MAJOR ARTICLE

Zinc Inhibits Cholera Toxin–Induced, but Not *Escherichia coli* Heat-Stable Enterotoxin–Induced, Ion Secretion in Human Enterocytes

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Background. Because zinc deficiency in malnourished children is associated with severe diarrhea, use of zinc supplementation has been proposed as an adjunct to oral rehydration. However, the effects of zinc on enterocyte ion transport are largely unknown. The objective of the present study was to investigate the effects of zinc on transepithelial ion transport under basal conditions and under conditions of enterotoxin-induced ion secretion.

Methods. Ion transport was investigated by monitoring electrical parameters in human intestinal Caco-2 cells that were mounted in Ussing chambers and exposed to increasing concentrations of zinc, both in the absence and presence of either cholera toxin (CT) or *Escherichia coli* heat-stable enterotoxin (ST). Intracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) concentrations were also determined.

Results. The addition of zinc to the luminal or basolateral side of enterocytes induced a chloride-dependent, dose-related decrease in short-circuit current, indicating ion absorption. It also resulted in a substantial reduction in CT-induced ion secretion and in cAMP concentration. *E. coli* ST–induced ion secretion and cGMP concentration were not affected. Ion absorption peaked at 35 μmol/L zinc, whereas excess zinc load induced active ion secretion.

Conclusions. By causing a decrease in cAMP concentration, zinc directly promotes ion absorption and substantially reduces CT-induced, but not *E. coli* ST–induced, ion secretion.

Worldwide, infectious diarrhea is still associated with high morbidity and mortality in persons of pediatric ages. The death rate has been estimated to be as high as 2.5 million children every year, with almost all deaths occurring in malnourished children in developing countries. Besides rotavirus, the major causal agents of diarrhea are *Vibrio cholerae* and enterotoxigenic *Escherichia coli* [1]. Cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT) induce secretory diarrhea by causing an increase in cAMP concentration, whereas *E. coli* heat-stable enterotoxin (ST) does so by activating the guanylate cyclase/cGMP system [2]. The use of oral rehydration solution (ORS) for treatment of diarrhea has become widespread and has resulted in reduced mortality from dehydration, but ORS does not decrease diarrheal duration and stool output. An active search for agents that are capable of inhibiting intestinal fluid losses has been ongoing for >2 decades. Although a number of candidate drugs have emerged, none has found a place in the routine management of acute diarrhea. Several clinical trials in developing countries have indicated that zinc is effective in the prevention and treatment of diarrhea in children [3–7]. A meta-analysis concluded that zinc supplementation given with appropriate fluids and foods during acute diarrhea reduces the duration and severity of illness in children in developing countries [8].

Zinc is an essential trace element in humans; it is a known constituent of important metalloenzymes, is involved in major metabolic pathways and DNA synthesis, helps to maintain the integrity of biological membranes and ion channels, and plays a major role in intestinal physiological processes [9]. Because there are no zinc stores in the body, its bioavailability is determined by a balance among food intake, intestinal absorption, and losses through urine, skin, and the intestinal tract. Intestinal losses of zinc are substantially

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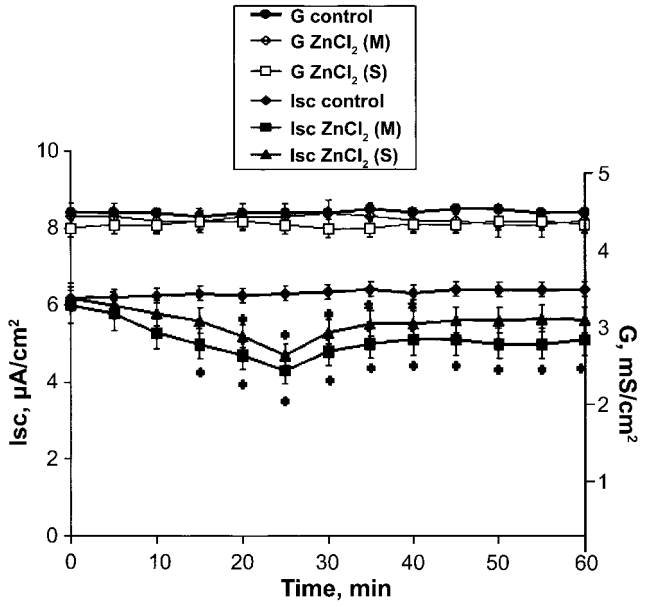

