Original Article

RhEPO protects against white matter damage in neonatal rats by increasing MOG expression

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Abstract: Background: Erythropoietin (EPO) is considered to be a promising treatment for many disorders that affect the premature developing brain. However, the molecular details of its interactions remain unclear. Methods: Intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) (n = 11) or saline (n = 5) were administered to 15-day old pregnant Wistar rats. Newborn rats of the LPS group received i.p. injection of recombinant human erythropoietin (rhEPO) (n = 32) or saline (n = 32). The pups of the saline group received injection of saline (n = 32). Placenta tissues and pup brain tissues were examined with hematoxylin and eosin staining. Levels of mRNA of myelin oligodendrocyte glycoprotein (MOG) and protein levels of EPOR were detected by RT-PCR and ELISA, respectively. Assessments of nerve behavior were performed two weeks after birth. Results: Infected newborn rats were characterized by white matter damage, which also exhibited a decrease in the mRNA levels MOG and an increase in the protein levels of EPOR in brain tissues, especially on postnatal days 3 and 7. RhEPO treatment resulted in a significant increase in MOG mRNA level and nerve behavior in the brains of pups. Conclusion: Administration of rhEPO protected against white matter brain damage, which might be mediated by increasing the MOG mRNA level. Early treatment with rhEPO following white matter damage may represent a promising therapeutic strategy.

Keywords: Erythropoietin, white matter brain damage, neonatal, MOG

Introduction

An intra-uterine infection can cause white matter damage in neonatal infants, and this can lead to long-term neurological sequelae such as cerebral palsy and sensory and cognitive impairments [1, 2]. The most deleterious effect of intra-uterine infections is periventricular leukomalacia (PVL), a condition which is thought to be linked with excess production of pro-inflammatory cytokines in response to the infection [3]. Many investigations have been conducted to reveal the mechanism(s) that mediate white matter damage in neonatal infants [4, 5]. More recently, research has focused on the therapeutic strategies for treating white matter damage [6-8]. However, to date, no clinical effective treatment has been identified.

The capacity for erythropoietin (EPO) to treat disorders of the central nervous system (CNS) in the developing brain has been reported [9]. EPO is a glycoprotein which is mainly produced by fetal liver and adult kidney tissues [10]. In animal models, a beneficial effect of recombinant human erythropoietin (rhEPO) on brain damage after perinatal asphyxia and hypoxia-ischemia has been reported [11, 12]. In addition, rhEPO has been shown to reduce inflammation in autoimmune encephalomyelitis [13], while Shen and colleagues reported that EPO administration exerted a neuroprotective effect towards white matter damage in developing rat brains following an intra-uterine E. coli infection [14]. In the premature brains of goats that were administered LPS, rhEPO reduced axonal injury [15]. Meanwhile, in a mouse model of multiple sclerosis, EPO exhibited a therapeutic effect by reducing both inflammatory reactions and axonal injury [16]. Taken together, these results indicate that EPO can mediate a protective effect in inflammatory pathologies following white matter damage. However, the detailed mechanism(s) responsible for these observations remain to be characterized.

To date, most research on the neuroprotective effect of EPO has focused on cortical neurons,
rather than white matter [17-19]. However, white matter is one of the most important components of the brain parenchyma. For example, white matter affects many brain functions and coordinates communication between different regions of the brain. White matter damage includes the responsive transformation of astrocytes into glial cells and injuries involving oligodendroglia (e.g., demyelination, axon damage). Myelin oligodendrocyte glycoprotein (MOG) is a 26-28 kDa transmembrane glycoprotein that is expressed in myelin sheaths and oligodendrocytes, and is thought to be important for the myelination of nerves in the CNS [20]. In addition, MOG is considered to be a marker of oligodendocyte maturation based on its expression profile [21]. Most investigations involving MOG focused on its role in demyelinating diseases, such as multiple sclerosis, which often represent an inflammatory state [22, 23].

In the present study, the effects of rhEPO on white matter damage in neonatal rats was investigated in an LPS-induced intra-uterine infection model. We found that rhEPO can protect against white matter damage in neonatal rats, which may involve the increase of MOG expression.

