**Tumor Necrosis Factor-α Expression and Intestinal ALP Activity in the Duodenum of Zinc-deficient Rats**

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**Summary**

Recently, it was reported that alkaline phosphatase (ALP) reduces the toxicity of lipopolysaccharide (LPS), a toxin of Gram-negative bacteria. Therefore, we examined the change in ALP activity and induction of tumor necrosis factor-α (TNF-α) in the duodenum of zinc-deficient rats. Fifty-six Sprague-Dawley rats were divided into two groups fed diets containing 0 % (zinc-deficient group) or 0.01 % (control group) zinc. Blood and duodenal mucosa samples (5 cm below the stomach) were obtained every week for four weeks, for ALP and TNF-α analyses. At one week, the serum ALP and intestinal ALP activities in the zinc-deficient group were significantly lower than in the control group, and decreased further over four weeks. TNF-α was detected in the duodenum of the zinc-deficient rats at four weeks, but not in the control group. This study shows that dietary zinc deficiency induces the expression of TNF-α protein in the duodenal mucosa; one cause of this response is a marked reduction in the detoxification of LPS by intestinal ALP.

**Key words:** zinc deficiency, alkaline phosphatase, tumor necrosis factor-α, small intestine

Zinc is a micronutrient in humans and animals, and dietary zinc deficiency can lead to dysgeusia and dermatitis¹,². Since, zinc is distributed widely throughout the organs and cells, there are many unknown symptoms of dietary zinc deficiency. Dietary zinc deficiency often has gastrointestinal manifestations, including diarrhea, abdominal pain, vomiting, and fever, indicating that the intestine is one of the tissues most sensitive to zinc deficiency¹. Zinc deficiency induces changes in intestinal morphology, including decreases in villous height and crypt depth, inflammatory cell infiltration of the lamina propria, and lesions of intestinal mucosa³.

Moreover, zinc is a factor in the formation of many enzymes, and is at the active center of alkaline phosphatase (ALP). ALP (EC 3.1.3.1) is a hydrolase that is responsible for removing the phosphate groups in the 5- and 3-positions of many molecules, including nucleotides, proteins, and alkaloids. However, not all the substrates of ALP in humans or animals are known. For example, the role of ALP in the small intestine remains largely unknown. Recently, liver ALP was shown to detoxify lipopolysaccharide (LPS), a toxin derived from the outer membrane of Gram-negative bacteria⁴. LPS is highly toxic, and two phosphate groups coupled to two glucosamines in the lipid A

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part largely determine its toxicity. Therefore, we tested whether decreased exogenous intestinal ALP might reduce the intestinal inflammatory defense against enterobacterial LPS in the intestinal lumen in dietary zinc deficiency. Using a rat model of zinc deficiency, ALP activity and tumor necrosis factor alpha (TNF-α) expression in the intestinal mucosa were examined.

**Materials and Methods**

1. **Chemicals and Reagent**

Anti-TNF-α antibody was purchased from eBioscience (San Diego, CA, USA). To obtain duodenal extracts, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA) was used. Disodium p-nitro-phenylphosphate hexahydrate (Wako Pure Chemicals) was used to measure ALP activity. The other chemicals used were of analytical grade.

2. **Dietary regimen**

This study was approved by the Animal Research Committee of Saitama Medical University. Male Sprague-Dawley rats (n = 56) weighing approximately 100 g were supplied by Clea Japan (Tokyo, Japan). The rats were maintained in a special cage in a temperature-controlled facility with a 12:12-h light-dark cycle. They were acclimatized to their environment for one week. Then, the rats were divided into two groups and pair-fed either a standard diet containing 0.01 % zinc (n = 28) or a zinc-deficient diet (n = 28), as previously reported, for four weeks. The protein, carbohydrate, fat, mineral, and vitamin compositions of the two diets were otherwise identical. The food consumption was monitored daily over the period of dietary conditioning. The two groups of rats ingested isocaloric diets each day. Distilled water was provided for drinking water and was available *ad libitum.*

3. **Sample preparation**

Blood and duodenal mucosa (5 cm from the stomach) samples were collected under ether anesthesia each week. The blood was centrifuged at 3,000 rpm for 20 min, and the serum was stored at -80 °C until the ALP activity was analyzed. The mucosal membrane from the duodenum was soaked in homogenate buffer (20 mM Tris-HCl buffer pH 7.5, containing 0.1 % Triton X-100, 1 % deoxycholate and protease inhibitor cocktail).

