Virulence Inhibition by Zinc in Shiga toxigenic *Escherichia coli*

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Running Title: Zinc against STEC

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Abstract

Previously our laboratories reported that zinc inhibited expression of several important virulence factors in enteropathogenic *Escherichia coli* (EPEC) and reduced EPEC-induced intestinal damage in vivo. Since EPEC is genetically related to Shiga toxigenic *E. coli* (STEC) we wondered if the beneficial effects of zinc extended to STEC as well. Treatment options for STEC infection are very limited since antibiotics tend to exacerbate disease via enhanced toxin production, so a safe intervention in this infection would be welcome.

In this study we report that in STEC strains zinc inhibits adherence to cultured cells as well as expression of EHEC secreted protein A (EspA). In addition, zinc inhibits the expression of Shiga toxin (Stx) at both the protein and RNA level. Zinc inhibits basal and antibiotic-induced Stx production, and inhibits both Stx1 and Stx2 by ≥90% at concentrations of 0.4 mM zinc. Rabbit EPEC strains were selected for acquisition of Stx-encoding bacteriophages and these rabbit STEC strains (designated RDEC-H19 and E22-stx2) were used to test the effects of zinc in vivo in ligated rabbit intestinal loops. In vivo, zinc reduced fluid secretion into loops, inhibited mucosal adherence, reduced the amount of toxin in the loops, and reduced STEC-induced histological damage (villus blunting).

Zinc has beneficial inhibitory effects against STEC strains that parallel those observed in EPEC. In addition, zinc strongly inhibits Stx toxin expression; since Stx is responsible for the extra-intestinal effects of STEC infection, such as hemolytic-uremic syndrome (HUS), zinc might be capable of preventing severe sequelae of STEC infection.

Abstract Word count = 250 words
Introduction

Shiga-toxigenic *Escherichia coli* (STEC), also called enterohemorrhagic *E. coli* (EHEC) or Verotoxigenic *E. coli* (VTEC) is a type of diarrhea-producing *E. coli* more common in developed countries than in developing countries. More severe cases are characterized by bloody diarrhea and some patients go on to develop severe systemic complications such as hemolytic-uremic syndrome (HUS) and encephalopathy. STEC is genetically and evolutionarily related to enteropathogenic *E. coli* (7), an *E. coli* pathotype known for causing long-lasting watery diarrhea in children in developing countries.

Zinc has been shown to reduce the duration and severity of acute diarrhea in children in field trials on several continents (1, 24). In earlier decades, these beneficial effects of zinc were usually attributed to the correction of zinc deficiency (29), which is unfortunately still seen in poor malnourished children. In more recent years, however, studies have shown that zinc can be beneficial against diarrhea even in patients without zinc deficiency (2, 21).

In a previous study, we showed that zinc reduced the expression of several important virulence factors in EPEC and reduced EPEC-induced intestinal damage and fluid secretion in ligated rabbit intestinal loops in vivo (5).

Since the locus of enterocyte effacement (LEE) is regulated in a similar way in STEC as in EPEC, we investigated whether zinc had any similar protective properties against STEC virulence factors in the LEE. We also tested whether the inhibitory effects of zinc on the expression of EHEC secreted protein A (EspA) were dependent on Ler, the LEE encoded regulator, as they were in EPEC. Next we tested the ability of zinc to inhibit production of Shiga toxins, which are not present in EPEC but are the hallmark of STEC strains. Zinc inhibited basal as well as antibiotic-induced expression of both Stx1 and Stx2 in a variety of STEC strains, with \( \geq 90 \% \) inhibition of Stx achievable with zinc concentrations of 0.4 to 0.5 mM. In vivo, zinc protected rabbit intestinal loops from STEC-induced histological damage, reduced STEC adherence to epithelium, and reduced Stx production in loop fluids in vivo. However, STEC bacterial numbers were similar in zinc-treated and control loops.

The anti-virulence effects of zinc extend to STEC as well as EPEC and are independent of the zinc nutritional status of the host. Further work is needed to understand the mechanism of Stx inhibition by zinc and to better define the protective dose of zinc in humans. In addition, we
wonder if zinc’s inhibitory effects are limited to EPEC and STEC, or if they extend to other bacterial enteropathogens as well.

Materials and Methods

Bacterial strains used. Bacterial strains used are listed in Table 1. Bacteria were grown overnight in LB broth at 37 °C with 300 rpm shaking, then subcultured into DMEM medium. In this report, when bacteria were subcultured in “DMEM” this refers to DMEM/F12 medium supplemented with 18 mM NaHCO₃ and 25 mM HEPES, pH 7.4, but without serum or antibiotics. DMEM/F12 medium contains 1.5 µM zinc. The ∆ler mutant of EDL933, CB49, was a gift from Dr. Alfredo Torres, Univ. of Texas Medical Branch, Galveston, TX.

Materials. The following reagents were obtained from Sigma-Aldrich Chemicals: zinc acetate, NiCl₂, MnCl₂, CuSO₄, Ga(NO₃)₃. FeSO₄ was from MP Biomedicals, Irvine, Calif.

Cell culture. HeLa cells were grown in DMEM/F12 medium supplemented with 7.5% fetal bovine serum (Gibco/Invitrogen, Grand Island, NY), 18 mM NaHCO₃, 20 µg/ml vancomycin, and 15 µg/ml gentamicin as previously described (4). According to the Gibco/Invitrogen catalog, the amount of zinc in DMEM/F-12 medium, not including serum, is 1.5 µM.