**Materials and methods**

**Animal experiments**

This study was performed in accordance with guidelines established by the Animal Ethics Committee of Qingdao Laboratory Animal Center (Approval ID: SCXK (Lu) 20130007). Adult Wistar rats were obtained from the Qingdao Laboratory Animal Center and were housed together in mating groups until the female rats became pregnant. All of the animals were maintained under standard conditions of 23°C, a 12 h/12 h light/dark cycle, and access to food and water ad libitum.

The rats that became pregnant were randomly divided into two groups. On day-15 of gestation, one group received an intraperitoneal (i.p.) injection of LPS (0.3 mg/kg; Sigma, St. Louis, MO, USA) (n = 11), while a second control group received an i.p. injection of saline (n = 5). Immediately after birth, the pups from the maternal intra-uterine infection group received daily i.p. injection of rhEPO at a dose of 5000 IU/kg (n = 32) or saline (n = 32) until they were euthanized. The pups born to the control pregnant rats continued as controls (n = 32). Eight pups from each group were euthanized on postnatal days 0, 3, 7, and 14. Their brains were immediately resected and stored at -80°C until analyzed by RT-PCR and ELISA assays. Assessments of nerve behavior were performed two weeks after birth.

**Hematoxylin and eosin (H&E) staining**

After delivery, the placentas from the pregnant rats (n = 5) and the brains from the newborn pups (n = 10) were subjected to H&E staining as previously described [24]. Briefly, formalin-fixed, paraffin-embedded placenta and brain tissues were sectioned (5-µm thick) and prepared on silanized slides with deparaffinization and hydration performed with xylene and graded alcohols, respectively. The tissue sections were then stained with hematoxylin (1-5 min) and eosin sequentially, followed by dehydration steps with degraded alcohols. Finally, the slides were sealed with Permount mounting media (Bio-Rad, USA). The staining was visualized with light microscopy. The standards of white matter damage include pale white matter, loose structure, and coarse nerve fibers. There is also a tendency of coagulation necrosis or cystic change.

**Real time-PCR (RT-PCR)**

Total RNA was extracted from pup brains by using a HiFi Tissue/Cell RNA Extract kit (Beijing BLKW Biotechnology Co., China) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed with a HiFi Reverse Transcript Kit (Beijing BLKW Biotechnology Co.). In the RT-PCR assays, separate primers were used to detect MOG (forward: 5’-CTCAGTCGCTCTCCCCGATG-3’, reverse: 5’-GGAAGGACTGAGAAACACAG-3’) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5’-TACTGAGTCGCGCCTGTGTTG-3’, reverse: 5’-GGATCTCGCTCGTCAAAGAG-3’). The PCR reactions were performed with a SYBR Green qPCR Mix (Beijing BLKW Biotechnology Co.) and an MJ Research Chromo4 Detector (BioRad, Hercules, CA, USA). A SYBR green fluorescence quantification system was used and relative expression levels were calculated according to the 2^ΔΔCt method.
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ELISA assay

Cytoplasmic proteins in pup brain tissues were extracted by using a Cytoplasmic Protein Extraction Kit (Beijing BLKW Biotechnology Co.) and EPOR protein levels were measured with the Mouse EPOR ELISA Kit (Abcam, England), according to the manufacturers’ instructions.

Behavior test

Six rats in each group (LPS+rhEPO, LPS+NS and NS+NS) were subjected to open field test, suspension test and resistance test two weeks after birth. Open field test was performed in a 36 cm×36 cm×36 cm carton box without a top. The arena was divided into 9×9 squares. During the one-session test, each rat was placed in the center of the arena and allowed to explore freely for 15 sec. One score was recorded when the mouse entered the adjacent squares.

The suspension test was performed as follows: rats are suspended by their forelegs on a horizontal glass rod with 0.5 cm diameter, 45 cm away from the table. The time of rat falling down was recorded and scored: 1, < 10 sec; 2, 10 sec-30 sec; 3, 30 sec-2 min; 4, 2 min-5 min; 5, > 5 min. The resistance test was performed by observing the rat response to the capture using gloves which rat has never been touched. The scoring system was as follows: 0, easy to capture; 1, screaming or avoiding; 2, screaming and avoiding; 3, escaping; 4, escaping and screaming; 5, biting or trying to tear the gloves; 6, jumping to attack.

