Fasl (protein and mRNA) in the cerebral cortex of the HIBD neonatal rats dynamically at the different time points, this study found the following characteristics: (1) the expression of Fas and Fasl (protein and mRNA) began to increase after 6 h of the cerebral ischemia-hypoxia; (b) the expression level of Fas and Fasl (protein and mRNA) reached the highest at 24 h of the cerebral ischemia-hypoxia and declined gradually later. In this study we observed that Fas/Fasl participated in the process of apoptosis of the brain cells in the early time after HIBD. After the EPO treatment, the expression of Fas/Fasl protein and mRNA decreased. Accordingly, the therapeutic window for effective EPO treatment after HIBD could be delayed up to 24 h, while the optimal time is with in 6 h after HIBD.

Thus, EPO therapy can protect neonatal rats against HIBD by inhibiting Fas or Fasl induced apoptosis. For neonatal rats inflicted with HIBD, EPO treatment within the optimal therapeutic window is of prime importance. Within 24 h of hypoxia-ischemic damage, it can be improved obviously by EPO in neonatal rats, with early treatment showing the greatest benefit. Hence, EPO might confer benefit as an efficient adjuvant neuroprotective strategy. This study provides a new idea for the treatment of neonatal HIBD in clinical.

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Conflicts of interest
There are no conflicts of interest.

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