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Effects of erythropoietin on neonatal hypoxia–ischemia brain injury in rat model

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HIGHLIGHTS

- EPO promoted weight gains, reduced brain edema, and improved neurological function in a preterm equivalent P2 rat HI model.
- A single dose of EPO at 5000 U/kg immediately or 48 h after HI had significant benefit for the P2 rats in brain injury recovery.
- Female rats expressed higher MBP at d14 post HI and higher mortality than the male ones.
- Female rats had improved neuronal behavior at one month post HI than the male ones.

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ABSTRACT

Background: Hypoxic–ischemic (HI) injury to the developing brain remains a major cause of morbidity. To date, few therapeutic strategies could provide complete neuroprotection. Erythropoietin (EPO) has been shown to be beneficial in several models of neonatal HI. This study examines the effect of treatment with erythropoietin on postnatal day 2 (P2) rats introduced with HI injury.
Method: Rats at P2 were randomized into four groups: sham, bilateral carotid artery occlusion (BCAO), BCAO + early EPO, and BCAO + late EPO groups. Pups in each group were injected with either saline or EPO (5000 U/kg) intraperitoneally once at immediately (early) or 48 h (late) after HI induction. Body weight was assessed at P2 before and day 7 after HI. Mortality Rate was assessed at 24 h, 48 h and 72 h after HI and brain water content was assessed at 72 h. Brain weight and expression of myelin basic protein (MBP) were assessed at day 7 and day 14. At day 31 to 35 following HI insult, neurological behavior function was assessed via Morris water maze (MWM) test.
Result: HI cause significant higher mortality in male than in female ($P = 0.0445$). Among the surviving animal, HI affect significantly the body growth, brain growth, MBP expression, and neurological behavior. EPO treatments at both early and late time points significantly benefit the rats in injury recovery, in which they promoted weight gains, reduced brain edema, as well as improved spatial learning ability and memory.
Conclusion: We demonstrated a single dose of EPO at 5000 U/kg immediately or 48 h after HI injury had significant benefit for the P2 rats in injury recovery, and there was no adverse effect associated with either EPO treatment.

1. Introduction

Hypoxic-ischemic (HI) encephalopathy is one of the most common types of brain damage due to lack of adequate oxygen and blood. Perinatal asphyxia causes about 23% of all neonatal death worldwide [1]. To the surviving infants, it can inflict profound neurological impairment that leads to severe developmental or cognitive delays. Premature birth itself is a major risk factor for cerebral complications [2]. In preterm infants, periventricular white matter injury as well as intracerebral and intraventricular hemorrhage accounts for most common HI injuries. The neurological deficits seen in the majority of surviving premature infants may be the result of cerebral white matter injury. The resulting

Abbreviations: WMD, White matter damage;; HI, hypoxic-ischemia;; EPO, erythropoietin;; BCAO, bilateral carotid artery occlusion;; MBP, myelin basic protein;; MWM, Morris water-maze;; PVL, periventricular leukomalacia;; HE, hematoxylin-eosin;; IHC, Immunohistochemistry;; IOD, integrated optical density;; EPO-R, EPO receptor; P2, Postnatal day 2;; P5, postnatal-5;; *P*1, postnatal-1;; NE, Northeast;; PBS, phosphatebuffered saline;; HRP, horseradish peroxide;; DAB, diaminobenzidine;; SNK, Student-Newman-Keuls;; AIF, apoptosis-inducing factor.

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cognitive and behavioral problems became a huge burden for both the family and society [3,4]. Although there is no established intervention that fully treat HI induced perinatal brain injury, many potential therapies that may prevent injury progression and enhance repair are under investigation [5].

Erythropoietin (EPO) is a 34-kDa glycoprotein that was originally identified for its essential role in erythropoiesis. It has since been discovered to have other functions in neural differentiation and neurogenesis in early development. Several different types of cells in the central nervous system express EPO and EPO receptor (EPO-R) with changing patterns during development [6]. The expression of EPO and EPO-R by a number of cells, including neurons, astrocytes, endothelial cells, and microglia, increases following brain injury [7,8]. These findings indicate the potential role of EPO in brain injury.

Subsequent studies have suggested that exogenous EPO treatment has a protective effect on a variety of brain injuries [9–11]. Studies in rodent models have demonstrated that EPO have protective effect on hypoxic-ischemic injury when treated before or after the injury. A single dose of EPO treatment at 1000 U/kg is shown to reduce infarct volume in a newborn hypoxic-ischemic brain injury rat model [12,13]. Exogenous EPO treatment is also shown to increase neurogenesis in the subventricular zone and promote neuronal progenitors migrating into the ischemic cortex and striatum [14]. Subsequently, EPO is shown to improve long-term spatial memory deficit in the neonatal hypoxia-ischemia rats [12,15]. EPO treatment has been found to promote reorganization of white matter and the increase of oligodendrocyte precursor cells, which may explain its effect on long-term improvement in some injury models [16,17].