4. **Measuring ALP activity**

The ALP activity was assayed using the *p*-nitrophenylphosphate method, as previously described. Briefly, duodenal extract was incubated with reaction buffer (250 mM carbonate-bicarbonate buffer pH 10.0, containing 5 mM *p*-nitrophenylphosphate, 1 mM MgSO₄), and the *p*-nitrophenol released was measured using the absorption at 405 nm.

5. **Western blot analysis**

Ten micrograms of duodenal extract were mixed with sample buffer and heated at 100 °C for 5 min. The heated sample was separated in 15 % polyacrylamide gels containing 0.1 % SDS. The separated proteins were transferred to poly(vinylidene fluoride) membrane (Millipore, Billerica, MA, USA), and blocked for 3 h at room temperature with Blocking One-P (Nacalai Tesque, Kyoto Japan). The blocked membrane was incubated with monoclonal antibody (diluted 2,000 times in Tris-HCl-buffered saline containing 10 % blocking reagent and 0.1 % Tween 20) against rat TNF-α overnight. The reacted membrane was rinsed five times with Tris-HCl-buffered saline containing 0.1 % Tween 20. Then, the rinsed membrane was reacted with horseradish peroxidase-conjugated sheep antibody against mouse IgG (Birmingham, England). After rinsing the membrane, the ECL Western blotting detection system (GE Healthcare Bio-science, Piscataway, NJ, USA) was used to detect the TNF-α using chemiluminescence.

6. **Statistical analysis**

The data are expressed as the means ± SE. The statistical analysis was based on unpaired Student’s *t*-tests or two-way analysis of variance (ANOVA).
Results

The observed changes in the body weights and serum zinc concentrations of the rats concurred with a previous report[6].

The serum ALP activity at one week was decreased significantly (48% of the control value) compared with the controls, as shown in Fig. 1. At four weeks, the serum ALP activity was reduced to 30% of the control value.

The ALP activity in the duodenum at one week was decreased significantly, to less than 50% of the control value, as shown in Fig. 2. The profile of intestinal ALP activity was identical to that of the serum ALP activity.

Using Western blot analysis, TNF-α protein with a molecular weight of 17 kDa was detected at four weeks in the zinc-deficient group, but not in the controls, as shown in Fig. 3.

![Fig. 1](image1.png) The serum ALP activity in the control and zinc-deficiency groups is indicated by open and closed columns, respectively. The values are expressed as the means ± SEM. *p < 0.001.

![Fig. 2](image2.png) The duodenal ALP activity in the control and zinc-deficiency groups is indicated by open and closed columns, respectively. The values are expressed as the means ± SEM. *p < 0.001.

Expression of TNF-α protein

![Fig. 3](image3.png) The band of TNF-α protein in the zinc-deficient rat.
Discussion

In this study, we showed that dietary zinc deficiency induces TNF-α expression in the duodenum. Furthermore, the ALP activity decreased quickly with dietary zinc deficiency. These results suggest that gastrointestinal manifestations of dietary zinc deficiency, including diarrhea, abdominal pain, vomiting, and fever, are often associated with a reduction in ALP activity and the induction of TNF-α in the intestinal mucosa.

Alkaline phosphatase contains zinc in its functional center. In our experiment, there was a sharp decrease in the serum and intestinal ALP (Figs. 1 and 2), which was likely caused by the decreased ALP activity. Consequently, serum ALP activity could be used as an index of the dietary zinc level.

On the other hand, reducing of serum ALP activity is accompanied with aging\(^{7}\), suggesting differentiation and proliferation of the cell can induce an activation of ALP enzyme. These cell factors may involve ALP enzyme activities in the intestinal mucosa of zinc-deficient rat.

LPS is a toxic molecule that is derived from the outer membrane of Gram-negative bacteria. Several diseases are associated with bacterial translocation from the gut\(^{8}\). When the LPS level in the gut increases, it is absorbed by the intestinal wall, and can elicit a systemic inflammatory response that is characterized by fever and diarrhea. The dephosphorylation of LPS by intestinal ALP reduces the toxicity of LPS. In our study, the intestinal ALP activity in the mucosa was markedly reduced in zinc deficiency. Consequently, the increased LPS toxicity induced TNF-α protein, which can itself induce an inflammatory response via the expression of inducible nitric oxide synthase.

In conclusion, our results show that dietary zinc deficiency induces the expression of TNF-α protein in the duodenal mucosa, via a marked reduction in the detoxification of LPS by intestinal ALP.

References