Study on the inflammatory intervention of erythropoietin on NEC

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Abstract. The aim of this study was to investigate the effect of erythropoietin (EPO) on the inflammatory response and the mechanism analysis of the Toll-like receptor 4 (TLR4)/nuclear factor-kB (NF-kB) signaling pathway of NEC. A total of 94 patients with necrotizing enterocolitis (NEC) were randomly divided into the control (42 cases) and observation (52 cases) groups. The control group received the standard medical treatment plan, whereas for the observation group this treatment plan was combined with the application of recombinant EPO for intramuscular injection treatment. The clinical effect was subsequently compared. The results showed that the complication and death rates in the observation group were significantly lower than those in the control group with statistically significant differences (P<0.05). Following treatments, the levels of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 in the observation group were significantly lower than those in the control group. The expression levels of mRNA of TLR4 and NF-kB in the observation group were significantly lower than those in the control group, with statistically significant differences (P<0.05). In summary, EPO was able to significantly lower than those in the control group. The expression levels of mRNA (IL)‑6 in the observation group were significantly lower than those in the control group with statistically significant differences (P<0.05). Following treatments, the levels of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 in the observation group were significantly lower than those in the control group. The expression levels of mRNA of TLR4 and NF-kB in the observation group were significantly lower than those in the control group, with statistically significant differences (P<0.05). In summary, EPO was able to reduce the levels of inflammatory response of TNF-α and IL-6 through the TLR4/NF-kB signaling pathway, and improve the NEC, thus providing a basis for the clinical treatment of NEC.

Introduction

The main biological effect of erythropoietin (EPO) has been manifested through the promotion of proliferation, differentiation, and maturation of erythroid progenitor cells, as well as an increase in the number of red blood cells in the circulation (1). Therefore, EPO is widely used in the treatment of neonatal, cancer and renal anemia. Recent findings have shown that EPO and EPO receptors are widely distributed (2). They are expressed in a variety of non-hematopoietic tissues, and are also equipped with a variety of non-hematopoietic biological effects, such as angiogenic promotion, anti-inflammatory, anti-apoptosis and neuroprotection and are considered to be a systemic protective cytokine (2). It has been found that breast milk contains EPO, and intestines of neonatals contain EPO receptors, which indicate that EPO may play an important physiological role in the growth and development of the gastrointestinal tract (3). Previous studies suggested that the recombinant EPO in a mouse model of neonatal necrotizing enterocolitis (NEC) can reduce the inflammatory reaction and improve NEC injury (4).

The aim of the present study was to examine the clinical effect of EPO on the inflammatory response and possible mechanism of action of the TLR4/NF-kB signaling pathway, through clinical trials, to provide a new basis for the clinical treatment of NEC.

Patients and methods

Patients. In total, 94 cases diagnosed with NEC between August, 2013 and August, 2015 were selected for the present study. The diagnosis of NEC conformed to the standard of the American Society for Parenteral and Enteral Nutrition (5). The study was approved by the Ethics Committee of The Fourth Hospital of Shijiazhuang. Informed consent from the guardian, according to the order of admission was obtained. The 94 patients with NEC were randomly divided into the control (42 cases) and observation (52 cases) groups. A comparison of the baseline information identified that differences were statistically significant (P<0.05). Table I shows that NEC staging was performed in accordance with the standard of Bell's NEC staging.

Research methods. The control group were given the standard medical treatment plan, which included fasting, no hydration, gastrointestinal decompression, parenteral nutrition, selection of sensitive antibiotics, early stage, full weight, appropriate amount of rehydration, maintenance of gastrointestinal tissue perfusion, correct anemia, electrolyte disorder, acid-base
imbalance, blood pressure, heart rate, blood glucose and respiratory support. The abdominal X-ray was regularly performed to observe changes of the disease. If the disease was not relieved or continued to deteriorate with conservative treatment of internal medicine, surgical resection was subsequently performed. The observation group was treated with a combined application of recombinant EPO (National Medical License no. S20000007, 2,000 IU/dose; Shandong Kexing Bioproducts Co., Ltd., Shandong, China) for intramuscular injection, at a dose of 200 IU/kg, twice a week for a total of 1 week. Any allergic reaction or other adverse reactions were observed closely.

**Observation index and detection technology.** The differences of the complication and death rates were compared between the two groups, including levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and the mRNA expression levels of Toll-like receptor 4 (TLR4) and nuclear factor-xB (NF-xB). Venous blood (5 ml) was collected on an empty stomach and centrifuged at 2,800 g at high speed and the supernatant was removed after 5 min. TNF-α and IL-6 were detected by quantitative ELISA kits purchased from Shanghai J&M Co. Ltd. (Shanghai, China). The ELISA method was applied and the experiment was performed according to the manufacturer's instructions. The expression of mRNA for TLR4 and NF-κB was quantified. The relative expression amount of TLR4 and NF-κB in each group was determined after correction of the housekeeping genes.

