Erythropoietin Inhibits the Increase of Intestinal Labile Zinc and the Expression of Inflammatory Mediators After Traumatic Brain Injury in Rats

Lin Zhu, MD, Wei Jin, MD, Hao Pan, MD, Zelan Hu, MD, Jing Zhou, MD, Chunhua Hang, MD, PhD, and Jixin Shi, MD, PhD

**Background:** The objective of this study was to determine the effect of erythropoietin (Epo) on the intestinal labile zinc and the inflammatory factor in rats after traumatic brain injury (TBI).

**Methods:** Male Sprague-Dawley rats were randomly divided into nine groups: (a) normal group; (b) sham-operation group; (c, d, e, f, and g) TBI group, killed at 1 hour, 6 hour, 24 hour, and 72 hour and 7 days postinjury, respectively; (h and i) TBI + saline and TBI + Epo, killed at 24 hour or 72 hour postinjury. Parietal brain contusion was produced by a free-falling weight on the exposed dura of the right parietal lobe. Intestinal labile zinc, the tumor necrosis factor-α, interleukin (IL)-8, and wet/dry weight ratio were investigated in different groups.

**Results:** The gut contains a certain amount of labile zinc in normal animals and TBI caused obviously gradual increment of intestinal labile zinc. The levels of inflammatory mediators and the gut wet/dry weight ratio were also found to increase in the trauma group ($p < 0.05$).

**Conclusions:** Epo can protect intestine from TBI-induced injury by attenuating intestinal inflammation and labile zinc accumulation in vivo.

**Key Words:** Traumatic brain injury, Intestine, Erythropoietin, Zinc, Cytokines.


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Erythropoietin (Epo) is a glycoprotein and cytokine of 34 kDa, which is produced mainly by the fetal liver and the adult kidney in response to hypoxia. The first identified main function of this pleiotropic factor is that it plays a crucial role in erythropoiesis. In recent years, Epo has been found to have important nonhematopoietic functions in the nervous system. Through attenuation of apoptosis, modulation of inflammation, and recruitment of stem cells, it protects neurons from glutamate toxicity. Furthermore, the first clinical trial using recombinant human erythropoietin (rhEpo) for patients suffering from acute stroke has obtained encouraging results. Also, a randomized phase II clinical trial is undergoing for traumatic brain injury (TBI) treatment in the United States.

TBI represents one of the most important causes of death and disability in the modern society. Acute gut mucosa injury is a common complication in comatose victims. Previous studies in our laboratory have demonstrated that TBI could induce marked damage of the intestinal mucosa structure in which inflammatory cytokines may play an essential role. These cytokines work together to promote not only a regional destructively inflammatory response, but also systemic inflammatory response syndrome and sepsis with subsequent multiple organ failure.

Zinc (Zn) is the second most abundant trace element in the human body. It participates in a wide variety of physiologic and pathologic processes in the body and exerts roles as an important immunoregulatory agent or growth cofactor. Using several fluorescent Zn$^{2+}$ indicators, previous studies concentrated mainly on histologic changes of brain structures associated with ischemia, seizure, and trauma. However, whether a TBI could alter intestinal zinc homeostasis and which pathologic process the zinc disturbance may lead have not been well elucidated to date.

The aim of the present study was to determine (i) temporal pattern of intestinal zinc and proinflammatory cytokine expression after TBI and (ii) the effect of Epo on intestinal proinflammatory factor and labile zinc after TBI.

**MATERIALS AND METHODS**

**Animal Preparation**

Adult male Sprague–Dawley rats (200–250 g) were purchased from Animal Center of Chinese Academy of Sciences, Shanghai, China. The rats were housed in temperature- and humidity-controlled room with a 12-hour light/dark cycle, room temperature was maintained at 23°C ± 1°C, and were given free access to drinking water and food throughout the experiments. Body weight was monitored as an index for...
evaluating nutritional state. All procedures were approved by the Animal Care and Use Committee of Nanjing University and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Weight Drop TBI and Administration of rhEpo**

After anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), animal head was fixed in the stereotactic device (ZH - Lanxing China), permitted to breathe spontaneously through a natural airway. A contusion injury of the right parietal cortex was produced using a modification of the Feeney model.\(^{13}\) The scalp and temporalis muscles were reflected and a 3.0-mm diameter burr hole was drilled through the skull 3.0-mm lateral to the midline and 4.0-mm caudal to bregma. Trauma was performed by letting a steel rod weighing 40 g with a flattened diameter of 5-mm fall onto a piston resting on the dura from a height of 25 cm. The piston was allowed to compress the tissue a maximum of 3 mm. The rats were randomly assigned to nine groups: (a) normal group (n = 10); (b) sham-operation group (n = 10); (c, d, e, f, and g) TBI group (n = 10/group), killed at 1 hour, 6 hour, 24 hour, and 72 hour and 7 days postinjury, respectively; (h and i) TBI + Epo and TBI + saline group (n = 20/group), killed at indicated time points. RhEpo at a dose of 5000 IU/kg or control saline was applied by systemic administration 30 minutes after TBI. This dose and time points of rhEpo administration have been widely used in analogous animal models described elsewhere.\(^{14,15}\) RhEpo was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