Bacteriophage Transduction of Rabbit EPEC Strains to Produce Stx-producing rabbit-adapted E. coli strains. The methods used to generate strain RDEC-H19A, in which bacteria carrying the antibiotic resistance tagged Stx1 phage from an O26 strain were co-cultured with the recipient RDEC-1 strain, have been previously described (22). Similar methods were used to select strain E22-stx2. Briefly, O157:H7 strain 86-24 (Stx2 only) was used as the phage source. A 1.5 kb kanamycin resistance marker was introduced 27 kb downstream from the Stx-2B coding sequence, and between predicted open reading frames. This insertion did not affect Stx-2 production (as determined by cytotoxicity analysis in vitro of cell lysates) and a lysogenic tagged strain was capable of being induced to produce entire phage particles. Following co-culture with the recipient O103 E22 strain, kanamycin resistant isolates were selected which had acquired the Stx2 phage as confirmed by PCR, and which were able to produce Stx without and with antibiotic induction. One such isolate was named E22-Stx2. Rabbits inoculated with this Stx2-producing lysogenic REPEC strain developed more severe histological lesions and inflammation as compared with those inoculated with the isogeneic non-toxin producing wild type strain E22.
Adherence Assay. Quantitative adherence assays were performed in duplicate or triplicate wells of HeLa cells grown in collagen-coated 24 well plates. HeLa cells were used at 90% confluency; cells were rinsed and changed into 0.25 ml per well of antibiotic-free DMEM medium, then infected with STEC at a multiplicity of infection (MOI) of 100:1. STEC bacteria were grown overnight in LB broth, then subcultured for 2 h in DMEM medium. To enhance adherence, STEC bacteria were centrifuged onto the HeLa cells at 500 g for 5 min at room temperature, then allowed to adhere for 2.5 h at 37 ºC in a 5% CO2 atmosphere. After 2.5 h of STEC adherence, the monolayers were washed twice with sterile phosphate-buffered saline (PBS) to remove unbound bacteria, then the monolayers were solubilized in 0.1 % Triton X-100 detergent in water. The Triton extracts were subjected to serial dilution and were spread on LB agar plates in duplicate and the number of colonies counted. Adherence was calculated as parts per thousand (º/oo) of the input inoculum.

Enzyme Immunoassay (EIA) for Shiga toxin protein. Shiga toxin protein was measured by EIA using the Premier EHEC EIA kit from Meridian Bioscience, Cincinnati, OH, which measures Stx1 and Stx2. Since different STEC strains produced different amounts of Shiga toxin, required different concentrations of antibiotics for induction of Stx, and so on, the optimal conditions for growth and EIA detection had to be determined by trial-and-error. The optimal antibiotic concentrations, culture duration, and dilutions of supernatant needed for successful Stx EIA detection for various STEC strains are summarized in Table 2. Bacterial culture supernatants were filter sterilized prior to Stx measurement by EIA; therefore, any toxin trapped in the periplasmic space, often a substantial proportion for Stx1 (31), would not be measured in our assay.

Analysis of RNA expression by reverse transcription and quantitative real-time PCR. Quantitative real-time PCR was carried out as described previously (5, 6). Primers for stx1 were those described by Jinneman et al. (11), while those for stx2, STEC espA, and the normalizing gene gnd were from the methods of Rashid et al. (18). Primers for measuring expression of espA and espB in rabbit EPEC strain E22 were as reported (6).

Normalizing genes for analysis of expression were rrsB, using the primers reported by Leverton & Kaper (14) and gnd as reported by Rashid et al. (18). Calculations of normalized expression using the two different normalizing genes gave quite similar results.
STEC infection in the ligated rabbit ileal loop model. Details of the surgical procedures used to create the ileal loops were included in the Supplemental Material of a previous article (5), including details of the methods used to provide analgesia and avoid pain. Experimental infection of rabbits was performed at the Boedeker laboratory in New Mexico. Animal use was approved by the Institutional Animal Care and Use Committee at the University of New Mexico. Briefly, 10 cm loops of ileum were created, then the loops injected with \(10^8\) to \(10^9\) cfu of RDEC-H19A (Stx1+) or E22-stx2 (Stx2+). Rabbits were re-anesthetized 16 h later and killed humanely using an overdose of pentobarbital. The abdomen was re-opened, the loop fluid recovered, and the rabbit intestinal tissues were preserved in 10 % buffered formalin for histological analysis. Bacterial cfu’s in the loop fluid were determined by dilutions and plate counts on LB medium + the appropriate antibiotic. For counting of RDEC-H19A, the medium used was LB agar + naladixic acid (50 mg/L) + tetracycline (25 mg/L), whereas for strain E22-stx2 it was LB + naladixic acid (50 mg/L) and kanamycin (50 mg/L). The amount of Shiga toxin in loop fluid was measured by EIA as mentioned above. Quantitative histological analysis of infected intestinal tissues was done by an observer unaware of the treatment groups. Villus-to-crypt ratios and percent enteroadherence were measured as previously described (5).