Figure 1. Time course of the effects of the mucosal (M) and serosal (S) addition of $ZnCl_2$ (35 μmol/L) on short-circuit current (Isc) and tissue ionic conductance (G) in Caco-2 cells mounted in Ussing chambers. The decrease in Isc induced by both the M and S addition of zinc indicates ion absorption. No effect on G values was observed. Each experiment was run in duplicate and was repeated at least 3 times. Results are expressed as means ± SD. $^*P < .05$, vs. control. mS, millisiemens.

increased during diarrhea [10]. In zinc-deficient animals, CT-induced ion secretion is increased, compared with that in control animals, and secretion is reduced by zinc replenishment [11]. However, the mechanisms that link zinc deficiency with severe diarrhea, as well as the mechanisms that explain the efficacy of zinc in reducing diarrhea, are not clear. We therefore investigated the effects of zinc on transepithelial ion transport under basal conditions and under conditions of CT- and *E. coli* ST–induced ion secretion.

We used a well-established in vitro model that is based on the human epithelial intestinal cell line Caco-2, which is capable of zinc uptake [12]. This model has been validated in recent studies that investigated the effects of enterotoxins and their antagonists [13–15].

MATERIALS AND METHODS

Transepithelial ion transport experiments. Caco-2 cells were grown on uncoated polycarbonate transwell filters and were used in intestinal ion transport experiments 15 days after confluence, as described elsewhere [15]. The filter area was 4.9 cm^2. Each filter was mounted in an Ussing chamber (WPI) as a flat sheet between the mucosal and the serosal compartments. Each compartment contained 5 mL of Ringer's solution with the following composition: NaCl (114 mmol/L), KCl (5 mmol/L), Na_2HPO4 (1.65 mmol/L), NaH_2PO4 (0.3mmol/L), $CaCl_2$ (1.25 mmol/L), $MgCl_2$ (1.1 mmol/L), $NaHCO_3$ (25 mmol/L), and glucose (10 mmol/L); the buffer was constantly gassed with 5% CO_2–95% O_2 and was maintained at 37°C. The following electrical parameters were measured as described elsewhere [16], both before and after mucosal or serosal addition of $ZnCl_2$ and in either the presence or absence of CT or *E. coli* ST: transepithelial potential difference (PD), short-circuit current (Isc), and tissue ionic conductance (G). Isc is expressed as microamperes per square centimeter, and G is expressed as millisiemens (mS) per square centimeter. Cell viability was checked by measurement of the electrical response to the serosal addition of theophylline (5 mmol/L) at the end of each experiment. In experiments performed to investigate the role played by Cl^- in the zinc-induced electrical response, SO_4^- was substituted for Cl^- at an equimolar concentration.

Determination of intracellular concentrations of cyclic nucleotides. After the Ussing chamber experiments were completed, each cell-containing filter was rapidly removed, transferred to ice-cold 5% trichloroacetic acid, and homogenized. The homogenate was centrifuged at 2000 *g* for 3 min at 4°C, and the supernatant was collected and evaporated to dryness under vacuum (Speed VAC 110; Savant Instruments). The dried sample was redisolved in 0.5 mol/L acetate buffer (pH 5.8) with 0.01% sodium azide, and cAMP concentrations were determined by a radioimmunoassay (Biotrak cAMP assay system; Amersham International). cGMP concentrations were measured by use of a commercial radioimmunoassay kit (cGMP ^{125}I assay system; Amersham International), in accordance with

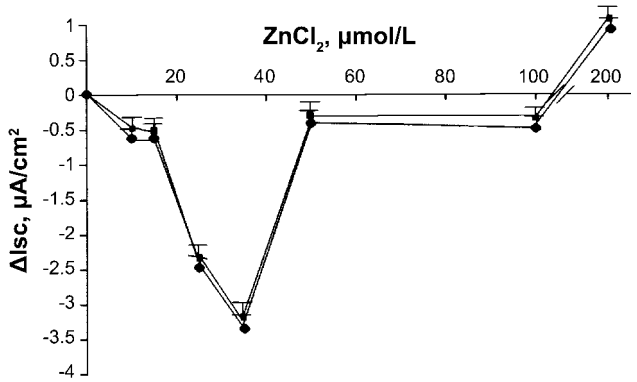

Figure 2. Changes in short-circuit current (Isc) in response to the mucosal (●) or serosal (■) addition of $ZnCl_2$ in increasing concentrations in Caco-2 cells mounted in Ussing chambers. Isc values are expressed as the difference (Δ) between measurements in cells exposed to $ZnCl_2$ for 60 min and measurements in untreated control cells. $ZnCl_2$ induced a dose-dependent decrease in Isc, which peaked at 35 μmol/L. The effect decreased at higher concentrations. A toxic concentration (200 μmol/L) of $ZnCl_2$ induced an increase in Isc to a value above that of the untreated control cells, indicating ion secretion. Each experiment was run in duplicate and was repeated at least 3 times. Results are expressed as means ± SD.