**Statistical analysis.** SPSS 20.0 statistical software (IBM SPSS, Armonk, NY, USA) was applied to determine statistical analysis. Measurement data were presented as mean ± standard deviation. The t-test was applied for comparisons between groups and the Kruskal-Wallis H was applied to make pathological scoring of intestinal tissues. Enumeration data were presented as a percentage (%). The χ² test was applied for comparisons between groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Comparison of complication and death rates.** The complication and death rates in the observation group were significantly lower than those in the control group and differences were statistically significant (P<0.05) (Tables I and II).

**Comparison of the expression levels of TNF-α and IL-6.** Prior to intervention, differences between the two groups with regard to the levels of TNF-α and IL-6 were not statistically significant (P>0.05). After intervention, the levels of TNF-α and IL-6 of the control group were increased while the levels of the observation group were reduced. The levels of the observation group were significantly lower than those of the control group and differences were statistically significant (P<0.05) (Table III).

**Comparison of mRNA expression levels of TLR4 and NF-xB.** Prior to intervention, differences between the two groups with regard to the mRNA expression levels of TLR4 and NF-xB were not statistically significant (P>0.05). After intervention, the mRNA expression levels of TLR4 and NF-xB in the two groups were significantly decreased and those in the observation were lower than those in the control group. Differences were statistically significant (P<0.05) (Table IV).

**Discussion**

EPO is a glycoprotein with a molecular weight of 30.4 kDa. In the fetus period, it is mainly synthesized by liver and post birth, and the kidney. Ledbetter and others (6) found from a retrospective study that the incidence of NEC on premature infants with EPO injection was significantly lower than that of infants not treated with EPO (7). To study the protective mechanism of EPO on NEC, Kumral and others (8) used EOP to prepro- cess hypoxia reperfusion NEC mice (EPO 750 U/kg/week, intraperitoneal injection in 3 shots for 2 weeks). On day 15, the animals were exposed to 100% CO₂ for anoxia for 5 min, followed by reoxygenation with 100% O₂ for 10 min, and then executed after 4 h. Compared to the result of the experimental group, the level of nitric oxide in the EPO pretreatment group was significantly decreased, and the injury of the intestinal

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**Table I. Comparison of baseline information between the control and observation groups.**

| Group                        | Control group, n=42 | Observation group, n=52 | t (χ² test) | P-value |
|------------------------------|---------------------|-------------------------|-------------|---------|
| Male/female                  | 23/19               | 28/24                   | 0.008       | 0.929   |
| Age, day                     | 13.4±2.5            | 12.5±2.4                | 0.624       | 0.337   |
| Weight, kg                   | 2.2±0.6             | 2.1±0.5                 | 0.421       | 0.564   |
| 1 min Apgar score            | 8.2±0.7             | 8.3±0.6                 | 0.519       | 0.768   |
| NEC staging                  |                     |                         |             |         |
| I                            | 6 (14.3)            | 6 (11.5)                | 0.920       | 0.631   |
| II                           | 26 (61.9)           | 37 (71.2)               |             |         |
| III                          | 10 (23.8)           | 9 (17.3)                |             |         |
| Premature birth section      |                     |                         |             |         |
| Caesarean section            | 20 (47.6)           | 23 (44.2)               | 0.107       | 0.743   |
| Breast feeding               | 29 (69.0)           | 33 (63.5)               | 0.373       | 0.830   |
| Milk powder feeding          | 6 (14.3)            | 8 (15.4)                |             |         |
| Breast + milk powder feeding | 7 (16.7)            | 11 (21.2)               |             |         |
| Hemoglobin, g/l              | 185.6±13.7          | 184.2±14.5              | 0.967       | 0.325   |
| Hematocrit                   | 62.4±1.7            | 62.5±1.6                | 0.624       | 0.857   |