Measurement of zinc concentrations in gastrointestinal contents of the rabbit after orogastric zinc administration. To determine what luminal concentrations of zinc were achieved in response to oral zinc, we measured zinc in various segments of the rabbit gastrointestinal (GI) tract after orogastric administration of three different zinc salts: zinc acetate, zinc sulfate, and zinc oxide. Zinc oxide (ZnO) is known to be less ionized and less bioavailable that the other 2 zinc salts. Since the 3 salts have different molecular weights, we adjusted the oral dose to achieve equal, 10 mg doses of elemental zinc and administered each zinc salt by orogastric tube to rabbits once daily for 3 consecutive days. Intestinal contents were collected from the small intestine, proximal cecum, mid-cecum, and large intestine. Rabbits used in this experiment had an average body weight of 2.4 kg, yielding a zinc dose of 4.1 mg/kg (mg elemental zinc per kg body weight). Rabbits were not surgically altered, nor were they infected with STEC. Zinc content of the samples was determined at Michigan State University, a national reference laboratory, by Inductively Coupled Plasma Mass Spectrometry (ICPMS). In addition to samples collected at the time of euthanasia, fecal pellets were collected daily, and the rabbits were killed humanely on the evening of the third day. Fecal pellets collected on the portion of day 3 were insufficient for
Data analysis. Error bars shown in graphs are standard deviations. Statistical analysis was by ANOVA using InStat 3.0 software (GraphPad Software, San Diego, Calif.) with the Tukey-Kramer post-test for multiple comparisons.
Results

Initially we tested whether zinc had any effect on the adherence of human STEC strains to cultured cells in vitro. Fig. 1 shows that 0.2 to 0.4 mM zinc almost completely abolished STEC adherence to HeLa cells in a short-term adherence assay. The molecular basis for STEC adherence to host cells is less well understood than for EPEC, with different laboratories reporting many putative adhesins, including intimin, EspA, H7 flagellae, outer membrane protein A (OmpA), long polar fimbriae, and hemolysin to name just a few (16, 18, 22, 23, 26, 27). Therefore, while the inhibition of STEC adherence by zinc is important, it does not immediately point to a molecular mechanism.

Zinc inhibited expression of the EPEC secreted proteins EspA and EspB in strain E2348/69, so we tested if zinc also inhibited expression of the esp genes in STEC strain EDL933. Zinc did inhibit esp expression in EDL933 (see Fig. 2A for espA). In EPEC, the inhibitory effect of zinc on the Esps showed an unexpected dependence on the LEE-encoded regulator, Ler, and was abolished in the ∆ler mutant. We compared the effect of zinc on the ∆ler mutant of EDL933, designated CB49, with its effect on the wild-type parental strain. Fig. 2A and Fig. 2B show that the inhibitory effect of zinc was lost in the ∆ler mutant of EDL933, just as it was in the EPEC ler mutant. To try to extend these findings even more, we compared the effect of zinc on the wild-type rabbit EPEC strain E22 with its effect on the ∆ler mutant of E22, strain ECB159. As with human EPEC and STEC, the inhibitory effect of zinc on espA and espB was lost in the ∆ler mutant of E22 (Fig. 2, panels C and D). The findings of Fig. 2, panels A-D, together with our previous study of human EPEC, shows that the presence of Ler is necessary for the inhibitory effects of zinc on the EPEC and EHEC secreted proteins (Esps).

Fig. 2, panels E and F, show experiments testing whether the presence of absence of Ler had any effect on Shiga toxin (Stx) production. Fig. 2E shows that the amount of Stx produced under basal and antibiotic-stimulated conditions was identical in the wild-type EDL933 and the ∆ler mutant CB49. Similarly, zinc inhibited Stx production with equal efficacy and potency in the ∆ler mutant as in the wild-type (Fig. 2F). To summarize, Fig. 2, panels A-D, shows that Ler is required for zinc’s inhibitory effects on the EspA and EspB, but is not required for its inhibitory effects on Stx (Fig. 2, E & F).

Stx is an important virulence factor in STEC because Stx1 and Stx2 are responsible for the bloody diarrhea and extra-intestinal complications observed in STEC infection. Therefore
we further tested zinc’s ability to inhibit Stx expression in various human-derived STEC strains, including strains that produce Stx1 alone, Stx2 alone, or both toxins. In addition, since several antibiotics are known to increase the expression and release of the Shiga toxins, we also tested whether zinc inhibited antibiotic-induced Stx expression. We measured Stx toxin protein expression by enzyme immunoassay (EIA) and stx RNA abundance by quantitative real-time PCR (qRT-PCR).

Fig. 3 shows the effect of zinc on Stx toxin expression as assessed by EIA performed on culture supernatants; the EIA used detects both Stx1 and Stx2. Fig. 3A shows that zinc inhibited basal toxin expression in strain EDL933 by 90%. In contrast, NiCl$_2$ and MnCl$_2$ stimulated Stx toxin production to about 50% above control at lower concentrations (0.05 to 0.2 mM) before Stx toxin fell off again at higher concentrations. We and others have noted the biphasic stimulatory effect of nickel on other virulence factors, including the bundle-forming pilus of EPEC (5, 17). Pilot experiments showed that ciprofloxacin induced Stx toxin production in all strains tested, so ciprofloxacin was used in tests of antibiotic-induced toxin production. Fig. 3 shows that zinc was able to inhibit ciprofloxacin-induced toxin production in strain Popeye-1 (Stx2-only, Fig. 3B) and in strain TSA14 (Stx1-only, Fig. 3C) as well as in EDL933 (data not shown, and Fig. 3E).

In order to test the effect of zinc in a rabbit intestinal model of STEC infection, two rabbit STEC strains were also tested for susceptibility to inhibition by zinc. Fig. 3D shows that zinc inhibited Stx2 production by rabbit strain E22-stx2. Toxin production from rabbit strain RDEC-H19A was also inhibited by zinc (data not shown).