**Obtaining Samples From Gut**

At indicated time points, rats were killed by an overdose of anesthesia and exsanguinated thoroughly from heart, then a 6-cm segment of the midjejunum was removed from freshly killed animals for determining cytokine levels (tumor necrosis factor [TNF]-\(\alpha\) and interleukin [IL-8]), labile zinc, or for the intestinal wet/dry weight ratio.

**Intestinal Wet/Dry Weight Ratio**

The intestinal wet/dry weight ratio was assessed as described in previous study.\(^{16}\) Briefly, after the gut tissue samples were taken, excess fluid was blotted from specimens, and wet weights were measured. Dry weights were measured after drying specimens at 80°C for 72 hour till constant weight. The gut wet/dry weight ratio was then calculated.

**Zinpyr-4 Staining and Zinpyr-4-Positive Cells Counting**

For preparation of gut sections subjected to zinc staining, the frozen, unfixed jejunal tissue were sliced into 10 \(\mu\)m sections at the temperature of \(-14\)°C using a cryostat (Leica CM 1900), and then mounted onto gelatin-coated slides and dried by gentle airflow. ZP4 (Zinpyr-4) fluorescence zinc staining was performed as described in previous study with slight modifications.\(^{17}\) ZP4 was freshly diluted in phosphate-buffered saline to a final concentration of 25 \(\mu\)mol/L and immediately pipetted onto these sections. The gut sections were incubated for 60 seconds at room temperature in dark and after rinsing in saline. Images were collected by a conventional compound fluorescence microscope (Zeiss Universal: exciter, 420–490 nm; dichroic beam splitter, 500 nm; barrier, 550 nm long pass). Tissue images were captured into a PC workstation by CCD camera (Retiga 1300R) and quantified. Ten villi of each section were observed under 100× objective lens. The average number of ZP4-positive fluorescence in the 10 villi was regarded as the data for each sample. ZP4 were purchased from NeuroBioTex (Galveston, TX).

**Enzyme-Linked Immunosorbent Assay**

The intestinal levels of inflammatory mediators were quantified using specific ELISA kits for rats according to the manufacturer’s instructions (TNF-\(\alpha\), Diaclone Research, France; IL-8, Biosource Europe SA, Nivelles, Belgium).\(^{18}\) Values were expressed as picogram per milligram of protein.

**Statistical Analysis**

All data were presented as mean ± SD. One-way analysis of variance with Tukey’s post test was performed using Graphpad Prism version 4.0 (Graphpad Software, San Diego, CA). Statistical significance was assumed for \(p < 0.05\).

**RESULTS**

No traumatic-related death was observed in all experimental groups.

**Intestinal Wet/Dry Weight Ratio**

The wet/dry weight ratio, which represents the percentage of tissue water, is a creditable index of tissue microvascular permeability. Compared with the normal or the sham group, the gut wet/dry weight ratio in the TBI group initially increased at 6 hour, and peaked at 72 hour postinjury. However, administration of rhEpo 30 minutes before insult markedly decreased the wet/dry weight ratio, compared with TBI or TBI + saline group at the time point of 72 hour. Quantification of gut wet/dry weight ratio shows that rhEpo could effectively inhibited TBI-induced increase of gut wet/dry weight ratio at 72 hour postinjury (Fig. 1).

**Intestinal ZP4 Fluorescent Staining**

In the normal group, there is certain amount of fluorescent “spots” in the lamina propria along the villus revealed by ZP4 staining (Fig. 2, A). The number of ZP4-positive cells and the intensity of fluorescent signals had no significant difference between normal and sham-operation group. After TBI, ZP4 fluorescence intensity in the gut started to increase at 6 hour and fluorescent cells was abundant at 24 hour. The maximum intensity of ZP4 fluorescence was observed at 72 hour after TBI (Fig. 2, B).
Enzyme-Linked Immunosorbent Assay

There was no significant difference in the level of TNF-α between normal and sham-operation groups (Fig. 3, A). Compared with those from the control group, intestinal TNF-α level was greatly increased at 6 hour after TBI, reached maximum at 24 hour postinjury, and remained elevated significantly at 72 hour postinjury. At the time point of 7 days, the level of TNF-α declined but was still higher than the levels of the control group. There was a highly positive correlation between the abundance of ZP4 fluorescent and TNF-α concentrations \((r = 0.7857, p < 0.05)\).

