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FULL PAPER

Bacteriology

Effects of zinc supplementation on Shiga toxin 2e-producing *Escherichia coli in vitro*

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ABSTRACT. Swine edema disease is caused by Shiga toxin (Stx) 2e–producing *Escherichia coli* (STEC). Addition of highly concentrated zinc formulations to feed has been used to treat and prevent the disease, but the mechanism of the beneficial effect is unknown. The purpose of the present study was to investigate the effects of highly concentrated zinc formulations on bacterial growth, hemolysin production, and an Stx2e release by STEC *in vitro*. STEC strain MVH269 isolated from a piglet with edema disease was cultured with zinc oxide (ZnO) or with zinc carbonate ($ZnCO_3$), each at up to 3,000 ppm. There was no effect of zinc addition on bacterial growth. Nonetheless, the cytotoxic activity of Stx2e released into the supernatant was significantly attenuated in the zinc-supplemented media compared to that in the control, with the 50% cytotoxic dose values of 163.2 ± 12.7, 211.6 ± 33.1 and 659.9 ± 84.2 after 24 hr of growth in the presence of ZnO, $ZnCO_3$, or no supplemental zinc, respectively. The hemolytic zones around colonies grown on sheep blood agar supplemented with zinc were significantly smaller than those of colonies grown on control agar. Similarly, hemoglobin absorbance after exposure to the supernatants of STEC cultures incubated in sheep blood broth supplemented with zinc was significantly lower than that resulting from exposure to the control supernatant. These *in vitro* findings indicated that zinc formulations directly impair the factors associated with the virulence of STEC, suggesting a mechanism by which zinc supplementation prevents swine edema disease.

KEY WORDS: edema disease, hemolysis, shiga toxin 2e, shiga toxin-producing *Escherichia coli*, zinc

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Swine edema disease is present worldwide [24]. Edema disease is a sporadically occurring illness observed primarily in piglets after weaning and causes systemic vascular damage as a result of intestinal infection with Shiga toxin (Stx) 2e–producing *Escherichia coli* (STEC) [20]. After STEC colonizes the intestine, Stx2e produced by STEC is absorbed into capillaries and damages endothelial cells [1, 12]. The manifestations of edema disease include palpebral edema, neurological impairment, lateral recumbency, and sudden death [1, 12]. Antimicrobial administration is one of the most common treatments of edema disease [16]. In some cases, however, antimicrobial treatment (such as a sulfamethoxazole-trimethoprim combination) has shown to be associated with increased frequency of piglet deaths [3, 15, 29]. In another case, antimicrobial treatment has been reported to be ineffective, on the basis of the emergence of resistant bacteria [26]. Additionally, Yuji *et al.* [28] have reported that some highly pathogenic STEC strains produce large quantities of Stx2e. Furthermore, we have reported that the exposure of STEC to some antibiotics that inhibit cell wall synthesis, such as ampicillin, can increase the release of Stx2e [27]. On the other hand, the integrated system used in modern pig farming imposes various stressors on piglets. These stressors may negatively affect the piglets' gastrointestinal tract, resulting in suboptimal growth, decreased feed efficiency, and an increased incidence of intestinal disturbances, including diarrhea. Postweaning diarrhea attenuates the average daily weight gain and thus leads to economic losses. To minimize these negative effects, antibiotics have been used extensively on pig farms as growth promoters, a trend that has in turn resulted in the selection of bacterial strains with increased antibiotic resistance. As a consequence, in 2003, the use of in-feed antibiotics in livestock diets was banned in the EU (Regulation No. 1831/2003). Some minerals and/or pre- and probiotics, acidifiers, and plant extracts may serve as alternatives to antibiotics used as growth promoters in weaned piglets [6]. Zinc oxide (ZnO) has been generally included in diets for weaned pigs since the early 1990s. Based on data published by Moreno [22], 57%

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of piglets in Spain are given ZnO during the preweaning stage of life, and 73% at the growth stage (27–75 days). There are several reports showing positive effects of ZnO addition to pig diets: a decrease in the incidence of diarrhea and an improvement in the growth rate of piglets [4, 10, 19, 21], promotion of the development of intestinal villi [17, 25], suppression of bacterial adhesion to intestinal epithelial cells [5], and inhibition of hemolysin production by *Brachyspira hyodysenteriae* [7]. Nevertheless, the exact mechanism via which zinc prevents edema disease remains unknown. We therefore investigated the effects of zinc supplementation on STEC growth, Stx2e production, and hemolysin activity *in vitro*.