In addition to its ability to inhibit ciprofloxacin-induced Stx, zinc also inhibited trimethoprim-induced Stx toxin production and release (Fig. 3E). Zinc also inhibited H$_2$O$_2$-induced Stx production as well (Fig. 3F). H$_2$O$_2$ has been implicated as an inducer of Stx toxin production in vivo in the absence of antibiotics (28). Fig. 3 showed that zinc was able to inhibit Stx production from a variety of different STEC strains, including strains that produce Stx1, Stx2, and both toxins; furthermore, zinc inhibited basal as well as antibiotic-induced toxin production.

Because of the promising results found using Stx toxin protein measurements, we also measured stx RNA by qRT-PCR. Fig. 4A shows that zinc inhibited the abundance of RNA
transcripts from \textit{stx2} in STEC strain Popeye-1. Basal \textit{stx2} expression was inhibited by 50%, while ciprofloxacin-stimulated \textit{stx2} expression was inhibited by 90% by zinc.

Using the same strain, we also compared the inhibitory ability of zinc with that of other divalent metals and gallium nitrate at 0.3 mM concentration (Fig. 4B). Gallium was tested because of its position adjacent to zinc on the periodic table and because Kaneko et al. reported that gallium has strong antivirulence and anti-biofilm activity against \textit{Pseudomonas aeruginosa} (12). Zinc and nickel chloride were the most inhibitory, followed by copper, then manganese and gallium. In contrast, addition of FeSO$_4$ actually stimulated \textit{stx2} RNA even more than that produced by ciprofloxacin alone. Although nickel strongly inhibited \textit{stx2} expression, nickel is also much more toxic to humans than is zinc (Table 3). In EPEC, nickel also had a paradoxical stimulatory effect on expression of the bundle-forming pilus and on the EPEC secreted proteins (5), and the adherence-enhancing ability of nickel was confirmed in another \textit{E. coli} strain (17). Because of these two undesirable features of nickel (increased toxicity and enhancement of pathogen adherence), we believe zinc is divalent metal with the best combination of desirable properties: inhibition of virulence expression in the pathogen and low toxicity toward the host.

Fig. 4C shows the effect of delaying the addition of zinc for varying amounts of time after adding an inducing antibiotic (ciprofloxacin in this case) on \textit{stx2}. Delay in zinc addition gradually reduced the inhibitory effect of zinc compared to immediate zinc addition. However, even after a 3 h delay zinc still inhibited ciprofloxacin-induced \textit{stx2} expression by ~50%. The fact that zinc can still have an inhibitory effect on \textit{stx2} RNA levels when added 3 h after the inducing antibiotic has implications for the molecular mechanism by which zinc inhibits Stx production. However, the loss in inhibitory efficacy when zinc is added hours after the inducing antibiotic means that zinc might not be very effective as a “rescue” treatment for patients who have already received antibiotics for STEC. Instead, it appears zinc would have to be given before or, at the latest, simultaneously with, an antibiotic if the Stx induction phenomenon is to be prevented.

Fig. 5 shows the effect of zinc in vivo against two different rabbit STEC strains in the rabbit ligated intestinal loop model. Rabbit ileal loops were infected with either the Stx1-producing strain RDEC-H19A or the Stx2-producing strain E22-stx2, with or without zinc. Fig. 5A shows that zinc inhibited fluid secretion into the loop, measured as the volume-to-length
ratio. For strain E22-stx2, zinc’s inhibitory effect on secretion reached statistical significance (p < 0.05), while for RDEC-H19A this reduction did not reach significance (p = 0.1).

Another measure of EPEC and STEC-mediated damage to the intestinal mucosa is the villus-to-crypt ratio. EPEC and STEC cause cell damage and death to villus enterocytes, resulting in villus blunting, while cells in the crypts compensate by proliferation. Fig. 5B shows that zinc strongly protected the rabbit intestines from the villus blunting induced by infection with both of the rabbit STEC strains, and Panels H & I show zinc’s protective effect against E22-stx2-induced blunting.

The percent of the mucosal surface with adherent mats of pathogenic E. coli is yet a third quantitative measure of infection. Zinc significantly reduced the percent enteroadherence for strain E22-stx2, which adheres avidly (Fig. 5, panels C, F, and G). For strain RDEC-H19A, which adheres less avidly, zinc’s inhibitory effect on adherence was not statistically significant (Fig. 5, panel C).

In addition to the parameters mentioned, zinc also inhibited the amount of Stx toxin that accumulated in the loops infected with rabbit STEC (Fig. 5D). In contrast to the inhibitory effects on fluid secretion, adherence, villus blunting, and Stx toxin production, zinc did not reduce the number of STEC bacteria recovered from the loops. For example, the number of E22-stx2 bacteria recovered from rabbit intestinal loops treated with 1 mM zinc acetate was 1.2 x 10^{12} cfu/ml, compared to 1.0 x 10^{12} cfu/ml recovered from the no-zinc control loops. Likewise, there was no difference in the numbers of RDEC-H19 bacteria recovered from the zinc-treated vs. control loops (p = 0.29). As with EPEC, the beneficial effects of zinc in vivo appear to be mediated via an inhibition of virulence rather than via an inhibition of bacterial growth. Lack of inhibition of bacterial growth has been cited as a possible advantage for other virulence inhibitors, such as quorum sensing inhibitors (19) because of a theoretical lower chance of emergence of resistance.