Data analysis

Data are presented as the mean ± standard error (S.E.M). Statistical analyses were performed by using independent-sample t-tests and one-way analysis of variance (ANOVA) with SPSS 17.0 software. Differences among the groups were considered to be significant when the P-values were less than 0.05.

Results

Intra-uterine infection induced a high death rate and premature delivery

Following LPS injection on eleven rats, two did not survive (death rate, 18.2%). The surviving rats all prematurely gave birth between day 19.0 and day 20.5 of gestation. The total number of pups born to the infected rats was 92, with 10 pups dying after birth (death rate, 10.9%). The rats in the control group gave birth between day 22.0 and day 22.5 of gestation and experienced normal deliveries. A total of 46 pups were born to the control rats, and 2

Figure 1. H&E staining of placenta and brain tissues. Pregnant rats received an i.p. injection of LPS (0.3 mg/kg) on day 15 of gestation. Saline injection was performed for the control rats. The placentas of the mother rats and the brains of the pup rats were fixed in 4% paraformaldehyde for H&E staining. Representative tissues are shown, including placenta tissues from infected (A) and control (B) rats and brain sections from infected (C) and control (D) pup rats. Tissue from infected pup rat with rhEPO treatment is also shown (E).
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died after birth (death rate, 4.3%). The death rates for the pregnant rats and the newborn pups were both much higher in the infection group than in the control group. Intra-uterine infections were associated with premature deliveries and a higher death rate for both the mother and pup rats.

**Intra-uterine infection induced white matter damage**

When the placenta tissues were examined with H&E staining, the tissues obtained from the rats that received injection of LPS exhibited congested and hydropic blood vessels compared to the control tissues, indicating the presence of hyperemia and edema (Figure 1A). More marked neutrophil infiltration was also observed in the infected placentas, thereby suggesting an inflammatory reaction had occurred. Brain white matter that was examined from the pups of the infection group was much looser than that of the control group. The structure of white matter of neonatal rats in experimental group was sparse and reticular (Figure 1C). In contrast, no obvious coagulative necrosis or cystic change were found in the control group. The white matter tissue was normal and the staining was clear (Figure 1D).

**MOG expression is reduced in the white matter damaged in newborn rats**

RT-PCR and ELISA assays were performed to detect mRNA levels of MOG and protein levels of EPOR, respectively. Initially, baseline levels of MOG mRNA and EPOR proteins were detected at P0, P3, P7, and P14 in the brains of pups born from the control pregnant rats. A time-dependent increase in MOG mRNA levels was observed (Figure 2A). The levels of EPOR also increased with time after birth, except there was no significant difference between the levels detected at P0 and P3 (Figure 2B).

Next, the levels of MOG mRNA and EPOR proteins were compared between the pups from the infection group and the pups from the control group. The level of MOG mRNA in the brains of the former group was significantly lower than that in the latter group at all four time points (P0, P3, P7, and P14) (P < 0.05, Figure 3). In contrast, there was no difference in the levels of EPOR at P0 and P14 between the infection group and control group. However, the EPOR levels at P3 and P7 were significantly higher in the infection group compared with the control group (P < 0.05, Figure 4).

**rhEPO rescued white matter damage and was accompanied by increased levels of MOG**

The effects of rhEPO on MOG mRNA and EPOR protein levels following intra-uterine infection were investigated. At P0, P3, P7, and P14, rhEPO treatment was accompanied by an increase in MOG mRNA levels in the brains of the pups from the intra-uterine infection group compared with the untreated infection group (P < 0.05, Figure 3). For EPOR, a significant increase in EPOR level was observed in the pup rat brains from the infection group treated with rhEPO at P7, yet not at P0, P3, or P14, compared with the untreated infection group (P < 0.05, Figure 4). H&E staining of brain tissue from the rhEPO treated group (Figure 1E) also showed more regular and clear periventricular white matter compared with brain tissues from the infection group.