Furthermore, we studied the concentration of IL-8 in gut after TBI. There was no significant difference of IL-8 concentrations between normal and sham-operation rats (Fig. 3, B). However, IL-8 concentration in the gut was significantly increased at 6 hour and maintained high until 7 days after TBI, compared with control group. The peak concentration of IL-8 was detected at 24 hour postinjury. The temporal profile of IL-8 after TBI was similar to that of the abundance of ZP4 fluorescent in the gut, which showed a highly positive correlation as well \((r = 0.7143, p < 0.05)\).

RhEpo Effect on Intestinal ZP4 Fluorescence and Inflammatory Factors after TBI

In rhEpo-treated group, the number of ZP4-positive cells decreased dramatically along the villus, compared with TBI or TBI + saline group 72 hour after TBI. Quantification of ZP4-labeled cells showed that rhEpo could effectively inhibit the increase of intestinal ZP4 fluorescence signal induced by TBI (Fig. 4). Systemic administration of rhEpo 30 minutes after TBI could also obviously reduce the expression level of the intestinal proinflammatory factors after TBI (Fig. 3).

DISCUSSION

Gastrointestinal dysfunction, such as gastrointestinal bleeding, gastric reflux, and decreased intestinal peristalsis, is a common complication in patients with TBI in surgical intensive care unit, which could influence the outcome after TBI. It is generally accepted that the intestine may serve as an important organ in the development of severe complications under critically ill conditions, including trauma, burns, and shock. Studies on nonneurologic complications associated with TBI concentrated mainly on the pulmonary edema and pneumonia, cardiac arrhythmia, electrolyte disturbance, and hematologic change. However, the effect of TBI on intestinal mucosa has not been studied extensively.

In this article, we used the ZP4, a second-generation member of the Zinpyr family of Zn\(^{2+}\)-selective sensors. ZP4 is far more specific and sensitive for detecting the free or more loosely bound (labile) pools of intracellular Zn than the quinoline-based reagents, such as TSQ, Zinquin, or TFLZn,
which are traditionally used for staining ionic Zn in biological samples.\textsuperscript{31,32} Using several histochemical techniques,\textsuperscript{33–35} it is found that the small intestine contains a certain amount of labile zinc within several regions of the normal gut. Our result is consistent with them in the normal or sham-operated group. However, there have been no studies of Zn redistribution in the gut after TBI. Zinc is essential for normal digestive tract physiology, though its precise contribution is not well understood.\textsuperscript{36} To date, the pathologic role of the increased chelatable zinc has been mainly examined in the central nervous system and pancreas, implicated in several acute or chronic diseases.\textsuperscript{12,37} For example, Zn\textsuperscript{2+} in pancreatic \( \beta \) cells is involved in the formation of insoluble insulin hexamer in secretory granules,\textsuperscript{38,39} which is also co-secreted with insulin after stimulation with secretagogues.\textsuperscript{40} Excessive secretion of zinc, however, has been linked to the death of these cells in a model of type I diabetes.\textsuperscript{41} Similar phenomena may occur in neurons in central nervous system.\textsuperscript{42} Chelatable zinc in the brain is involved in modulating synaptic activity at excitatory and inhibitory synapses and is also thought to be a key element in events leading to cell death during episodes of excessive release. In the present study, we demonstrated for the first time that TBI could induce a rapid and persistent increment of labile zinc in gut, which indicated that the importance of zinc homeostasis in normal gut function and the role of disrupted zinc metabolism in pathogenesis of acute gut mucosal injury.

At present, we can only formulate conjectures to explain our main findings. Increased chelatable zinc might be accumulated through several sources, including the mobilization and redistribution from metallothioneins or mitochondria\textsuperscript{43,44} as well as the recruitment of the granulocytes (3 types) which contain high level of zinc in their cationic protein.\textsuperscript{45} In general, cytokines are not stored in intracellular apparatus, and their secretion depends on new protein synthesis. As a consequence, release of cytokines in response to an inflammatory stimulus, such as trauma, sepsis, and hemorrhagic shock, is predominantly regulated by the transcription rates of cytokine genes. Kim et al.\textsuperscript{46} have demonstrated that zinc could induce IL-8 expression through mitogen-activated protein kinase (MAPK) and activator protein 1 (AP-1) activation in human airway epithelial cells. The MAPKs and AP-1, which play critical roles in regulating gene transcription during the processes such as cell proliferation and differentiation, apoptosis, inflammation, and the immune response, are important trans-
ducers of extracellular stimuli to the nucleus. Because transcriptional regulation is important for the production of many cytokines, it is not strange that zinc may play a key role in regulating cytokine-mediated inflammation. However, there has no direct evidence showing that zinc could induce intestinal inflammation cytokine expression both in vitro and in vivo. The current study showed that intestinal labile zinc, TNF-α, and IL-8 were significantly up-regulated as early as 6 hour after TBI and there was a positive relationship among them. These findings suggest that a modulation may exist in the interaction of zinc with proinflammatory cytokines.