Fig. 5E shows massive submucosal edema in loops infected with E22-stx2, an effect also observed with strain RDEC-H19A (photos not shown). Since submucosal edema is not observed with the parental REPEC strains (E22 and RDEC-1), the edema appears to be an effect of Stx toxins. Fig. 5F shows that infection with E22-stx2 causes villus blunting. Fig. 5G, at higher (600 X) power, shows E22-stx2 adhering in thick mats or biofilms (arrow; E. coli bacteria stain blue with H&E stain). In contrast, loops infected with E22-stx2 in the presence of 1 mM zinc
acetate (Fig. 5H and 5I) show histology that is difficult to distinguish from normal, uninfected ileum (uninfected not shown), with normal slender villi and almost no adherent pathogenic *E. coli*.

In our previous work with zinc on EPEC, and in the present study of zinc on STEC, we observed that fairly high concentrations of zinc, in the 0.1 to 0.5 mM range, were required to inhibit virulence. We were unsure if such high concentrations of zinc were achievable in the lumen of the gastrointestinal tract. To determine what concentrations of zinc were produced in response to oral zinc, we measured zinc in various segments of the rabbit gastrointestinal (GI) tract after orogastric administration of three different zinc salts: zinc acetate, zinc sulfate, and zinc oxide.

As shown in Fig. 6A, the 10 mg dose of zinc resulted in levels of zinc in the intestinal tract that were significantly higher than those in the control, non-zinc-treated rabbits. Zinc levels we measured in intestinal and cecal lumen were in the 0.3 to 0.4 mM range for both of the more bioavailable zinc salts (acetate and sulfate), ranges that we have shown are inhibitory for both EPEC and STEC virulence. In the fecal pellets, zinc levels were even higher, reaching 0.5 to 0.6 mM (Fig. 6B); higher levels are expected due to reabsorption of water from intestinal contents during passage through the colon. Furthermore, increased levels of zinc were observed in the fecal pellets after just 1 day of administration, showing that zinc supplements would not have to be given for a prolonged period to achieve protective levels in the distal GI tract. In contrast to the large, ~3-fold increase in zinc levels in the intestinal and fecal contents, zinc levels in rabbit plasma increased only 28 % in the zinc acetate- and zinc sulfate-supplemented groups, compared to control rabbits. This finding is in accord with the nutrition literature (15) in that the ability of the upper GI tract to absorb zinc is limited, like its ability to absorb iron. High doses of oral zinc can apparently exceed the absorptive ability of the upper GI tract and thereby reach therapeutic concentrations in the lower GI tract where EPEC and STEC adhere and cause disease. The doses of zinc required for this latter purpose well exceed the amounts of zinc needed to maintain normal zinc nutritional status, so that high-dose zinc should be viewed as acting more like a drug and less as a nutrient in this context.

Discussion
Our previous work showed that zinc blocked the production of virulence factors in EPEC, with strong inhibition by zinc of the production of the bundle-forming pilus, several EPEC secreted proteins (Esp), intimin, and Tir. Since EPEC is considered the evolutionary progenitor of most STEC strains (7, 30), we wondered if zinc’s inhibitory effects extended to STEC virulence factors as well.

As in EPEC, zinc inhibited expression of the Esp in STEC (Fig. 2), which is not surprising considering the similarity in the operons of the locus for enterocyte effacement (LEE) between EPEC and STEC, including the regulation of the LEE4 operon which encodes the Esp. In our previous work, we noticed that the inhibitory effects of zinc were abolished in an isogenic strain with a deletion mutation in ler, the LEE-encoded regulator. Here we show again that in the Δler mutant of STEC strain EDL933, and in the Δler mutant of rabbit EPEC E22, zinc’s inhibitory effects on espA and espB were lost (Fig. 2, Panels A-D). Therefore in human EPEC, rabbit EPEC, and in STEC O157:H7, Ler is needed to observe the inhibitory effects of zinc on the EPEC and EHEC secreted proteins (Esp). Further work is needed to determine the molecular basis of this interesting observation.

Production of the Shiga toxins is the main characteristic which distinguishes STEC from EPEC. The Shiga toxins are encoded on lysogenic bacteriophages within the STEC chromosome, and regulation of Stx toxin production is quite different from that of other STEC virulence factors. For example, Stx production is independent of Ler (9), quorum sensing (10), bicarbonate, and other environmental signals and transcription factors which regulate virulence gene expression in EPEC and STEC. Although Stx production is regulated differently than the Esp, zinc nevertheless inhibited Stx production (Figs. 3 and 4), and even inhibited Stx in the Δler mutant (Fig. 2F). Therefore, although the principle of parsimony would make it desirable to postulate a single, unifying target for zinc action, our current data forces us to hypothesize that there are at least two independent targets for zinc in STEC: one which involves Ler, and a separate Ler-independent target that mediates inhibition of Stx.

Zinc’s inhibitory effects on Stx were observed in strains expressing Stx1 only, Stx2 only, and both toxins. Zinc inhibited basal as well as antibiotic-induced Stx toxin expression, and its effects were measurable at the level of toxin protein as well as RNA expression. Zinc-mediated inhibition of Stx generally achieved or exceeded 90% inhibition of Stx at 0.3 to 0.5 mM zinc. In
addition to inhibition of ciprofloxacin-induced Stx production, zinc also inhibited Stx induced by trimethoprim and by H$_2$O$_2$.

Zinc and other divalent metals have been noted to be able to inhibit protein and RNA synthesis at concentrations similar to those used in our study (0.1 to 0.3 mM) (3, 8). There appears to be some general ability of divalent metals (zinc, nickel, copper, cobalt, etc.) to inhibit *E. coli* growth and protein synthesis, but among this group of metals zinc appears to stand out as the most potent inhibitor of EPEC and STEC virulence factor expression (Figs. 3A, 4B, and Ref. 5).

