Original Article

Zinc influences innate and adaptive immune responses in mice with enterotoxicogenic Escherichia coli-induced diarrhea

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Abstract: Enterotoxigenic Escherichia coli (ETEC) is a major cause of diarrheal disease. Information is limited on the effect of zinc on immune responses to diarrhea induced by ETEC. We investigated the immunological effects of zinc treatment in mice with diarrhea caused by ETEC. Our study included a total of 6 C57BL/6 mice aged 7-8 weeks that were treated with a high zinc diet for 2 weeks as well as 6 mice with ETEC-induced diarrhea that were not treated with zinc (UT). Six control mice (CON) of the same age group were also studied. The UT group showed more severe disruption to intestinal morphogenesis than ZT group. Serum zinc, complement C3, IgG, and IgM concentrations were higher in the ZT group than those in UT group but did not differ from those in the CON group. Phagocytic activity in mice in ZT group was greater than that in the UT group. However, oxidative burst capacity was lower in ZT group than in the UT group. The CD4/CD8 T-cell ratio in ZT group was higher than that in the UT group. Increased responses including complement C3, phagocytic activity, and changes in T-cell phenotypes suggests that zinc treatment enhances innate and adaptive immunity against ETEC infection in mice.

Keywords: Diarrhea, enterotoxigenic Escherichia coli, zinc deficiency, immune responses

Introduction

Enterotoxigenic Escherichia coli (ETEC) is the major bacterial pathogen causing diarrheal disease worldwide, in both children and adults [1]. EAEC is acquired through consumption of contaminated food or water. Diarrheal disease caused by EAEC can present either acutely or persistently, with mucoid and/or watery stool.

Previous studies have demonstrated that malnutrition increases the severity of symptoms of EAEC infection [2, 3] and is associated with recurrent diarrhea [4]. Zinc deficiency can increase the risk of a child developing a more severe diarrheal illness following the ingestion of pathogens [5] and has been associated with longer duration and increased morbidity [6]. In turn, diarrhea also leads to substantial zinc loss and abnormalities of zinc metabolism [7, 8]. The benefits of zinc on diarrhea are widely recognized [9] and supplemental zinc has been shown to improve intestinal barrier function and decrease duration of diarrhea [10-12].

Previous studies have been carried out in an attempt to understand immune responses to ETEC infection and mostly have been centered on understanding the specific humoral immune response [13, 14]. However, less is known about innate and adaptive cellular immune responses to this infection. The objective of this study was to investigate both the innate and adaptive immune responses of mice with ETEC-induced diarrhea that were given a high zinc diet as treatment for 14 days and to determine whether zinc supplementation had any beneficial effect.

Materials and methods

Animals and experimental design

All experimental procedures were performed according to the guidelines of the Laboratory
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Animal Ethical Commission of the Sichuan Animal Science Academy. Enterotoxigenic Escherichia coli were isolated from piglet diarrhea. The ETEC infection model was established according to the method described by Allen et al. [15]. Female C57BL/6 mice (7-8 weeks old), weighing 20-22 g were purchased from SLAC Laboratory Animal Central (Shanghai, China) and were housed under a controlled environment at a temperature of 25 ± 2°C and a 12 hour light/dark cycle at 50-70% humidity. All animals had free access to food and water. Animals were acclimatized for 1 week before the experiments were conducted. Thirty healthy C57BL/6 female mice were randomly divided into 3 treatment groups (10 in each group). Control mice were administered 0.4 mL sterile PBS. For the ETEC (un-treatment, UT) and zinc treatment (ZT) groups, mice were inoculated by oral gavage with the prepared ETEC strain K88 suspension (0.4 mL, 3.41 × 10^9 CFU/mL). The control group and UT group were fed up with a zinc deficient diet (content of zinc < 1 mg/kg) for 2 weeks, respectively. ZT group were fed with a high zinc diet (500 mg/kg). Mice were monitored and weighed daily. Studies ranged from 1-14 days in duration. Following completion of the study, animals were humanely euthanized by intraperitoneal administration of sodium pentobarbital followed by manual cervical dislocation. Samples collected from these animals included blood (for serum), jejunum, and ileum sections. Weight at the time of euthanasia was also recorded. Tissues were flash frozen in liquid nitrogen and stored at -80°C for further analysis.