Excessive cytokine-mediated inflammation is likely to play a fundamental role in the pathogenesis of a variety of disease states, including alterations of intestinal mucosa structure, the sepsis syndrome, and the adult respiratory distress syndrome. Our present study has been developed to evaluate the role of inflammatory reaction in the gut after TBI. It has been shown that many proinflammatory cytokines have a cytotoxic effect by inducing apoptosis of intestinal epithelium, thus resulting in the destruction of intercellular tight junctions and increased permeability. TNF-α, regarded as the most important proinflammatory cytokine, is considered a major initiator of inflammation and is released early after an inflammatory stimulus. IL-8 is a chemokine that stimulates migration of neutrophils from intravascular to interstitial sites and can directly activate neutrophils and regulate the expression of neutrophil adhesion molecules. Elevated serum level of IL-8 is associated with necrotizing enterocolitis. Our previous study demonstrated that TBI could lead to significant intestinal mucosal damage and the present study provided the direct evidence that TBI could apparently up-regulate tissue water, because of increased microvascular permeability. Therefore, we concluded that the intestinal inflammation response may be mainly mediated by zinc and proinflammatory cytokines might contribute to the acute intestinal injury after TBI.

Epo is a growth factor produced by the kidney in response to anemia. It is well recognized for its erythropoietic effect mediated by Epo receptor binding and JAK2 signal transduction to arrest the constitutive apoptosis of erythrocyte progenitor cells allowing maturation and differentiation of nascent erythrocytes. More recently, it has been recognized that Epo and Epo receptors are also expressed in other tissues and organs, including the brain, heart, intestine, and lung. Although Epo receptors are reported to be expressed in human fetal and postnatal intestine, the role of Epo has been less well characterized in the intestine, thus we used rhEpo to study if it is able to protect the gut against TBI-induced damage.

In the article, we demonstrated for the first time that through inhibiting the increased intestinal labile zinc and inflammation factors expression, rhEpo may protect gut from damage induced by TBI. Cuzzocrea et al. have demonstrated that Epo is protective in experimental colitis and that inhibition of TNF-α formation (among other myeloperoxidase effects that include inhibition of neutrophils infiltration) in the colon probably accounts for its beneficial effects. Claud et al. have found that enterocytes have an exaggerated IL-8 secretion in response to TNF-α, whereas Epo is able to decrease this stimulated IL-8 secretion, which may partially explain the protective effect of Epo in necrotizing enterocolitis. Using in vitro models, Epo has been shown to decrease TNF-α-induced intestinal epithelial cells apoptosis. Our result of Epo’s effect on proinflammatory cytokines is consistent with their results in the TBI-induced acute gut mucosa injury. Additionally, previous studies have demonstrated that Epo can maintain mitochondrial membrane potential and inhibit caspase activity. Loss of ΔΨm through the opening of the mitochondrial permeability transition pore represents a significant determinant for cell injury and the subsequent induction of apoptosis. Thus, it is possible that rhEpo may protect enterocytes through preventing the zinc outflow from mitochondria and preventing the release of cytochrome c that ultimately can trigger cellular apoptosis. Reactive oxygen metabolites and nitric oxide also play an important role in direct injury against the intestinal mucosa. It has been proposed that suppressing formation of NO-mediated free radicals or antagonizing their toxicity may underlie the tissue-protective effect of Epo. In brief, it is possible that Epo undertakes multiple mechanisms in combination to protect the intestine tissue against TBI.

During the past decades, our understanding of the pathophysiology of TBI has greatly increased. Based on this understanding, numerous pharmacological therapies have been developed, tested, and proven effective in the treatment of experimental TBI. To date, however, promising experimental results have not been fully translated into successful clinical trials. Our results indicated that administration of Epo during TBI has protective effect, not only in brain, but also in gut. Thus, to intervene the pathophysiology process in TBI, an overall therapy for TBI is emphasized. Indeed, additional studies are required to clarify the exact underlying mechanisms of the protective effect on intestinal mucosa after TBI as well as the dosage of Epo on potential clinical trials.

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REFERENCES