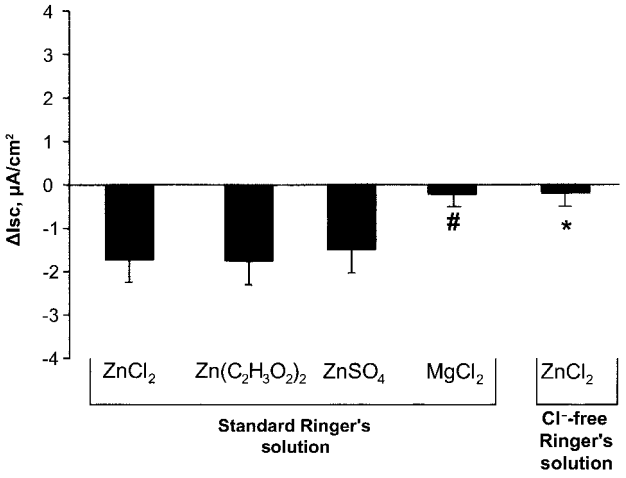

Figure 3. Comparative effects of $ZnCl_2$, $Zn(C_2H_3O_2)_2$ (zinc acetate), $ZnSO_4$ (zinc sulfate), and $MgCl_2$ on short-circuit current (Isc) in Caco-2 cells mounted in Ussing chambers. To test the hypothesis that the proabsorptive effect of $ZnCl_2$ was related to zinc ions, cells were probed in parallel with $ZnCl_2$, zinc acetate, zinc sulfate, and $MgCl_2$. A decrease in Isc was observed with all 3 zinc compounds but not with $MgCl_2$, indicating that zinc was directly responsible for the observed electrical changes. To test the hypothesis that Cl^- transport is the target of zinc, the experiments were performed in Cl^--free Ringer's solution, and no electrical effects were observed. Each experiment was run in duplicate and was repeated at least 3 times. Results are expressed as means ± SD. $^{\#}P < .001$, for $MgCl_2$ vs. $ZnCl_2$ and $MgCl_2$ vs. $Zn(C_2H_3O_2)_2$; $^{*}P < .001$, for $ZnCl_2$ in Cl^--free Ringer's solution vs. $ZnCl_2$ in standard Ringer's solution. Δ, difference between measurements in untreated control cells and cells exposed to the substances for 60 min.

the manufacturer's instructions. Results are expressed as picomoles of cGMP per square centimeter.

Reagents and cell culture. Chemicals were obtained from Sigma Chemical. Culture medium was obtained from Life Technologies GIBCO BRL. Transwell filters and supports were obtained from Costar. Caco-2 cells were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle medium that had a high glucose concentration (4.5 g/L) and that was supplemented with 10% fetal calf serum, 1% nonessential amino acids, penicillin (50 mU/mL), and streptomycin (50 mg/mL) and were incubated in 5% CO_2–95% air. The medium was changed daily.

Statistical analysis. Each experiment was run in duplicate and was repeated at least 3 times. Results are expressed as means ± SD. Significance was evaluated by use of the nonparametric 2-tailed Mann-Whitney *U* test. $P < .05$ was considered to be significant. The SPSS software package for Windows (version 12.0.1; SPSS) was used for statistical analysis.

RESULTS

Effects of Zinc on Transepithelial Ion Transport in Caco-2 Cells

The addition of $ZnCl_2$ at a final concentration of 35 μmol/L to the mucosal side of Caco-2 cell monolayers mounted in Ussing chambers induced a decrease in Isc entirely due to an effect on PD, without affecting G values. The lowest peak was observed 25 min after the addition of $ZnCl_2$ (figure 1). The addition of $ZnCl_2$ to the serosal side induced a decrease in Isc entirely similar to that observed when $ZnCl_2$ was added to the mucosal side, although the magnitude of the response was slightly reduced (figure 1). The effect on Isc was dose dependent; it was detected at a $ZnCl_2$ concentration as low as 10 μmol/L, peaked at 35 μmol/L, and decreased at higher concentrations. To investigate the effects of excess zinc concentrations, cells were loaded with 200 μmol/L $ZnCl_2$. The supraphysiological concentration was based on previous results on the cytotoxic effects of zinc in Caco-2 cells [17]. An ion-secretion pattern was observed in response to zinc overload, as evidenced by the increase in Isc (figure 2).

