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Zinc Deficiency Elevates Fecal Protein, But Not Electrolyte and Short-Chain Fatty Acid, Levels in Enterotoxigenic *Escherichia coli*-Induced Diarrhea in Rats

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ABSTRACT

Purpose: To determine the effect of zinc deficiency on fecal protein, electrolyte, and short-chain fatty acid levels in both heat-stable (ST) and heat-labile (LT) enterotoxigenic *Escherichia coli* (ETEC)-induced diarrhea in rats.
Methods: Albino rats, weighing 100 to 150 g, were divided into 2 groups, with 15 animals each: non-zinc and zinc-deficient. These two groups were sub-divided into three sub-groups with five rats each: control (saline); LT-ETEC; and ST-ETEC. Sodium phytate (30 mmol/L) was added to the animals' water to induce zinc deficiency, while diarrhea was induced using 5×10^9 ETEC cells/mL. Fecal protein levels were estimated using the Bradford method, while sodium and potassium levels were determined using atomic absorption spectrophotometry. Short-chain fatty acids were measured using gas chromatography-mass spectrometry.
Results: Among the non-zinc and zinc-deficient groups, there were significant increases (p=0.04), (p=0.03) in fecal protein concentrations (mg/mL) in the LT-ETEC- (4.50±0.33), (6.50±0.26) and ST-ETEC- (3.85±0.19), (5.98±0.32) induced groups compared to the control groups (2.60±0.52), (3.50±0.11) respectively. Fecal sodium and potassium levels (mg/L) were significantly (p=0.029) increased in non-zinc-deficient rats induced with LT-ETEC (9.35±0.95, 1.05±0.48), and ST-ETEC (9.96±1.02, 1.21±0.45) compared with the control group (8.07±0.44, 0.47±0.17) but the increase were not statistically significant (p=0.059) in the zinc deficient rat groups. Fecal acetate and propionate levels (mg/g) significantly (p=0.032) increased when induced with LT-ETEC and ST-ETEC in non-zinc and zinc-deficient groups compared with the control groups.
Conclusion: Zinc deficiency among rats with ETEC-induced diarrhea elevated fecal protein loss but may not have an effect on fecal sodium, potassium and short-chain fatty acid levels.

Keywords: Zinc; Enterotoxigenic *Escherichia coli*; Diarrhea; Fatty acids, Volatile

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Conflict of Interest
The authors have no financial conflicts of interest.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is a pathogenic subtype of *E. coli* known to produce diarrheagenic heat-labile (LT) and heat-stable (ST) enterotoxins. These bacteria, identified as a cause of cholera-like watery diarrhea, represent a major global health threat, particularly among young children in resource-limited areas of the world [1]. ETEC virulence is associated with colonization of the intestine and production of ≥1 toxins that induce the secretion of electrolytes and water into the lumen. The two classes of toxins—LT and ST—act to stimulate the expression of adenylate and guanylate cyclase, respectively, with each strain possessing one or both of LT and/or ST [2].

Diarrhea and its underlying enteric infections lead to morbidity, malnutrition, and mortality, partly due to the loss of protein and nutrients [3]. Enhanced fecal protein loss has been observed in children with acute and persistent diarrhea caused by different pathogens, including ETEC [4]. The severity of electrolyte abnormalities, concomitant with dehydration, has been reported to be consistent with acute diarrhea [5]. An increase in the fluidity and volume of wet stool in acute diarrhea also leads to the loss of water and electrolytes [6]. Fecal short-chain fatty acid (SCFA) levels have been found to be altered in patients with diarrhea-predominant irritable bowel syndrome (IBS) [7] and rotavirus-induced diarrhea [8].

Zinc has been reported to exert therapeutic efficacy through the resolution of small bowel damage and shortening the duration of diarrhea in several trials investigating prolonged diarrhea [9]. Interestingly, zinc supplementation significantly increased both ETEC shedding and intestinal burden in stool samples [10]. Although zinc supplements and low-osmolarity oral rehydration solutions are recommended for the treatment of childhood diarrhea [11], research data regarding the effect of zinc deficiency on fecal nutrient loss in ETEC-induced diarrhea remain limited. As such, this study investigated the effect of zinc deficiency on fecal protein, electrolyte, and SCFA levels in ETEC-induced diarrhea in rats.