In non-surgically altered rabbits, zinc levels in GI tract contents achieved levels in the range (0.3 to 0.5 mM) that our in vitro experiments showed were sufficient to inhibit adherence, Esp expression, and Stx production. The data in Fig. 6, however, were obtained in rabbits which were not infected with EPEC or STEC. The zinc levels achieved in the presence of active infection could be different from those we measured in uninfected rabbits. EPEC is known to induce a malabsorptive state in humans and animals (20, 25), so it is possible that zinc absorption would be reduced during infection and that higher amounts of zinc would therefore reach the ileum, cecum, and colon. On the other hand, zinc concentrations could be reduced by the dilutional effect of diarrheal fluid secretion. Determining the concentrations of zinc achievable in the intestinal lumen in the presence of active infection is therefore a goal for future research.

For many years there has been a strong presumption in the nutrition literature that the beneficial effects of zinc supplementation in infectious diseases, including infectious diarrhea, were due to correction of a pre-existing zinc deficiency. This view has unfortunately persisted despite that fact that field studies of zinc have shown benefits even in subsets of children with normal plasma zinc levels (21). That “zinc deficiency” view has led to the assumption that zinc supplements would offer no benefit in diarrheal illness in previously well-nourished subjects. The present study, our previous work (5), and the work of others (13) strengthens our belief that zinc, by acting directly against pathogen virulence, could be therapeutic even in humans and animals with completely normal zinc nutritional status. The doses of zinc required for this protective effect are supra-physiologic, meaning that zinc is acting in a drug-like fashion. Furthermore, zinc can act within the lumen of the gastrointestinal tract, meaning that its antimicrobial effects do not necessarily require systemic absorption. To gain acceptance and to be
translated into practical benefit, these new concepts require a very small but important paradigm shift in understanding zinc’s actions in acute infectious diarrheal disease.

References


Figure Legends

Fig. 1. Effect of zinc on the adherence of two STEC strains to HeLa cells. HeLa cell monolayers were infected with STEC, then the multiwell plates were centrifuged at 500 g for 5 min to enhance adherence. Adherence was allowed to proceed for 2.5 h, then adherent bacteria were measured by dilution and plate counts as described in “Materials and Methods.” STEC bacteria were only exposed to the zinc during the 2.5 h adherence period itself, i.e., no pre-incubation with zinc. Adherence was calculated as parts per thousand (%oo) of the input inoculum. Adherence was significantly reduced at concentrations of zinc of 0.1 mM and greater for both strains (p <0.05 by ANOVA, using Tukey-Kramer post-test for multiple comparisons).

Fig. 2. Role of Ler in the effect of zinc on expression of the EHEC secreted proteins (Esps). Expression of RNA transcribed from esp genes of STEC and rabbit EPEC was measured by quantitative real-time polymerase chain reaction (qRT-PCR) as described in Materials and Methods. Panels A and B show the results of the same experiment with the data normalized in two different ways. In Panel A, espA expression was normalized to the no-zinc control for each strain separately. In Panel B, both data sets were normalized to the no-zinc samples of the wild-type strain. Panel B shows that the expression of espA is reduced in the Δler mutant CB49; Ler is known to be a positive regulator of LEE gene expression. Panels C and D, comparison of espA and espB expression in the wild-type rabbit EPEC strain E22 and the isogenic Δler mutant, with each strain normalized to its own no-zinc control. Panels E and F, lack of effect of Ler on the production of Stx toxin protein as measured by enzyme immunoassay (EIA).

Fig. 3. Inhibition by zinc of Shiga toxin production in various STEC strains and under various conditions, as measured by EIA in supernatant medium. Optimal conditions, including antibiotic concentrations and supernatant dilutions used, are summarized in Table 2. Zinc acetate was used as the zinc salt in all panels. Panel A, effect of zinc on basal Stx production in strain EDL933. Panel A, Inset, shows that unlike zinc, NiCl₂ and MnCl₂ have a biphasic effect on Stx toxin production, with a stimulatory effect at lower concentrations (0.05 to 0.2 mM), followed by a fall-off at higher metal concentrations; *, significant at p <0.05. Panels B - D, effect of zinc on
ciprofloxacin-induced Stx in strains Popeye-1, TSA14, and E22-stx2. In Panels B – D, zinc at concentrations of 0.2 mM and greater showed significant inhibition of Stx toxin (p < 0.05 by ANOVA). E and F, effect of zinc on trimethoprim-stimulated and H$_2$O$_2$-stimulated Stx production in strains EDL933 and Popeye-1. Panel E, TMP, trimethoprim; *, significantly inhibited compared to trimethoprim-induced levels. Panel F, *, significantly reduced compared to basal (p < 0.05); **, , significantly reduced compared to H$_2$O$_2$-stimulated levels.

Fig. 4. Inhibition by zinc of stx2 RNA by qRT-PCR in strain Popeye-1. Panel A, inhibition by zinc of stx2 RNA under basal and ciprofloxacin-stimulated conditions at 4 h. Panel B, comparison of various metals and the semi-metal gallium on ciprofloxacin-stimulated stx2 expression. All metals were added to a final concentration of 0.3 mM. FeSO$_4$ significantly increased stx2 RNA while all the other metals shown significantly inhibited ciprofloxacin-induced stx2. Panel C, effect of time delay in zinc addition after ciprofloxacin induction of stx2 expression. 0.3 mM zinc significantly inhibited ciprofloxacin-induced stx2 RNA at all times of zinc addition (15 min to 3 h, p < 0.05).