MATERIALS AND METHODS

Bacterial strain

E. coli strain MVH269 was isolated in 1999 from a piglet with signs of edema disease; a stock of the strain that had been stored at −80°C after the isolation was used in the present study. This strain, serotyped as O139, harbors the *stx2e* gene and produces Stx2e [26, 27]. The presence of the *fedA* gene was demonstrated by polymerase chain reaction with previously reported *fedA*-specific primers (5′-GTGAAAAGACTAGTGTTTATTTC-3′ and 5′-CTTGTAAGTAACCGCGTAAGC-3′) [13]. This strain yielded β-hemolysis when grown on brain-heart infusion agar (BHIA; Becton Dickinson and Co., MD, U.S.A.) supplemented with 5% of defibrinated sheep blood (Nippon Bio-Test Laboratories, Inc., Tokyo, Japan) (B·BHIA). Unless otherwise noted, liquid cultures were incubated with shaking at 37°C in brain-heart infusion broth (BHIB; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented as indicated. Initial cultures of the strain were incubated for 15 hr in BHIB; an aliquot (5 μl) of this primary culture then was inoculated into 5 m*l* of fresh BHIB and incubated for another 6 hr. The resulting actively growing culture, which had a concentration of approximately 10^9 colony-forming units (cfu)/m*l* in terms of viable bacteria, was used for each experiment.

Zinc formulations

Zinc oxide (ZnO; Nacalai Tesque, Inc., Kyoto, Japan) and zinc carbonate ($ZnCO_3$; Nacalai Tesque, Inc.) were employed in the experiments.

Effect of zinc on the growth of strain MVH269 in BHIB

A culture suspension was diluted with BHIB supplemented with 3,000 ppm ZnO (group ZnO+BHIB) or $ZnCO_3$ (group $ZnCO_3$+BHIB) to obtain a final concentration of approximately 10^4 cfu/m*l* and was incubated for 24 hr. Samples were collected at 0, 4, 6, 16 and 24 hr postinoculation (PI). The concentration of viable bacteria was confirmed by subjecting the samples to serial 10-fold dilution, by spreading 100-μl aliquots onto Mueller–Hinton agar (Oxoid, Hampshire, U.K.), by incubating the plates at 37°C overnight, and by counting the colonies.

Inhibition of Stx2e production by zinc

The procedure of the examination is shown in Fig. 1. Each culture suspension was centrifuged at 2,840 *g* for 10 min at room temperature, and the supernatant was discarded to eliminate secreted Stx2e. The bacterial pellet was washed 3 times with phosphate-buffered saline (PBS) and resuspended in BHIB at approximately 10^5 cfu/m*l*. This suspension was diluted with BHIB (zinc-free control), ZnO+BHIB, or $ZnCO_3$+BHIB to a final concentration of approximately 10^4 cfu/m*l* and incubated for 24 hr. Samples were collected at 0, 4, 6, 16 and 24 hr PI and centrifuged at 5,950 *g* for 10 min at 4°C. These supernatants were passed through membrane filters with a pore size of 0.20 μm and then tested for cytotoxic activity in a Vero cell assay [27]; the resulting data were applied to determine the 50% cytotoxic dose (CD_{50}) as previously described [20]. The above process was performed a total of 3 times.

Next, to test whether the zinc compounds inactivated Stx2e, the supernatants from each culture (BHIB, ZnO+BHIB, and $ZnCO_3$+BHIB) were mixed with the respective zinc compounds: 3,000 ppm of ZnO was added to the supernatant from group ZnO+BHIB, 3,000 ppm of $ZnCO_3$ to the supernatant from group $ZnCO_3$+BHIB, and none to the supernatant from BHIB, and incubated for 2 hr. Each mixture was then centrifuged at 5,950 *g* for 10 min at 4°C, and the supernatants were filtered and tested for their cytotoxic activities.

Hemolysis on blood agar supplemented with zinc

Zone of hemolysis on sheep blood agar: For qualitative analysis, a culture containing approximately 10^8 cfu/m*l* was subjected to a serial 10-fold dilution, and 100 μl of each dilution was spread on no-zinc B·BHIA, B·BHIA supplemented with 3,000 ppm ZnO, or B·BHIA supplemented with 3,000 ppm $ZnCO_3$. After incubation at 37°C for 20 hr, the widths of the hemolytic zones of 10 colonies on each plate type were measured using the ImageJ software (http://rsbweb.nih.gov/ij/).