MATERIALS AND METHODS

ETEC samples

ST-ETEC and LT-ETEC used in the present study were isolated from children presenting with acute diarrheal disease. The organisms were identified using standard biochemical tests and a polymerase chain reaction method [12-14].

Animals, diet, diarrheal induction, and sample collection

Albino rats, weighing between 100 and 150 g, obtained from the National Institute of Health, Pakistan, were first divided into two groups, with 15 animals each: non-zinc deficient; and zinc-deficient. These two were further sub-divided into three sub-groups (A, B, and C), with five rats each: A (control group [normal saline]); B (diarrhea induced with LT-ETEC); and C (diarrhea induced with ST-ETEC). The rats were acclimatized for 7 days before the start of the experiment and housed in a temperature-and humidity-controlled room. The animals had *ad libitum* access to food and water throughout the acclimatization period. Sodium phytate (30 mmol/L) was added to the water to induce zinc deficiency in the assigned groups. Fecal and serum zinc levels were monitored until deficiency was confirmed. Twelve hours after the induction of diarrhea, food and water were withdrawn. Diarrhea was induced using approximately 5×10^9 LT/ST-ETEC cells/mL, corresponding to

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a density of 4 McFarland standards in 1 mL normal saline. After 18 hours, diarrhea was observed as watery stools. The rats were anesthetized and euthanized using chloroform. Fecal samples were collected, weighed, and refrigerated in Tris-HCl buffer before analysis. The care and treatment of experimental animals adhered to an approved protocol specified by ARRIVE animal care guidelines.

Fecal protein estimation

Fecal protein levels were estimated using the Bradford method. Five grams of feces was weighed, homogenized, and centrifuged in Tris-HCl buffer. The supernatants were used for protein estimation. Bovine serum albumin (BSA) and relevant standards (Sigma Aldrich, St. Louis, MO, USA) were prepared using 2 mg/mL in Tris-HCl diluents. Approximately 5 µL of each standard and samples were pipetted into a microwell plate, and 250 µL of Coomassie reagent (Sigma Aldrich) was added. The microwell plates were placed on a plate shaker for 30 seconds, then incubated at room temperature for 10 minutes. Absorbance was measured at a wavelength of 595 nm using a plate reader (FLUOstar Omega; BMG LabTech, Ortenberg, Germany). The average measurement for the blank replicates was subtracted from the readings of other individual standards and sample replicates. A standard calibration curve was plotted against concentrations in mg/mL using the average blank-corrected BSA standard. The standards were used to determine protein concentrations in the samples. The protein concentrations obtained using the Bradford method were compared with those determined using an automated spectrometer (Nanodrop Colibri Titertek; Berthold Detection Systems GmbH, Pforzheim, Germany).

Fecal electrolyte level determination

Fecal sodium and potassium levels were determined using a method described by Palma et al. [15]. The digestion solution was prepared using nitric acid and perchloric acid (Sigma Aldrich) at a ratio of 2:1 v/v in a one-step digestion procedure. Approximately 5 mL of digestion solution was added to the sample and heated to 200°C until the solution became translucent and brownish-color smoke stopped being released. This indicated complete digestion of the sample; the tubes were then allowed to cool to room temperature. The digested samples were transferred to a 50 mL volumetric flask using filter paper. The volumes of the solutions were made up to 50 mL using deionized water. Electrolytes were evaluated using an atomic absorption spectrophotometer (AAnalyst 700; Perkin-Elmer, Waltham, MA, USA).

Fecal gas chromatography-mass spectrometry analysis

SCFA levels (i.e., acetate, propionate, and butyrate) in the feces were measured using gas chromatography-mass spectrometry (GC-MS), as previously described by Guard et al. [16] with some modifications. Briefly, fecal samples were weighed, lyophilized (cryodos-50; Telstar, Barcelona, Spain), and diluted 1:5 in extraction solution, ethyl acetate. After homogenization for 30 minutes at room temperature, the fecal suspensions were centrifuged (5810 R; Eppendorf, Hamburg, Germany) for 20 minutes at 2,100×*g* and 4°C. Supernatants were collected using sterile syringe filters (Corning Inc., Corning, NY, USA). A gas chromatograph (Clarus 600; Perkin-Elmer) coupled to a mass spectrometer (Clarus 600 C; Perkin-Elmer) was used for chromatographic separation and detection of SCFAs in the samples. The GC temperature program was as follows: 40°C for 0.1 minute, increased to 70°C at 5°C/min, 70°C for 3.5 minute, increased to 160°C at 20°C/min, and finally increased to 280°C for 3 minute at 35°C/min. The total run time was 20.53 minute, with a solvent delay of 5 minute.