**Histopathological analyses**

For light microscopic observation, jejunum tissues were fixed with 10% formalin at 4°C, dehydrated in a graded series of ethanol, then embedded in paraffin. Tissue sections (5 μm) were mounted on slides, dewaxed, hydrated, and then stained with hematoxylin and eosin.

**ELISA analysis**

The titers of IgM and IgG in the serum samples were measured using ELISA (BD Pharmingen, San Diego, CA, USA). Levels of complement C3 in serum were measured using C3 ELISA kit (Kamiya Biomedical, Seattle, WA, USA). The serum zinc concentration was determined using atomic absorption spectrophotometry [16].

**Phagocytosis assay**

The phagocytic capacity of polymorphonuclear granulocytes (PMN) and monocytes was measured by flow cytometry, as described previously [17]. Briefly, 4 × 10^7 FITC-labeled E. coli (molecular probes) was added to 100 μL heparinized whole blood and incubated at 37°C for 10 minutes in the dark. At the end of incubation, samples were kept on ice and 100 μL of quenching solution was added. DNA staining was performed to exclude aggregation artifacts from bacteria or cells. The reaction was stopped by the addition of lysing solution, which removed erythrocytes and partially fixed leukocytes. After washing, cells were acquired by FACSCalibur flow cytometer (BD Biosciences) using CellQuest software (Becton Dickinson) for data acquisition and Flowjo software (Tree Star version 4.3) for analyses.

**Oxidative burst assay**

The oxidative burst activity of PMN and monocytes was assessed using flow cytometry to measure the intensity of redox indicator dye 29 79-dichlorodihydrofluorescein (DCF), as described earlier [17]. DCF-diacetate (Sigma) was added to 0.1 mL heparinized blood at a
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Figure 2. Histological appearance of representative hematoxylin and eosin-stained intestinal tissue sections (× 200 magnification) in mice. A. The intestinal architecture was disrupted in UT mice challenged with EAEC. Normal-appearing colonic crypts are uniform and evenly placed, with no increase in mucosal inflammatory cells. Infected mice had more severe epithelial damage with crypt architectural distortion, submucosal edema, goblet cell depletion, and infiltration of acute and chronic inflammatory cells deep to the crypts. B. Villus to crypt ratio was significantly altered in these mice compared to either uninfected control. C. Histological damage score, which is the summation of scores for epithelial integrity, submucosal edema, mucodepletion, crypt hyperplasia, and neutrophil and mononuclear infiltration. Values are mean ± SD, n = 6/group. *P < 0.05, compared with CON group. #P < 0.05, compared with UT group.

**Statistical analysis**

Statistical analysis was performed with SPSS software version 13.0. Results are expressed as mean ± standard deviation (SD). Significance of differences between the two groups was tested by Student’s t-test or ANOVA. A P value of < 0.05 was considered statistically significant.
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Results

Zinc treated mice have lower weight loss after EAEC challenge

Mice maintained on a zinc deficient diet (UT) or high zinc diet (ZT) for 2 weeks were pretreated with an antibiotic cocktail as described above, prior to infection. As shown in Figure 1, after a single oral challenge of 10⁹ EAEC, UT mice had significant weight loss on days 2-6 (P < 0.05 compared to CON group). Conversely, ZT mice infected with EAEC had an insignificant amount of weight loss.

Zinc treatment prevents intestinal architecture disruption

Ileum sections from all treated mice were isolated to assess histopathology. Inflammation infiltration score and the villus to crypt ratio was significantly altered in UT mice compared to ZT or uninfected controls (Figure 2A-C; P < 0.05).

Mice fed a high zinc diet have higher serum zinc, complement C3, IgG and IgM levels

To determine whether we could elevate zinc concentrations in mice, we fed mice either a high zinc diet or a diet without any added zinc for 2 weeks and then assayed serum for zinc levels. As expected, the UT mice had significantly lower serum zinc levels (Figure 3A). More importantly, we saw a significantly lower level of zinc in serum of mice fed a zinc deficient diet compared to a high zinc diet (Figure 3A; P < 0.05).

Whole blood phagocytic and oxidative burst activity

Phagocytic activity and oxidative burst capacity of phagocytes were compared among the different groups. The phagocytic capacity of PMN to ingest bacteria increased in mice receiving zinc (ZT group, P < 0.05) compared with that seen in UT. Levels in the CON group were similar to those in ZT group but higher than those in UT group (P < 0.05) (Figure 4A). The phagocytic activity of monocytes followed a similar trend as PMN (Figure 4B). Oxidative burst capacity of PMN (Figure 4C) and monocytes (Figure 4D) were lower in CON and ZT groups than in the UT mice (P < 0.05).