Effects of zinc on hemolysis in broth cultures: Defibrinated sheep blood was centrifuged at 600 *g* for 5–10 min at room temperature, and the supernatant was discarded. Blood cells were washed 3 times with saline supplemented with 0.4 mM $MgSO_4$, followed by centrifugation at 600 *g* for 10 min at room temperature. BHIB supplemented with these washed red blood cells (B·BHIB) to 5% (v/v) was used for the hemolysis assay. To confirm the quality of B·BHIB, 0.5 m*l* of this medium was vigorously mixed with 7.0 m*l* of distilled water to attain complete hemolysis. In each experiment, optical density (OD) of the resulting mixture was measured at a wavelength of 541 nm (OD_{541}) on a spectrophotometer (Gene Quant 100, GE Healthcare, Tokyo, Japan); in a total of 3 experiments, the resulting OD_{541} was 0.62 ± 0.03 (mean ± standard error [SE]).

Bacterial cultures to be tested in the hemolysis assay were centrifuged at 2,840 *g* for 10 min at room temperature, and the

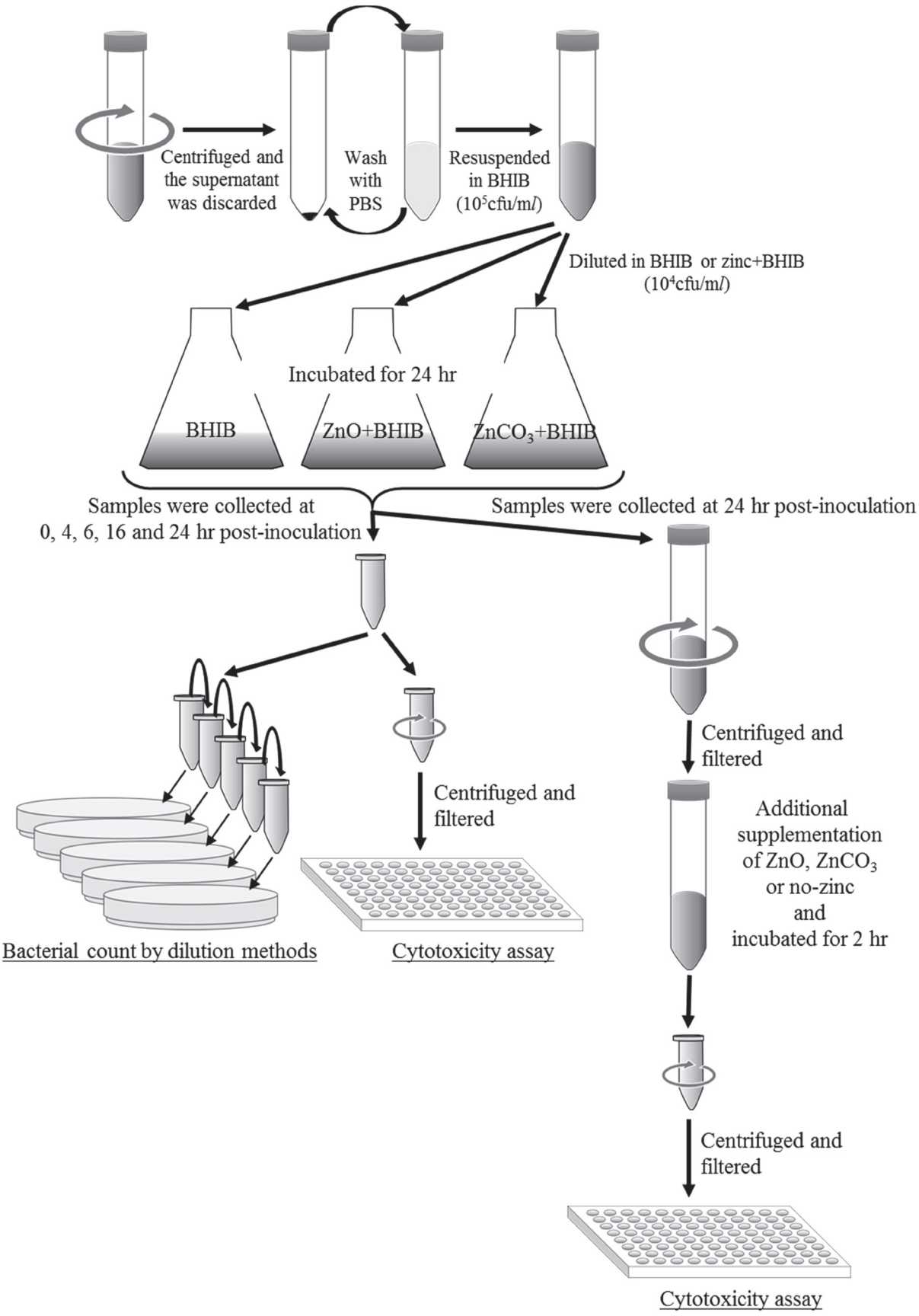

Fig. 1. Procedure for investigation of the effect of zinc on Stx2e production.

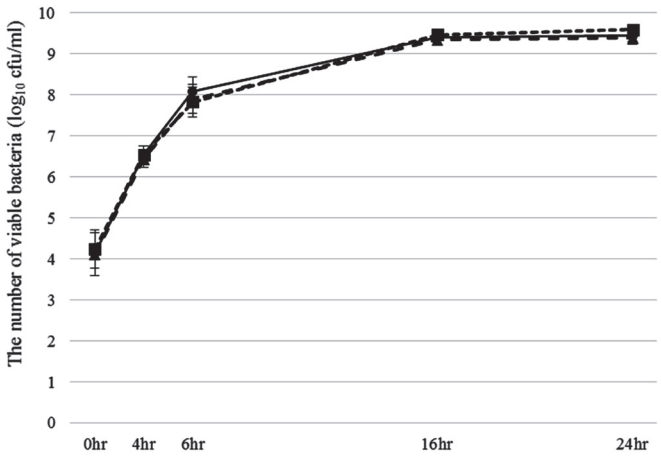