The same experiments were repeated in Cl^--free buffer. Under these conditions, neither the mucosal nor the serosal addition of $ZnCl_2$ induced changes in Isc. These findings suggest that the changes in Isc observed in the first experiments involved transepithelial Cl^- movement.

To determine whether the proabsorptive effect of $ZnCl_2$ was specifically related to zinc ions, we performed the same experiments in parallel with $ZnCl_2$, zinc acetate, zinc sulfate, and $MgCl_2$. The addition of zinc acetate or zinc sulfate to the mucosal side induced a decrease in Isc entirely similar to that observed when $ZnCl_2$ was added to the mucosal side. In contrast, the addition of $MgCl_2$ at an equimolar concentration had no effect on the electrical parameters, indicating that the proabsorptive effect was selectively related to zinc (figure 3).

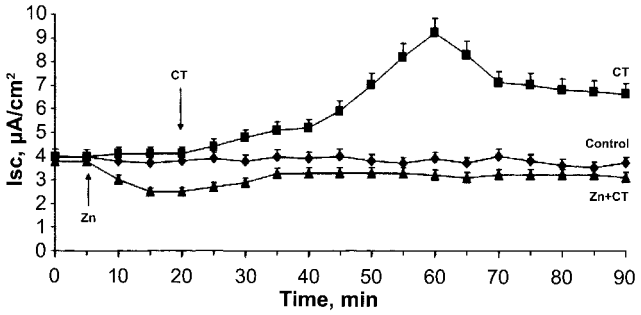

Figure 4. Time course of the effect of cholera toxin (CT), alone or in the presence of $ZnCl_2$, on short-circuit current (Isc) in Caco-2 cells mounted in Ussing chambers. The arrows indicate the time of addition of each agent. CT induced an increase in Isc, consistent with ion secretion. The latter was virtually abolished in the presence of $ZnCl_2$ (35 μmol/L). Preload with $ZnCl_2$ was associated with a decrease in Isc, consistent with ion absorption, which was followed by a modest increase in Isc after addition of CT, reaching the baseline values for the untreated control cells. Each experiment was run in duplicate and was repeated at least 3 times. Results are expressed as means ± SD.

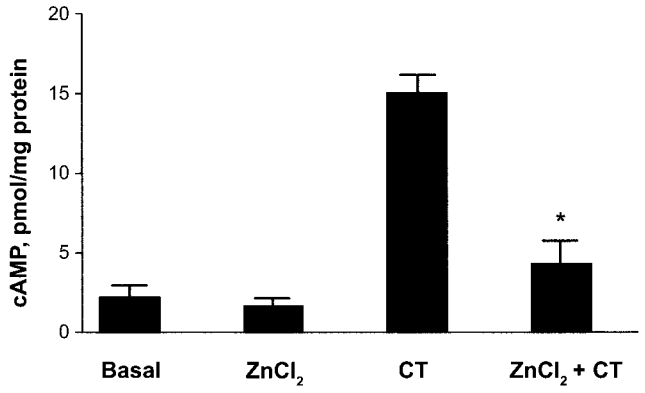

Figure 5. Changes in intracellular cAMP concentration in Caco-2 cells after a 1-h incubation with $ZnCl_2$, cholera toxin (CT), or both. A modest decrease in cAMP concentration was observed in the presence of $ZnCl_2$, whereas CT induced a marked increase in cAMP concentration. The CT-induced increase in cAMP concentration was substantially reduced in the presence of $ZnCl_2$. Data are the means ± SD of 3 different observations. $^*P < .001$, for CT alone vs. $ZnCl_2$ + CT.

Effects of Zinc under Conditions of Active Secretion

CT-induced ion secretion. To investigate the effects of zinc under conditions of CT-induced ion secretion, Caco-2 cell monolayers were exposed to the maximal effective dose of CT (6×10^{-8} mol/L), which was added to the mucosal side in the absence or presence of $ZnCl_2$. As shown in figure 4, preincubation with $ZnCl_2$ at its maximal effective concentration resulted in the complete inhibition of CT-induced ion secretion.