Fig. 5. Effect of zinc in vivo on STEC infection in ligated rabbit ileal loops. 10 cm loops of ileum were created as described in “Materials and Methods” and infected with 10$^9$ cfu of RDEC-H19A (Stx1+) or 10$^8$ cfu of E22-stx2 (Stx2+). Infection was allowed to proceed for 16 h, then loops were recovered and various parameters measured as shown in the panels. Panel A, effect of zinc on fluid secretion as measured by the volume-to-length ratio for rabbit STEC strains RDEC-H19A and E22-stx2. Panel B, effect of zinc on the villus-to-crypt ratio, a measure of intestinal damage. *, significant at p= 0.03 compared to infected with no zinc. Panel C, effect of zinc on the percent enteroadherence; ¥, non-significant; *, significant compared to infected, no zinc. . Panel D, effect of zinc on the amount of Stx toxin protein in loop fluid, as measured by EIA. †, non-significant trend toward lower Stx toxin in RDEC-H19A plus zinc; *, significant reduction in Stx toxin in loops receiving zinc compared to E22-stx2 alone, p < 0.01. Panels E – I, histology of STEC E22-stx2 infection in ileal loops in the absence and presence of 1 mM zinc. Panel E, low power (100X) view showing submucosal edema (double-headed arrow). Panel F, intestinal damage caused by E22-stx2 infection includes villus blunting, and production of an exudate of acute inflammatory cells, mostly heterophiles, a type of rabbit.
leukocyte. Panel G, adherent E22-stx2 bacteria form mats or biofilm on the epithelium (arrow; 600 X; bacteria stain blue with H&E stain). Panel H, histology of intestine infected with E22-stx2 with 1 mM zinc acetate, showing preservation of normal slender villous architecture. Panel I, higher power (200X) view showing lack of adherent bacteria and normal villous architecture in the presence of zinc.

Fig. 6. Concentrations of zinc achieved in intestinal lumenal contents and rabbit fecal pellets after orogastric administration of zinc at 10 mg elemental zinc daily. Panel A, concentrations of zinc in intestinal lumenal contents after 3 days of daily zinc administration, by mass spectrometry. Small int, small intestine; prox cec, proximal cecum; large int, large intestine. Zinc concentrations were significantly higher in all 3 zinc-supplemented groups compared to the same intestinal segment with no added zinc. Note that standard rabbit chow included a mineral supplement with zinc. Panel B, zinc concentrations in rabbit fecal pellets. Fecal pellets collected from cage trays on the morning of the second day were counted as fecal pellets passed on day 1, and so on.
<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Serotype</th>
<th>Clinical Setting and Geographic Origin</th>
<th>Toxins Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human-derived</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Popeye-1; TW14359; SNP genotype 30</td>
<td>O157:H7</td>
<td>10 y.o. girl, Buffalo, NY; Spinach-associated outbreak</td>
<td>stx2, stx2c</td>
</tr>
<tr>
<td>EHEC 987</td>
<td>O157:H7</td>
<td>Argentina</td>
<td>sx1+ stx2+</td>
</tr>
<tr>
<td>TSA14</td>
<td>O26:H11</td>
<td></td>
<td>stx1+</td>
</tr>
<tr>
<td>EDL933</td>
<td>O157:H7</td>
<td>U.S.; Reference Strain</td>
<td>stx1+ stx2+</td>
</tr>
<tr>
<td>CB49</td>
<td>O157:H7</td>
<td>∆ler mutant of EDL933</td>
<td>Gift of Alfredo Torres, UTMB</td>
</tr>
<tr>
<td><strong>Rabbit Strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E22</td>
<td>O103: H2</td>
<td>wild-type, virulent rabbit EPEC</td>
<td>none</td>
</tr>
<tr>
<td>ECB159</td>
<td></td>
<td>∆ler mutant of E22</td>
<td>none</td>
</tr>
<tr>
<td>RDEC-H19A</td>
<td>O15: H-</td>
<td>Rabbit EPEC strain RDEC-1 transduced with bacteriophage H19A</td>
<td>stx1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>conferring Stx1 toxin expression</td>
<td></td>
</tr>
<tr>
<td>E22-stx2</td>
<td>O103: H2</td>
<td>Rabbit EPEC strain E22 transduced with bacteriophage 933W</td>
<td>stx2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>conferring Stx2 expression</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.
Conditions for Detecting Stx Production in STEC by Enzyme Immunoassay (EIA)

All strains were subcultured into DMEM medium 1:100 from overnight growth in LB broth and grown at 37 °C with 300 rpm shaking.