T-cell populations are altered in zinc treated mice infected with EAEC

CD4+/CD8+ T-cells were also altered in EAEC infected mice (Figure 5); specifically the ratio

Figure 3. Serum zinc (A), complement C3 (B), IgG (C), and IgM (D) concentrations in mice with ETEC-induced diarrhea in the CON, UT, and ZT groups. Values are mean ± SD, n = 6/group. *P < 0.05, compared with CON group. #P < 0.05, compared with UT group.
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Discussions

EAEC is a major cause worldwide of diarrheal disease primarily affecting travelers, immuno-compromised individuals, and people from developing countries [18-20]. Repeated episodes of diarrhea or EAEC infection in children may lead to growth or cognitive shortfalls later in life [21]. Zinc deficiency can result in a variety of clinical manifestations [22] and diarrhea can lead to loss of dietary zinc through malabsorption [23].

Our aim in this study was to examine the effects of zinc supplementation on EAEC infection in a murine system and to investigate innate and adaptive immune responses to ETEC infection and zinc supplementation. We found lower effective activation of the complement activation pathway [25, 26]. Zinc, an essential micronutrient, is involved in the regulation of multiple cellular functions. It fulfills several key functions such as being a catalytic cofactor for enzymes or as a structural cofactor for proteins. The role of zinc in increasing levels of complement needs further in-depth attention.

In ZT mice, concentrations of C3 elevated after 2 weeks, similar to those in the CON group. The reason why complement C3 levels decrease in diarrhea mice could be due to a number of factors. Acute infections can induce protein energy malnutrition which can lead to decreased levels of C3. In addition, inhibitory bacterial factors can depress C3 levels [24]. Depression of levels in diarrheal mice may result in less efficient bacterial killing because of less inflammation infiltration scores in mice given zinc than in the UT group and speculated that intervention led to a decrease in the severity of diarrhea. Acute disease significantly lowered the level of complement C3 in sera of ETEC-infected mice. Phagocytic activities of both PMN and monocytes were depressed in mice not treated with zinc. An increase in oxidative burst response was observed in phagocytes obtained from UT mice compared with the controls.

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Figure 4. Effect of zinc on phagocytic activity of PMN (A) and monocytes (B) and oxidative burst capacity of PMN (C) and monocytes (D) in mice with ETEC-induced diarrhea in the CON, UT, and ZT groups. Values are mean ± SD, n = 6/group. *P < 0.05, compared with CON group. #P < 0.05, compared with UT group.
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Superoxide dismutase may be activated in the zinc treatment groups of diarrheal mice, thereby decreasing generation of reactive oxygen species. This decrease is probably enough to maintain bactericidal killing activity but not enough to abolish the killing effect of phagocytes in mice, as we observed. However, there are additional oxygen-independent killing mechanisms functioning via granzyme, perforin, or granulysins that may also be important for phagocytic activity [29].

Zinc deficiency has been associated with adverse effects on immune system function including thymic atrophy and function of T-cells [30]. Zinc is thought to improve thymic function, most likely due to increased thymulin activity [31, 32], a zinc-dependent thymic hormone involved in differentiation and maturation of T-cells. Experimental zinc deficiency in humans has been shown to alter T-cell populations (lower CD4/CD8 ratio) [33, 34]. Moreover, low levels of Zn have been shown to affect cellular responses [35] and are associated with increased systemic inflammation [36]. In the current study, we observed altered numbers of CD4+ and CD8+ cells in the serum of EAEC-infected mice, while zinc treatment increased the CD4/CD8 ratio. Further studies are currently underway to elucidate the role of T-cells in EAEC infection and zinc supplementation.

Overall, our study demonstrates that ETEC diarrhea induces changes in immune responses in mice and that some of these effects are further...
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modulated by zinc. Innate factors, including complement C3 and phagocytic activity, are enhanced after zinc treatment and probably help to stimulate quick protection. Adaptive factors, including CD4/CD8 ratio, are increased in zinc treated mice. In summary, zinc is a critical micronutrient for prevention and treatment of EAEC infection and enhances innate and adaptive immunity.

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Disclosure of conflict of interest

None.

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