Because CT induces intestinal Cl^- secretion by causing an increase in intracellular cAMP concentration [2], we investigated the effect of zinc on this intracellular cyclic nucleotide concentration. To do this, we measured intracellular cAMP concentrations before and after exposure of the cell monolayers to $ZnCl_2$ (35 μmol/L) and CT (6×10^{-8} mol/L), both alone and in combination. Basal cAMP concentration was slightly reduced by the addition of $ZnCl_2$; however, $ZnCl_2$ was effective in substantially inhibiting the increase in intracellular cAMP concentration induced by CT (figure 5). Therefore, similar to what was observed in the ion-transport experiments, the effect of zinc on intracellular cAMP concentration was much more evident under conditions of active secretion than under basal conditions.

E. coli *ST–induced ion secretion.* To investigate whether zinc is effective in inhibiting *E. coli* ST–induced ion secretion, Caco-2 cells were exposed to 10^{-7} mol/L *E. coli* ST, the maximal effective ST concentration [18]. The addition of $ZnCl_2$ did not modify the increase in Isc induced by *E. coli* ST. In addition, $ZnCl_2$ did not affect either the basal or the *E. coli* ST–induced intracellular cGMP concentration (figure 6).

DISCUSSION

We have obtained evidence that zinc promotes ion absorption through a direct effect on enterocytes. The zinc-induced decrease in Isc is consistent with an increased flux of anions from the mucosal to the serosal side of enterocytes, as a consequence of their increased absorption or decreased secretion. The negation of the Isc response observed in the experiments with Cl^--free Ringer's solution indicates that Cl^- transport is the target of zinc. Because the same response was obtained with different zinc salts but not with $MgCl_2$, the absorptive effect must be entirely zinc specific.

Zinc was able to stimulate ion absorption after addition to either the mucosal or serosal side of epithelial monolayers. Several clinical and experimental data have shown that diarrhea is more severe in zinc-deficient subjects [19]. The absorptive effect induced by the serosal addition of zinc provides an explanation for the mechanism that allows zinc deficiency to be associated with severe diarrhea. The results of the present study are in agreement with previous results that showed decreased net water and electrolyte absorption in the small and large intestines of zinc-deficient rats [20]. The increased volume of stool observed in zinc-deficient children with infectious diarrhea [5–7, 19] may well be the consequence of a reduced intestinal basal absorptive tone and of a limited enterocyte compensatory absorptive capacity due to zinc deficiency.

However, the effects of zinc on intestinal ion transport, al-

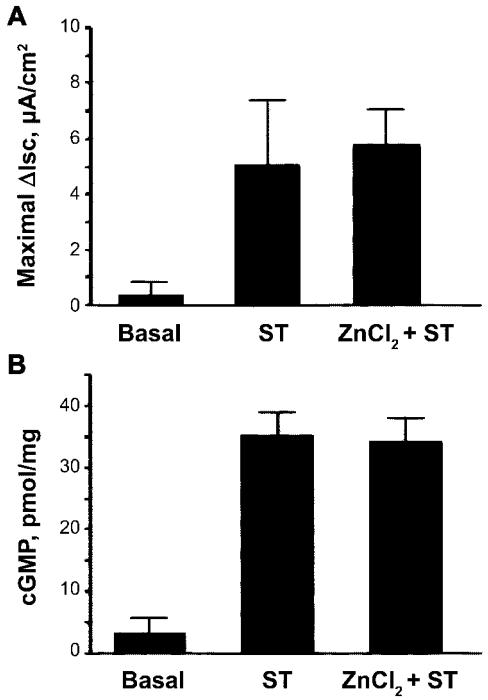

Figure 6. Effect of the addition of *Escherichia coli* heat-stable enterotoxin (ST), alone and in the presence of $ZnCl_2$, on short-circuit current (Isc) *(A)* and cGMP concentration *(B)* in Caco-2 cells. *E. coli* ST induced an increase in both Isc and cGMP concentration, neither of which was changed by the presence of zinc. Data are the means ± SD of 3 different observations. $^*P < .001$, for ST alone vs. $ZnCl_2$ + ST.

though observed under basal conditions, were maximal under conditions of active ion secretion induced by CT. At the maximal effective concentration, zinc was effective in preventing virtually all CT-induced ion secretion, and its effect on intestinal ion transport was paralleled by changes in cAMP concentration. These findings are consistent with previous findings from an animal model that showed that zinc supplementation is able to reduce intestinal cAMP-dependent ion secretion induced by theophilline [21]. In addition, zinc-induced inhibition of cAMP production through a reversible inhibition of adenylate cyclase activity has been reported in neuroblastoma cells, suggesting that zinc plays a wider—and previously unidentified—role in the regulation of intracellular cyclic nucleotide concentration [22].