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Statistical analysis

Statistical analysis was performed using SPSS version 20.0 (IBM Co., Armonk, NY, USA) and GraphPad Prism version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). The Student's *t*-test was used for comparisons between the groups. Differences with $p<0.05$ were considered to be statistically significant.

RESULTS

Among the non-zinc-deficient groups, there were significant ($p=0.04$) increases in fecal protein concentrations (in mg/mL) in the LT-ETEC (4.50±0.33) and ST-ETEC (3.85±0.19) induced groups compared with the control group (2.60±0.52). This increase was higher in the LT-ETEC-induced group than in the ST-ETEC-induced group. Similarly, the LT-ETEC (6.50±0.26) and ST-ETEC (5.98±0.32) -induced groups exhibited significantly higher ($p=0.03$) fecal protein concentrations compared to the control group (3.50±0.11) in zinc-deficient rats. A higher increase in protein concentration was again recorded in the LT-ETEC-induced group than in the ST-ETEC-induced group. Generally, fecal protein concentrations observed in zinc-deficient rats were significantly higher than those in non-zinc-deficient control rats (**Table 1**).

Among the non-zinc deficient control rat groups (**Table 2**), induction with LT-ETEC and ST-ETEC significantly ($p=0.029$) increased fecal sodium (in mg/L) (9.35±0.95, 9.96±1.02) and potassium (in mg/L) (1.05±0.48, 1.21±0.45) concentrations compared to the control groups (8.07±0.44, 0.47±0.17). The ST-ETEC-induced group exhibited a greater increase than the LT-ETEC induced group. Among the zinc-deficient rat groups, induction with LT-ETEC and ST-ETEC increased fecal sodium (8.95±0.46, 9.26±0.18), and potassium (0.98±0.08, 0.86±0.49) concentrations compared with the control groups (8.23±0.38, 0.58±0.41), respectively, but without a statistically significant difference ($p=0.059$). While the LT-ETEC-induced group exhibited a greater increase in potassium concentration, the ST-ETEC-induced group exhibited a greater increase in sodium concentration. The general trend demonstrated that zinc deficiency did not significantly increase fecal sodium and potassium levels in the LT-ETEC and ST-ETEC-induced diarrhea compared with the non-zinc-deficient groups (**Table 2**).

As shown in **Table 3**, acetate (in mg/g) was found to be significantly ($p=0.032$) increased when induced with LT-ETEC (5.52±0.02) and ST-ETEC (5.81±0.11) in the non-zinc deficient groups compared with the control group (4.00±0.12). The greater increase observed in the ST-ETEC-induced group was not significant ($p=0.056$) compared with the LT-ETEC group. Acetate was also significantly ($p=0.032$) increased in LT-ETEC (6.01±0.03) and ST-ETEC (6.20±0.01) induced groups in zinc-deficient rats compared with the control group (4.51±0.21). The greater increase observed in the ST-ETEC-induced group compared with the LT-ETEC group, however, was not significant ($p=0.056$). The LT-ETEC- (6.51±0.01, 7.50±0.00) and ST-ETEC-

Table 1. Fecal protein concentrations according to treatment group

Group	Rats	
	Non-zinc deficient	Zinc deficient
Control	2.60±0.52	3.50±0.11†
LT-ETEC	4.50±0.33*	6.50±0.26†
ST-ETEC	3.85±0.19*	5.98±0.32†

Protein concentrations expressed as mg/mL (mean±standard deviation).
LT-ETEC: heat-labile enterotoxigenic *Escherichia coli*, ST-ETEC: heat-stable enterotoxigenic *Escherichia coli*.
*Significantly different ($p<0.05$) compared to non-zinc deficient control group; †Significantly different ($p<0.05$) compared to the zinc-deficient control group.