Fig. 2. Growth curve of *E. coli* strain MVH269. Viable cell numbers in control (—●—) or ZnO-supplemented (–■–) or $ZnCO_3$-supplemented (- -▲- -) culture media. Data are plotted as mean ± SE (n=3).

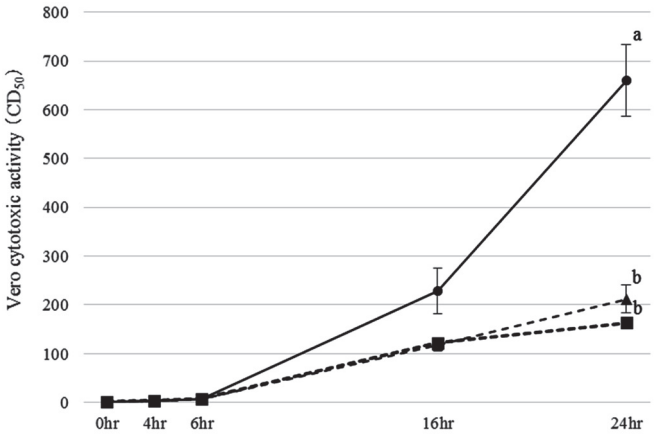

Fig. 3. Vero cytotoxic activities (CD_{50} values) for *E. coli* strain MVH269 cultures in control (—●—) or ZnO-supplemented (–■–) or $ZnCO_3$-supplemented (- -▲- -) culture media. Data are plotted as mean ± SE (n=3). Different letters indicate values that are significantly different (P<0.05) for a given time point, as assessed by the Tukey–Kramer multiple comparison test.

supernatant was discarded to eliminate secreted hemolysin. The bacterial pellets were washed 3 times with PBS and resuspended in BHIB at approximately 10^9 cfu/m*l*. The suspension was diluted with B·BHIB containing 100, 1,000 or 3,000 ppm ZnO or $ZnCO_3$ to a final concentration of approximately 10^8 cfu/m*l* and was incubated without shaking at 37°C for 240 min. Samples were collected at 0, 5, 15, 30, 60, 120, 180 and 240 min PI and centrifuged at 1,138 *g* for 10 min at room temperature. OD_{541} of the resulting supernatants was measured spectrophotometrically.

Samples taken at 0 and 240 min PI were also assessed for viable-bacteria cell counts.