<table>
<thead>
<tr>
<th>STEC Strain/ Serotype/ Stx types produced</th>
<th>Minimum inhibitory concentration (MIC) of ciprofloxacin, ng/ml a</th>
<th>Concentration of ciprofloxacin used to induce Stx toxin, ng/ml</th>
<th>Usual duration of subculture</th>
<th>Usual dilution of supernatant before EIA</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDL933 O157:H7 Stx1+, Stx2+</td>
<td>24</td>
<td>4-6</td>
<td>4-5 h</td>
<td>1: 25 for basal toxin production; 1:100 for cipro-induced</td>
<td></td>
</tr>
<tr>
<td>Popeye-1 O157:H7 Stx2+, Stx2c+</td>
<td>16</td>
<td>4</td>
<td>4-5 h</td>
<td>1: 50</td>
<td></td>
</tr>
<tr>
<td>TSA14, O26:H11 Stx1+</td>
<td>8</td>
<td>4</td>
<td>5 h</td>
<td>1: 10 for cipro-induced expression</td>
<td>1: 4 for basal toxin</td>
</tr>
<tr>
<td>E22-stx2 b O103:H2 Stx2+</td>
<td>250</td>
<td>60</td>
<td>6 h</td>
<td>3- to 5-fold dilution for cipro-induced expression</td>
<td>Sonication of bacterial suspension for 30 sec increased toxin detection</td>
</tr>
<tr>
<td>RDEC-H19A b O15:H(-) Stx1+</td>
<td>64</td>
<td>32-64</td>
<td>4 h</td>
<td>1: 5 dilution</td>
<td></td>
</tr>
</tbody>
</table>

a, by E-test, AB Biodisk, Piscataway, NJ; AB Biodisk has now been acquired by Biomerieux.
b, The parental rabbit strains E22 and RDEC-1 had previously been selected for nalidixic acid resistance. The phage-transduced strains also expressed nalidixic acid resistance, resulting in increased MICs for ciprofloxacin.

EIA was by Premier EHEC EIA kit, Meridian Bioscience, Cincinnati, OH, which detects both Stx1 and Stx2.
Table 3
Comparative Toxicity of Selected Metals in Humans

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Daily Reference Intake <em>(mg)</em></th>
<th>Tolerable Upper Limit (TUL)</th>
<th>Toxicity Relative to Zinc, based on inverse ratios of the TUL’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc</td>
<td>11</td>
<td>40</td>
<td>--------</td>
</tr>
<tr>
<td>Copper</td>
<td>0.9</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Nickel</td>
<td>Not determined †</td>
<td>1.0</td>
<td>40</td>
</tr>
<tr>
<td>Manganese</td>
<td>2.3</td>
<td>11 mg</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* Daily Reference Intake values, previously called the Recommended Dietary Allowance (RDA), are based on a healthy adult 19-70 years. Source: Food and Nutrition Board, Institute of Medicine, National Academies, and USDA. † Nickel has never been shown to be an essential nutrient in humans. [http://www.iom.edu/Object.File/Master/21/372/0.pdf](http://www.iom.edu/Object.File/Master/21/372/0.pdf)
Fig. 1

A. Popeye-1

HeLa cells 2.5 h

Popeye-1 Adherence (\% of)

B. EDL933

HeLa cells 2.5 h

EDL933 Adherence (\% of)
Fig. 2

**A**

STECEspA

Normalized Expression Ratio (% of wild-type no-zinc control)

normalized to the no-zinc control separately for each strain

**B**

STECEspA

both strains normalized to the wild-type no-zinc

**C**

espA in rabbit EPEC

**D**

espB in rabbit EPEC

**E**

EDL933, wt  CB49, Δler mutant

**F**

Stx toxin

basal toxin production

in culture supernatant, at 5 h
Fig. 3

A. Basal toxin release in DMEM.

B. Strain Popeye-1.

C. Strain TSA14 O26:H11, Stx1-only.

D. Strain E22-stx2.

E. Trimethoprim-induced Stx production.

F. H$_2$O$_2$-stimulated Stx.

Legend:
- **: p < 0.05
- ***: p < 0.001

Table:

<table>
<thead>
<tr>
<th>Condition</th>
<th>[Stx Toxin Protein in supernatant], EIA A450</th>
</tr>
</thead>
<tbody>
<tr>
<td>no cipro</td>
<td>0.0</td>
</tr>
<tr>
<td>+ 0.1 mM Zn</td>
<td>0.1</td>
</tr>
<tr>
<td>+ 0.2 mM Zn</td>
<td>0.2</td>
</tr>
<tr>
<td>+ 0.3 mM Zn</td>
<td>0.3</td>
</tr>
<tr>
<td>+ 0.4 mM Zn</td>
<td>0.4</td>
</tr>
<tr>
<td>+ 0.5 mM Zn</td>
<td>0.5</td>
</tr>
<tr>
<td>+ 0.6 mM Zn</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Note: TMP, trimethoprim; 0.1 µg/ml.
Fig. 4

A

Strain Popeye-1

$stx2$ Normalized Expression Ratio
(compared to no-cipro, no-Zn control)

B

Popeye-1

$stx2$ Normalized Expression Ratio
(compared to no-metal, no-cipro control)

C

Effect of time delay in zinc addition on $stx2$ expression

Samples collected for RNA analysis at 4 h
**Fig. 5**

**A** Fluid Secretion: Volume-to-Length Ratios

**B** Villus-to-Crypt Ratio

**C** Mucosal Adherence

**D** Zinc on Stx Toxin in vivo

**E** Stx Toxin by EIA

**F** 75 μm

**G** 30 μm

**H** 75 μm

**I** 75 μm

* statistically significant inhibition by zinc by linear regression for strain E22-stx2; p = 0.003

* significant at p = 0.03

† p = 0.05; † p = 0.1; note that the higher Stx concentrations in RDEC-H19-infected loops reflect the lower amount of fluid secretion triggered by this strain.
Zinc in intestinal segments

Segment of GI Tract

10 mg of elemental zinc of each salt administered daily for 3 days

Fig. 6

A

Zinc in intestinal segments

B

Zinc in Rabbit Fecal Pellets

Day of zinc administration