Enterocyte cAMP is the signaling molecule for CT and other heat-labile bacterial enterotoxins [2]. It is 1 of the 3 intracellular mediators of active ion secretion, the other 2 being cGMP and intracellular calcium concentration [2]. We have previously shown that cAMP plays a central role in the regulation of ion secretion in the enterocyte, in concert with NO released by the activation of constitutive NO synthase (cNOS). In the enterocyte, cNOS becomes activated in the presence of CT-induced ion secretion and decreases cAMP concentration [23]. Thus, there is a cNOS/NO/cAMP pathway acting in the enterocyte as a breaking force to limit active ion secretion such as that induced by CT, and cAMP is the target of the breaking force. In the present study, we found evidence that cAMP is also under the control of extracellular zinc through a direct interaction with the enterocyte. In contrast with the observed effect zinc had on the cAMP/CT pathway of intestinal secretion, zinc had no effect on *E. coli* ST–induced secretion and on its effector cGMP. However, we cannot rule out the possibility that *E. coli* ST–induced diarrhea may be more severe in zinc-deficient children or that zinc may exert some beneficial effect during *E. coli* ST–induced diarrhea in children. Nonetheless, it is possible to hypothesize that these zinc-related positive regulatory actions on intestinal fluid transport could be further reinforced in vivo by 1 or more of the previously observed inhibitory effects that zinc has on intestinal permeability, responses to histamine and serotonine, inducible NOS (iNOS) activity, and production of uroguanylin (UG) [24–26]. At least in part, these effects are related to zinc regulation of specific gene expression. Specifically, overexpression of both the UG and iNOS genes has been previously demonstrated in a zinc-deficient–animal model. Interestingly, repletion with zinc reversed up-regulation of the iNOS gene within 1 day, whereas 3–4 days of up-regulation of the UG gene was required to achieve normal concentrations; this suggests that the mechanisms of UG and iNOS gene dysregulation are different [27]. Dysregulation of these genes may contribute to the severity of zinc-responsive diarrheal disease, as well as to the severity of intestinal inflammatory diseases.

It is known that zinc should be used cautiously in children, because of the risk of overdose. Increased mortality has been reported in malnourished children receiving as much as 6 mg/kg/day of zinc [28]. In the present study, an increase in Isc was observed in response to excess zinc load, indicating that further ion secretion may be induced by administration of zinc and providing direct proof of the danger of the administration of excessive amounts of zinc. However, most clinical trials and meta-analyses have shown that, at lower doses (such as 1.5 mg/kg/day), zinc is safe and effective [4–8]. Here, we have demonstrated that zinc does affect basal ion transport when used in concentrations (10–22 μmol/L) that are within normal plasmatic ranges and are very similar to the plasmatic concentrations reported in clinical studies in patients with diarrhea [7, 8, 29]. Furthermore, we have demonstrated that different zinc salts exert the same effects on intestinal ion transport—this suggests that different zinc formulations could be successfully used in clinical practice.

In conclusion, the results of the present study have provided evidence that zinc has direct effects on enterocyte ion transport. Zinc promotes ion absorption and prevents active secretion induced by CT, with a direct effect on cAMP concentration. Although the addition of zinc does not affect cGMP-mediated ion secretion, zinc may still have a protective effect that is associated with its action on basal ion transport.

There is an ongoing debate on the efficacy and risks of the new universal ORS, whose formulation was released by the World Health Organization/UNICEF in 2002. The new ORS has a reduced sodium concentration and is recommended for treatment of adults and children with cholera and noncholera diarrhea. On the one hand, some scientists believe that, because of the reduced sodium concentration, there is an increased risk of hyponatremia in patients with cholera diarrhea [30]. On the other hand, hypoosmolar ORS may substantially reduce childhood deaths by reducing the need for intravenous fluids [31]. We are well aware of the immense benefits of having a universal ORS [32], and we suggest that zinc should be considered as one of its components.

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