Statistical analysis

With the exception of the zone of hemolysis assay on sheep blood agar, all the experiments were carried out in triplicate; the results are expressed as mean ± SE. All the statistical analyses were conducted in the SAS software (SAS Institute, Inc., Cary, NC, U.S.A.). The effects of zinc supplementation on STEC growth, hemolysin activity, and Stx2e release were analyzed using the MIXED procedure for repeated measures. Zinc supplementation groups, time, and interactions were regarded as categorical fixed effects, with each sample number as the subject. All the significant main effects and interactions were tested by Tukey-Kramer multiple comparisons. Data with P values <0.05 were considered significant.

RESULTS

Effect of zinc on the growth of strain MVH269 in BHIB

The numbers of viable bacterial cells in BHIB, BHIB with ZnO, and BHIB with $ZnCO_3$ at 16 hr PI were 9.40 ± 0.02, 9.47 ± 0.03 and 9.34 ± 0.01 log_{10}cfu/m*l*, respectively. At 24 hr PI, the respective values were 9.45 ± 0.15, 9.60 ± 0.01 and 9.39 ± 0.13 log_{10}cfu/m*l*. No significant differences in the bacterial growth curves were observed between the experimental and control groups (Fig. 2).

Inhibition of Stx2e production by zinc

The CD_{50} values increased with culture duration. These values did not significantly differ among the 3 groups through 16 hr PI, when the values for zinc-free BHIB, ZnO+BHIB, and $ZnCO_3$+BHIB were 228.5 ± 53.6, 121.9 ± 12.1, and 118.8 ± 15.3, respectively. Nevertheless, the CD_{50} values at 24 hr PI were significantly lower in groups ZnO+BHIB (163.2 ± 12.7) and $ZnCO_3$+BHIB (211.6 ± 33.1) than in the control (659.9 ± 84.2; Fig. 3).

To examine the possibility that the zinc compounds may directly interact with Stx2e released from STEC and reduce its activity, we tested the culture supernatants at 24 hr PI for their cytotoxic activities in the presence of the additional respective zinc compound as described above. The addition of the extra zinc compound to the supernatant did not significantly alter the CD_{50} values. That is, the CD_{50} values were 666.7 (before) and 709.7 (after addition) in group BHIB, 142.9 (before addition) and 132.7 (after addition) in group ZnO+BHIB, and 165.8 (before) and 191.1 (after addition) in group $ZnCO_3$+BHIB, indicating that the effect of the zinc compounds was not mediated by a direct interaction with Stx2e, but possibly by a reduction in the production of Stx2e by STEC.

Additionally, no significant differences in bacterial growth were observed among the 3 groups in each experiment, as tested by the cytotoxicity assays (data not shown).

Inhibition of hemolysis by zinc

A clear β-hemolysis zone was observed around colonies of this STEC strain on B·BHIA. There was no significant difference in colony numbers among the 3 types of agar. Nonetheless, the widths of the hemolytic zones around colonies on B·BHIA with ZnO (0.02 ± 0.05 mm) and B·BHIA with $ZnCO_3$ (0.02 ± 0.04 mm) were smaller than those on B·BHIA without zinc (1.03 ± 0.06 mm; Fig. 4).

In the hemoglobin absorbance test, the OD_{541} values (measuring the release of hemoglobin upon hemolysis) increased with time in both the test and the control group (Table 1). There was a significant difference in OD_{541} between the groups for each dose of zinc at 120 min PI. The OD_{541} value of the control increased after 60 min PI, reaching 6.93 ± 0.32 at 240 min PI. OD_{541} of the supernatant in the culture with 3,000 ppm ZnO increased after 120 min PI, reaching 4.37 ± 0.36 at 240 min PI; OD_{541} of the supernatant in the culture with 3,000 ppm $ZnCO_3$ reached 1.40 ± 0.29 at 240 min PI. In contrast, in the bacteria-free control, the OD_{541} value did not change with time.

No significant difference in the number of STEC cells was observed among 7 cultures at 240 min PI (data not shown).