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Table 2. Fecal electrolyte concentrations according to treatment group

Group	Non-zinc deficient rats			Zinc-deficient rats		
	Control	LT-ETEC	ST-ETEC	Control	LT-ETEC	ST-ETEC
Sodium	8.07±0.44	9.35±0.95*	9.96±1.02*	8.23±0.38	8.95±0.46†	9.26±0.18†
Potassium	0.47±0.17	1.05±0.48*	1.21±0.45*	0.58±0.41	0.98±0.08†	0.86±0.49†

Electrolyte concentrations expressed as mg/L (mean±standard deviation).
LT-ETEC: heat-labile enterotoxigenic *Escherichia coli*, ST-ETEC: heat-stable enterotoxigenic *Escherichia coli*.
*Significantly different (p<0.05) compared to control group; †Not significantly different when compared to control groups.

Table 3. Fecal short-chain fatty acid (SCFA) concentrations according to treatment group

SCFA	Non-zinc deficient rats			Zinc-deficient rats		
	Control	LT-ETEC	ST-ETEC	Control	LT-ETEC	ST-ETEC
Acetate	4.00±0.12	5.52±0.02*	5.81±0.11*	4.51±0.21	6.01±0.03*	6.20±0.01*
Propionate	5.3±0.00	6.51±0.01*	7.32±0.00*	6.00±0.02	7.50±0.00*	8.10±0.14*
Butyrate	3.2±0.00	3.6±0.00†	3.5±0.00†	4.0±0.01	4.21±0.02†	4.5±0.00†

SCFA concentration expressed as mg/g (mean±standard deviation).
LT-ETEC: heat-labile enterotoxigenic *Escherichia coli*, ST-ETEC: heat-stable enterotoxigenic *Escherichia coli*.
*Significantly different (p<0.05) compared to control group; †Not significantly different (p≥0.05) compared to control group.

(7.32±0.00, 8.10±0.14) induced groups were compared with zinc-deficient groups. Fecal butyrate levels (in mg/g) exhibited no significant difference (p=0.056) in LT-ETEC- (3.6±0.00, 4.21±0.02) and ST-ETEC- (3.5±0.00, 4.5±0.00) induced groups compared with the control groups (3.2±0.00, 4.0±0.01) in both non-zinc- and zinc-deficient rats, respectively.

DISCUSSION

In this study, LT-ETEC and ST-ETEC were used to induce diarrhea in non-zinc-deficient and zinc-deficient rats. Diarrheal stools were used to evaluate protein, sodium, potassium, and SCFA loss. There were significant increases in fecal protein levels in the LT-ETEC and ST-ETEC-induced groups compared to the control group, and fecal protein levels observed in zinc-deficient rats were significantly higher than those in non-zinc-deficient groups. This implied that zinc deficiency may have led to increased protein loss, suggesting that diarrhea is associated with impaired reabsorption of endogenous nitrogen, resulting in a cycle of protein depletion that ultimately leads to an intractable stage. Significant amino acid loss through the stool as a result of impeded reabsorption of water, fat, and electrolytes has been observed in infantile diarrhea [17]. Using a mouse model, Bolick et al. [10] demonstrated that zinc deficiency reduced growth and upregulated virulent gene expression of ETEC. As such, increased fecal protein levels were observed in all ETEC-infected mouse groups regardless of diet, with the strongest induction in zinc-deficient mice [10]. In rats, zinc deficiency has been shown to decrease the absorption of proteins by altering enterocyte peptidase activity, thereby potentiating diarrhea and increasing protein loss [18]. It has been reported that zinc deficiency can cause diarrhea and, conversely, chronic diarrhea can lead to zinc deficiency. This has led to the use of zinc supplementation in infants and children with diarrheal illnesses in most developing nations, where malnutrition often results in zinc deficiency [19].