respectively. Subsequently, the standard curves of the target genes (TLR4 and NF-xB) and housekeeping genes (GAPDH) were created. By means of the standard curves, the expression of the target and housekeeping genes in the samples was quantified. The relative expression amount of TLR4 and NF-xB in each group was determined after correction of the housekeeping genes.
tissue was reduced, indicating that EPO retarded the occurrence of NEC in mice by inhibiting the excessive production of nitric oxide (9). Akisu and others (10) used the same method to study the role of EPO in the production of intestinal malondialdehyde (MDA) and platelet activating factor (PAF). Their study results showed that although EPO cannot inhibit the production of PAF in the necrotic intestine tissues resulting from hypoxia reperfusion, it can significantly decrease the MDA content in the intestinal tissues of mice, indicating that EPO may play a role in the prevention and treatment of NEC by inhibiting the lipid peroxidation mediated by oxygen-free radicals (11). Shiou and others (12) have shown that supplementing EPO via the intestinal tract upregulated the expression of ZO-1, a tight junction protein in the enterocytes of NEC mice, which protected the intestinal mucosal barrier function (13). Halpern and Denning (7) used 3-day SD mice and constructed a NEC model after artificial feeding and hypoxia cold stimulation, and intervened by supplementing EPO of a similar physiological concentration as breast milk through the intestinal tract. Their study results showed that EPO downregulated the expression of TLR4 in intestinal tissues of mice and inhibited the inflammatory cascade reaction, reduced the intestinal tissue damage, retarded the malignant progression, and finally reduced the occurrence of bacterial translocation (3,12).

The role of TLR4 in the pathogenesis of NEC has aroused increasing attention (14). The expression of TLR4 in normal intestinal epithelial cells is low, whereas in NEC children and animal models is high. The high expression of TLR4 appeared even before NEC tissue injury. High-risk factors of NEC, including premature birth, artificial feeding, hypoxia ischemia, and bacterial factors can lead to an increase of TLR4 expression (4). The role of TLR4 in the pathogenesis of NEC was mainly associated with inflammation and repair (15). Activated TLR4 can activate NF-κB, and induce the upregulated expression of downstream inflammatory cytokines. NF-κB in intestinal tissues removed from animal models and NEC children showed a sustained high expression, while the expression of the inflammatory cytokines in the lower reaches, such as TNF-α, IL-6 and IL-8, also increased. A large number of inflammatory mediators leads to damage in the intestinal

### Table II. Comparison of complication and death rates.

| Group              | Cases | Septicemia | Acute intestinal perforation | Infectious peritonitis | Others | Complication rate | Death rate |
|--------------------|-------|------------|-------------------------------|------------------------|--------|-------------------|------------|
| Control group      | 42    | 7          | 6                             | 6                      | 2      | 21 (50.0)         | 11 (26.2)  |
| Observation group  | 52    | 5          | 5                             | 4                      | 2      | 16 (30.8)         | 5 (9.6)    |
| $\chi^2$ test      |       |            |                               |                        |        | 3.868             | 4.519      |
| P-value            |       |            |                               |                        |        | 0.049             | 0.034      |

### Table III. Comparison of the levels of TNF-α and IL-6 between the observation and control groups.

| Group       | Pre-intervention | Post-intervention | Pre-intervention | Post-intervention |
|-------------|------------------|-------------------|------------------|-------------------|
| Control     | 56.7±6.4         | 78.4±10.2         | 241.3±36.9       | 287.5±48.7        |
| Observation | 61.2±6.8         | 32.5±4.8          | 259.6±42.3       | 143.2±32.5        |
| t-test      | 0.529            | 6.498             | 0.629            | 6.213             |
| P-value     | 0.423            | 0.012             | 0.415            | 0.018             |

TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

### Table IV. Comparison of mRNA expression levels of TLR4 and NF-κB.

| Group       | Pre-intervention | Post-intervention | Pre-intervention | Post-intervention |
|-------------|------------------|-------------------|------------------|-------------------|
| Control     | 3.3±0.5          | 2.6±0.3           | 2.4±0.6          | 1.8±0.2           |
| Observation | 3.6±0.7          | 1.2±0.4           | 2.7±0.5          | 0.7±0.1           |
| t-test      | 0.924            | 7.436             | 0.825            | 8.125             |
| P-value     | 0.721            | <0.001            | 0.364            | <0.001            |

TLR4, Toll-like receptor 4; NF-κB, nuclear factor-kB.
epithelial cells and mucosal defense mechanism, which would ultimately develop into NEC. Inhibiting of the TLR4 pathway can significantly reduce the expression of inflammatory mediators, alleviate macrophage infiltration, and reduce the occurrence of bacterial translocation (16,17).

Results of the present study show that the complication and death rates in the observation group were lower than those in the control group. The levels of TNF-α and IL-6 in the observation group were significantly lower than those in the control group. The mRNA expression levels of TLR4 and NF-κB in the observation group were significantly lower than those in the control group, and differences were statistically significant. In conclusion, EPO can reduce the levels of inflammatory response of TNF-α and IL-6 through the TLR4/NF-κB signaling pathway and improve NEC, thereby providing a basis for the clinical treatment of NEC.

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