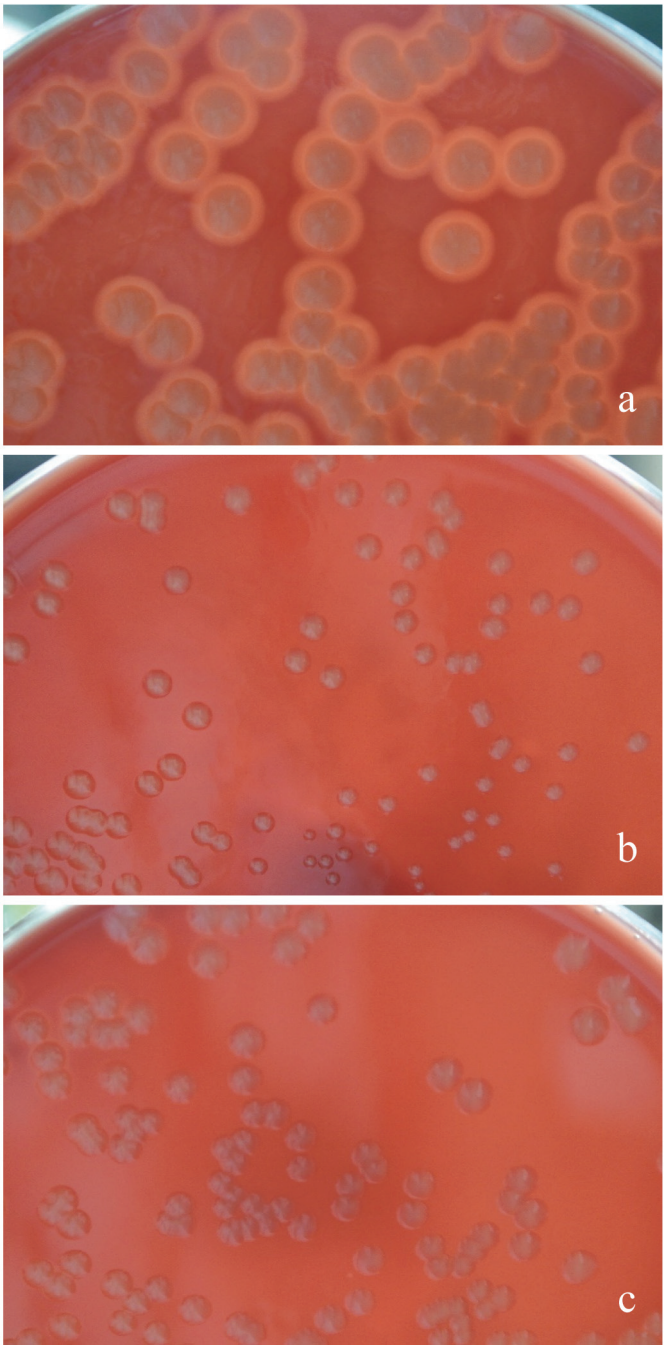

Fig. 4. *E. coli* strain MVH269 colonies and β-hemolysis on sheep blood agar. Panels show representative images of colonies growing on a) control agar, b) agar with ZnO added, and c) agar with $ZnCO_3$ added.

DISCUSSION

The results revealed that the growth of bacteria was not inhibited in the medium supplemented with zinc. Indeed, no antimicrobial effects of zinc on *E. coli* have been reported [18]. In one experiment, the numbers of coliform bacteria cells in the intestinal tract of newly weaned piglets given a high ZnO dose were the same as or higher than those in piglets receiving no ZnO [11]. Thus, our results are similar to those in other reports, indicating that the preventive effect of zinc against edema disease does not reflect an antibacterial effect. Although zinc did not inhibit the growth of STEC in broth media, the sizes of the bacterial colonies were smaller when grown on blood agar supplemented with zinc. The reason for this result is unknown. Nonetheless, there was no difference in the size of the STEC cells from the colonies grown on the solid medium with or without zinc when examined under an optical microscope (unpublished data).

The present results show that the hemolysis caused by STEC was significantly reduced for cells cultured in the medium supplemented with zinc. Most Stx2e-producing *E. coli* strains produce α-hemolysins and form zones of β-hemolysis on blood agar [2]. Moxley *et al.* [23] have reported that the incidence of septicemia in the gnotobiotic piglet model does not decrease even if the infecting bacterium harbors an inactivated hemolysin gene (*hlyA*). On the other hand, Inukai *et al.* [14] have suggested that the production of hemolysin by *E. coli* O139 is closely related to the metabolic activity of the pathogen. Zinc is an essential transition metal, required in either the catalysis or maintenance of the structural integrity of *E. coli*; this element also serves as an essential structural cofactor for many proteins [8]. Dietary doses of 2,500 ppm ZnO reduce bacterial activity (assessed as adenosine triphosphate accumulation) in the gastrointestinal tract of newly weaned piglets [11]. Furthermore, a high concentration of zinc ions disrupts the metabolic pathways of *B. hyodysenteriae*, the causative pathogen of swine dysentery, and causes complete inhibition of the hemolytic activity of this microorganism [7]. Therefore, the decrease in STEC hemolysis associated with zinc supplementation may be a result of the inhibition of hemolysin production by metabolic dysfunction. Hemolysis was inhibited in a dose-dependent manner by either $ZnCO_3$ or ZnO, with the potency of $ZnCO_3$ apparently exceeding that of ZnO. HCO_3^- stimulates zinc uptake activity of the zinc transporter, zinc-regulated transporter, and iron-regulated transporterlike protein (ZIP) in cultured mammalian