In the non-zinc-deficient groups, induction of diarrhea with LT-ETEC and ST-ETEC significantly increased fecal sodium and potassium concentrations compared with the control group. This observation supports the use of a glucose-electrolyte solution to treat active sodium and potassium loss in infectious ETEC-induced diarrhea in the treatment of childhood diarrhea. The World Health Organization recommended a physiological concentration of glucose, sodium, potassium, and bicarbonate as an oral rehydration therapy

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to reduce the mortality rate in diarrheal illnesses among developing countries [20]. A slightly different pattern in fecal sodium-potassium loss has been reported in colonic pseudo-obstruction complicated by diarrhea. A high fecal output of potassium was observed due to stimulation of active colonic potassium secretion, while low fecal excretion of sodium indicates that active sodium absorption was not inhibited [21]. However, in this study, zinc deficiency increased fecal levels of sodium and potassium in the LT-ETEC- and ST-ETEC-induced groups compared with the control group, although the increase was not statistically significant (i.e., $p>0.05$). In rats, zinc deficiency has been shown to upregulate the expression of intestinal uroguanylin, a peptide that triggers electrolyte secretion and subsequent water secretion, thereby increasing diarrheal output and subsequent loss of electrolytes [18]. *Vibrio cholera* causes diarrhea by increasing cyclic adenosine monophosphate (cAMP) production, inhibiting the absorption of sodium, and inducing the intestinal secretion of water and chloride [22]. Interestingly, zinc deficiency increased cAMP-regulated chloride *secretion via* basal-lateral potassium (K^+) channels. This explains its role in increasing the duration of cholera-induced diarrhea, an effect that may involve basal-lateral zinc action on basal-lateral membrane K^+ channels [19], which further supports the effect of zinc on electrolyte output in acute infectious diarrhea. In this study, the non-significant ($p>0.05$) increase in fecal sodium and potassium levels observed in the zinc deficient LT-ETEC- and ST-ETEC-induced groups compared with the control group suggests that zinc exerts a selective effect against intestinal pathogens under varying zinc status (**Table 2**). This was supported by an in vitro model that demonstrated that zinc prevents active ion secretion induced by cholera toxin by directly inhibiting the elevation in intracellular cAMP concentration, but has no effect on ST-ETEC-induced secretion [23].

SCFAs are compounds produced during fermentation in the gut microbiota. Acetic, propionic, and butyric acids are the most important SCFAs produced from non-digestible foods [24]. These three acids act by preserving gut barrier functions, and their anti-inflammatory and immunomodulatory effects [7], which are severely mitigated in infectious diarrhea. In this study, although fecal acetate and propionate levels were found to be significantly increased when induced with LT-ETEC and ST-ETEC in non-zinc-deficient and zinc-deficient groups compared with control, a statistical difference was not observed ($p>0.05$) when non-zinc deficient groups were compared with zinc-deficient groups. This may imply that zinc deficiency has no influence on fecal SCFA levels in ETEC-induced infectious diarrhea.

In rotavirus-induced diarrhea, celiac disease, and adenomatous polyposis, the levels of all SCFAs were not significantly different [8,25], which may rule out increased SCFA synthesis. A significant increase was observed for fecal propionate and butyrate among IBS patients compared with normal controls [26], with fecal propionate being significantly higher, thus implicating non-absorbed and undigested carbohydrates [27]. Increased fecal acetate and propionate levels in ETEC-induced diarrhea may be related to increased synthesis of SCFAs from non-absorbed carbohydrates by the colonic microbiota [28]. A recent study concluded that chronic zinc deficiency alters the chick colonic microbiota and function by significantly lowering phylogenetic diversity [29]. The same study observed a concomitant decrease in SCFAs. This indicates that zinc deficiency may alter fecal SCFAs in diarrheal diseases under chronic conditions.

In conclusion, ETEC-induced acute diarrhea resulted in intestinal loss of protein, sodium, potassium, and major SCFAs. This may be the result of impaired reabsorption of nutrients

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due to inflammatory outcomes normally observed in infectious diarrhea, while zinc deficiency increased protein loss as a result of upregulation of ETEC virulent genes. Its non-significant effect on sodium and potassium loss supports a selective effect on different intestinal pathogens. The non-significant increase observed in fecal sodium/potassium loss in the zinc-deficient state suggests that zinc deficiency has no effect on gut electrolyte transport. While ETEC-induced diarrhea increased fecal sodium and propionate levels, zinc deficiency demonstrated no such effect. Zinc deficiency may have no effect on the function of SCFAs in preserving gut barrier function in acute conditions.

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