Rat is most commonly used animal for models of perinatal asphyxia. Introduced in early 1980s, a combination of unilateral common carotid artery ligation with 8% oxygen method was used to introduce hypoxic ischemic injury in a 7-day rat pup [18]. Over the years, this model has been well characterized for studying the gray matter of brain injury. Recently, modified models have been established to study the white matter injury, including transient bilateral carotid artery ligation in the 7-day rat pup [19]. Permanent bilateral common carotid artery ligation in postnatal-5 (P5) rats [20], as well as in postnatal-1 (*P*1) rats [21] were developed to study specifically white-matter structure damage due to ischemia insult.

In the present study, we performed bilateral carotid artery occlusion (BCAO) procedure on the P2 rats to introduce the hypoxic ischemic injury and studied the short and long term effect of EPO treatment at two different time points after injury.

2. Methods

2.1. Animal

The experiment protocol was approved by the Institutional Animal Care and Use Committee of Liaocheng People's Hospital (Liaocheng, China), and performed in accordance with the guidelines established for human handling of animals. Sprague-Dawley P2 rats ($n = 120$ male/female 61/59) were obtained from the Experimental Animal Center of Anhui Medical University and divided randomly into four groups: sham-treated, BCAO-treated, BCAO + early EPO-treated, and BCAO + late EPO-treated.

2.2. Induction of HI injury and EPO administration

BCAO was conducted. The bilateral common carotid artery was permanently ligated under anesthesia to induce HI injury, but separated without ligation in sham control (male/female 12/18). EPO (diluted with saline at the concentration of 500 U/ml) was administered intraperitoneally at 5000 U/kg dose immediately (early EPO male/female 17/14) or 48 h (late EPO male/female 15/12) after surgery respectively. Animals in HI group (male/female 17/15) and sham group were injected intraperitoneally with saline solution (0.1 ml/10 g) immediately after the surgery. Rats were then allowed to recover 1 h and returned to the mother squirrel cage kept in 37 °C incubator.

2.3. Weight monitoring

The body weight of the rat was measured on P2 before procedure and d7 (between 8 and 10 a.m.) after operation. The body growth rate (%) was calculated as follow: (bodyweight at d7 − bodyweight at P2)/bodyweight at P2. At postoperative d7 and d14, 4 rats of each group were euthanized and their brains were separated and weighted. The brain growth rate (%) was calculated as follow: (brain weight at d14 – brain weight at d7)/ brain weight at d7.

2.4. Hematoxylin-eosin (HE) and immunohistochemistry (IHC) staining

At postoperative 72 h, 3 rats of each group were euthanized and their brains were separated and fixed for 48 h in 4% paraformaldehyde at room temperature. Then the brain tissue was embedded in paraffin and sliced into 5-μm sections. Paraffin sections were dewaxed, hydrated, and stained with HE. The cells were observed with a light microscope.

At d7 and d14 post HI injury, the separated brain was fixed for 48 h in 4% paraformaldehyde at room temperature after the weight measuring. Paraffin embedded and continuous coronary sliced tissues were studied with IHC to observe the expression of MBP. Briefly, the sections were de-waxed with xylene and incubated with 3% peroxide for 30 min to deactivate the endogenous catalase. Sections were then washed three times with PBS for 5 min each, heated for antigen retrieval, and incubated in normal goat serum working solution at 37 °C for 30 min. The incubation of MBP monoclonal antibody (1:300) (Boster Biological Technology, Wuhan, China) was carried out overnight at 4 °C. The sections were washed three times with phosphatebuffered saline (PBS) before incubated with horseradish peroxide (HRP)-labeled rabbit-*anti*-goat IgG working solution at 37 °C for 30 min. After washed with PBS, the sections were stained in diaminobenzidine (DAB) staining solution for 3–5 min, then counter stained in hematoxylin, differentiated with hydrochloric alcohol, and gradient dehydrated. After the sections were sealed with neutral balsam, images were taken and analyzed with ImageJ software. The results were presented as integrated optical density (IOD).

2.5. Brain edema measurement

Tissue-water percentage content in brain was determined by comparing the wet and dry weight at 72 h after operation. Eight rats from each group were randomly selected, euthanized, brain was surgically removed from the skull. After the cerebellum was discarded, the right and left hemispheres were separated along the anatomic midline, and the wet weight of each hemisphere was measured. The tissue was then completely dried in an oven at 80 °C for 72 h, and the dry weight of each hemisphere was recorded. The percentage of water content (% water) was calculated for each hemisphere as follows: %water = (wet weight-dry weight)/wet weight × 100% [22–25].

2.6. Neurological behavior testing

Rats were assessed from d31 to d35 after HI using the Morris water maze (MWM) test. For behavioral studies, following rats were subjected for testing: sham group (male/female 4/6), HI group (male/female 3/4), early EPO group (male/female 5/3), and late EPO group (male/female 4/4). The procedure was modified from previous version [26] and has been found to be useful for chronic spatial memory assessment in rats and mice with brain injury [27]. All tests were conducted by investigators blinded from the treatment status of the rats. The computerized tracking system was applied for automatic video and data acquisition.