Table 1. Zinc dose dependence of hemolysin production by *E. coli* strain MVH269

		Time of culture (min)						
		0	15	30	60	120	180	240
Control (zinc-free)		0.09 ± 0.01*	0.10 ± 0.00	0.12 ± 0.01	0.60 ± 0.04	5.98 ± 0.27[a)]	6.37 ± 0.19[a)]	6.93 ± 0.32[a)]
ZnO	100 ppm	0.09 ± 0.00	0.11 ± 0.01	0.11 ± 0.01	0.42 ± 0.05	4.46 ± 0.20[b)]	5.71 ± 0.26[b)]	6.29 ± 0.37[a)]
	1,000 ppm	0.09 ± 0.00	0.10 ± 0.00	0.11 ± 0.01	0.17 ± 0.01	2.05 ± 0.33[c)]	4.46 ± 0.48[c)]	5.82 ± 0.45[a)]
	3,000 ppm	0.11 ± 0.01	0.11 ± 0.00	0.12 ± 0.01	0.15 ± 0.02	1.24 ± 0.15[c)]	3.00 ± 0.21[d)]	4.37 ± 0.36[b)]
$ZnCO_3$	100 ppm	0.10 ± 0.01	0.11 ± 0.00	0.12 ± 0.01	0.35 ± 0.03	4.57 ± 0.29[b)]	5.15 ± 0.22[b)]	6.37 ± 0.29[a)]
	1,000 ppm	0.10 ± 0.00	0.10 ± 0.00	0.11 ± 0.01	0.13 ± 0.01	0.79 ± 0.33[d)]	2.59 ± 0.52[d)]	4.20 ± 0.46[b)]
	3,000 ppm	0.10 ± 0.01	0.11 ± 0.00	0.11 ± 0.00	0.12 ± 0.01	0.13 ± 0.02[d)]	0.28 ± 0.07[e)]	1.40 ± 0.29[c)]
Bacteria-free		0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.00	0.03 ± 0.00[d)]	0.02 ± 0.01[e)]	0.03 ± 0.01[d)]

*Data are presented as mean ± SE of the OD_{541} values (n=3). Different letters indicate values that are significantly different ($P<0.05$) for a given time point, as assessed by the Tukey-Kramer multiple comparison test.

cells [8]. *E. coli* has a zinc transporter (ZupT) belonging to the ZIP family [9]. Thus, (bi) carbonate may promote the uptake of zinc by *E. coli* via a mechanism similar to that used by mammalian cells.

Furthermore, in the present study, Stx2e production by STEC was significantly lower in the media containing supplemental zinc. Stx2e is a toxin that induces the host symptoms of edema disease [20]. When certain classes of antimicrobials (such as ampicillin) are applied to treat edema disease, the accumulated Stx2e can be released after bacterial-cell body destruction, resulting in sudden worsening of symptoms [15, 26, 27, 29]. Therefore, to avoid the mass release of Stx2e, it is important to understand the antibacterial mechanism of action of proposed drug treatments. Stx2e is produced by and accumulates in the cell body and is then released from the cell [27]. As shown in the present study, supplementation with zinc decreases Stx2e and hemolysin production. These results suggest that zinc inhibits the production of Stx2e and hemolysin by impeding the synthesis of these proteins within the bacterial cytoplasm.

Dietary zinc has been used empirically for the prevention and treatment of swine edema disease although the mechanism of these effects remains unknown. As demonstrated here, suppression of the release of virulence factors from STEC is a possible mode of action of zinc supplementation in prevention of the disease. These results suggest that zinc supplementation is a scientifically valid treatment of swine edema disease.

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