**Behavior test**

The rats in the infection+rhEPO group showed more exploratory behavior on an open field active assay than the infection rats (P = 0.035,
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Figure 3. The effect of rhEPO on MOG mRNA levels in pup brains following their birth to mothers with intra-uterine infections. The pups from the maternal intra-uterine infection group received daily i.p. injection of rhEPO (infection+rhEPO group) or saline (infection group) after birth. The pups born to the control pregnant rats continued as controls (ctrl group). At P0 (A), P3 (B), P7 (C), and P14 (D), MOG mRNA levels were detected in pup brain tissue by RT-PCR. *P < 0.05, #P < 0.01.
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The behavioral activity of rats in the infection group was similar to the control group (3.33 ± 1.03 vs 3.83 ± 0.75, Figure 5). The rats in the infection+rhEPO group lasted for a longer time in grasping the glass rod in the suspension test. The scores of the rats in the infection+rhEPO group, infection group, and control group were 4.17, 3.67 and 3.17, respectively (Figure 5). The resistance test showed that the infection group had less resistant reactions to being picked up than the experimental group. The scores of the rats in the infection+rhEPO group, infection group, and control group were 4.67, 4.00 and 3.50, respectively (Figure 5).

Discussion

Recently, the survival rate for premature infants has increased markedly due to advances in obstetrics and intensive care for newborns. However, these premature infants are still at risk for compromised long-term neurodevelopment [25]. White matter damage has become a leading cause of newborn brain injury instead of hypoxia-ischemia injury, with approximately 25-40% of premature newborn infants developing white matter damage [26]. Intra-uterine infection has been identified as a cause of perinatal brain injury [27], mainly due to the triggering of an inflammatory response in the newborn infant brain that stimulates the release of cytokines such as IL-1 and TNF-α. As a result, permeability of the blood brain barrier is increased and this allows microorganisms, cytokines, and/or other products (e.g., endotoxins) to gain access to the CNS. The subsequent activation of microglia and astrocytes additionally stimulates the release of cytokines such as IL-6, IL-8, TNF-α, TOLL-like receptors, and reactive oxygen [28]. These cytokines can directly induce a toxic effect, affect myelination, and give rise to neurotoxicity and cytotoxicity of excitatory amino acids, thereby resulting in cell apoptosis and death. In the present study, an intra-uterine infection model was established in rats with intraperitoneal injection of LPS. H&E staining of placenta tissues from this model showed hyperemia and edema, the white matter was sparse and reticular changed compared to the control group.

Previously, it was confirmed that EPO can inhibit neuron apoptosis, inflammation, and reactive oxidative reactions, while also promoting angiogenesis and neurogenesis and mediating neurotrophic effects [29-31]. In these studies, the effects of EPO were mainly mediated th-
rough EPOR. Interactions between EPO and EPOR have been shown to induce the expression of myelin genes in oligodendrogial cells, including MOG and myelin basic protein (MBP), to facilitate the differentiation of oligodendrocyte precursor cells into oligodendrocytes, and to enhance the regeneration of myelin [16, 32]. Oligodendrocyte injury is one of the most important pathological features of hypoxic-ischemic encephalopathy (HIE). EPO induces a neuroprotective effect in HIE, and it may have a protective effect on oligodendrocytes as well [33, 34]. Here, LPS infection induced white matter damage in rats, and the offspring had significantly lower levels of MOG mRNA in their brains compared with control rats. These results suggest that inflammation may inhibit expression of myelin genes. With rhEPO treatment, MOG mRNA levels increased in the brain, and scores in the behavioral assessment were improved in the neonatal rats, indicating that rhEPO can promote regeneration of neurons and myelin expression. Thus, rhEPO may improve long-term outcome, consistent with previous results [32]. In addition, rhEPO treatment resulted in an increase in EPOR protein levels as detected by ELISA. It is hypothesized that EPOR may facilitate neuronal regeneration via an increase in MOG expression.

In conclusion, the results of the present study demonstrate that inter-uterine infections can induce premature birth and are closely related to the presence of white matter damage. Thus, reducing the incidence of perinatal infection is very important for the prevention of premature brain damage. In addition to early brain imaging examinations, regular histological biopsies on the placenta of premature newborns is necessary to discover the presence of inter-uterine infections and to provide early intervention and alleviation of related sequelae. RhEPO exhibited a protective effect in white matter damage induced by intra-uterine infection in the present model, and these results suggest that administration of rhEPO may represent a promising therapeutic approach for the treatment of brain injury in newborn infants. However, further investigations are needed to confirm these results and to optimize dosage and administration time of rhEPO.

Disclosure of conflict of interest

None.

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