Growth Inhibition of Enteric Pathogens by Zinc Sulfate: An in vitro Study

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Key Words

Growth inhibition · Enteric pathogen · Zinc

Abstract

Objective: To determine the inhibition effect of zinc sulfate on isolates of enteric bacteria. **Materials and Methods:** Mueller-Hinton agar containing different concentrations of zinc sulfate was prepared. Isolates used in this study were obtained from local clinics. They were *Salmonella typhi*, *Salmonella* groups A, B, C, D and E, *Escherichia coli*, *Enterobacter*, *Shigella* and *Vibrio cholerae*. Overnight cultures of test organisms in brain-heart infusion broth were adjusted to 0.5 McFarland standard turbidity by adding normal saline with 1 µl of each adjusted broth culture inoculated onto zinc sulfate agar plates. The plates were incubated for 18–20 h at 37 °C. The inhibition concentration was recorded as the lowest concentration of zinc sulfate that completely inhibited growth. **Results:** All enteric pathogens tested were inhibited by zinc sulfate. Of the isolates, *S. typhi* was most sensitive since 20% of the strains were inhibited by zinc sulfate of 0.8 mg/ml. *Salmonella paratyphi* A was inhibited at 1.2 mg/ml. Other *Salmonella* spp. were inhibited at concentrations between 1.4 and 2.0 mg/ml. *V. cholerae* O1 and *Shigella flexneri* demonstrated a similar pattern of inhibition as *S. paratyphi* A. A higher concentration of zinc sulfate (1.6 mg/ml) was required to completely

inhibit *Shigella sonnei*. **Conclusion:** The findings indicate that zinc sulfate has an antimicrobial effect on enteric pathogens and may contribute to the treatment of diarrhea.

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Introduction

Infectious diarrheal diseases are a leading cause of morbidity and mortality worldwide. Between 1978 and 1987, global mortality due to diarrhea among children less than 5 years of age was estimated at 3.3 million deaths per year, with a case fatality rate of 0.3% [1]. In Indonesia, similar mortality rates associated with diarrheal disease (1–8 per 1,000 children less than 5 years old), with the highest death rate among infants (<12 months) have been observed [2]. Household surveillance conducted by the Indonesian Ministry of Health in 1995 [3] showed that approximately 15% of deaths among infants and 25% among children between 1 and 4 years old were caused by severe diarrhea. Although the use of oral rehydration solution has been successful and substantial global progress has been made in reducing mortality due to diarrhea, current rates of morbidity as a result of infectious diarrhea remain a major public health problem [4]. Studies on zinc supplementation in children with acute diarrhea have been shown to be effective in reducing the duration of

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diarrhea and preventing persistent diarrhea [5–7]. Results of pooled analyses showed that children taking zinc supplements had a 15% lower probability of continued symptoms on a given day during an episode of acute diarrhea [6]. Although results from several studies support the beneficial effects of zinc in preventing prolonged diarrhea, the mechanism of such action is not completely understood [8, 9]. It is thought that zinc enhances the recovery and regeneration of impaired intestinal epithelial cells caused by diarrhea. Effects of zinc deficiency on cellular immunity response are well known, and zinc supplementation has been shown to improve the symptoms of lymphoid atrophy and increase the number of T and B lymphocytes [10]. However, the effectiveness of zinc administration should be appropriately investigated before it is recommended routinely as an adjunct therapy for acute childhood diarrhea.

Therefore, the aim of this study was to investigate the inhibition effects of zinc on enteric bacterial isolates.

Methods and Materials

Zinc Sulfate Agar

A stock solution of zinc sulfate was prepared by dissolving 0.2 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 10 ml of distilled water to obtain a concentration of 20 mg/ml. The solution was sterilized by filtration, using a Millipore filter (Millipore Co., Bedford, Mass., USA). To prepare agar media with various concentrations of zinc sulfate (zinc sulfate agar; ZSA), different volumes of zinc sulfate stock solution (20 mg/ml) between 0.2 and 2.0 ml were added into the tubes containing 20 ml melted (50°C) Mueller-Hinton agar as described in table 1. The Mueller-Hinton ZSA was poured into a sterile Petri plate and set at room temperature.

Bacterial Cultures

Isolates of enteric pathogens used in this study were wild-type strains obtained from local clinics in Jakarta, Indonesia, including *Salmonella typhi*, *Salmonella paratyphi* A, *Salmonella* groups A, B, C, D and E, *Escherichia coli*, *Enterobacter*, *Shigella* and *Vibrio cholerae* O1. Cultures were maintained in tryptic soy broth with 20% glycerol and stored at –70°C. Before testing, cultures were thawed and inoculated onto blood agar medium, incubated at 37°C for 18–20 h. Colonies were picked and identified by biochemical reactions for confirmation of purity. Pure culture was transferred into 5 ml of brain-heart infusion broth and incubated at 37°C overnight.

Inoculation of ZSA

Overnight bacterial cultures in brain-heart infusion broth were adjusted to 0.5 McFarland standard turbidity by adding normal (0.85%) saline. The ZSA was inoculated using an inoculum replicating apparatus transferring 32 inocula per microtiter plate (Corning Costar, Corning, N.Y., USA). A 10-μl sample of broth culture was dispensed into the well of a microtiter plate, and the inoculum replicating apparatus was used to transfer 1 μl of the broth culture to ZSA. The broth cultures were also inoculated onto plain (no zinc sulfate)

Table 1. The addition of zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg/ml) to Mueller-Hinton agar to make ZSA with various concentrations of zinc sulfate

Medium No.	Volume of zinc sulfate added, ml	Mueller Hinton volume, ml	Concentration in ZSA of zinc sulfate, mg/ml
1	0	20	0
2	0.2	19.8	0.2
3	0.4	19.6	0.4
4	0.6	19.4	0.6
5	0.8	19.2	0.8
6	1	19	1
7	1.2	18.8	1.2
8	1.4	18.6	1.4
9	1.6	18.4	1.6
10	1.8	18.2	1.8
11	2	18	2

Mueller-Hinton agar as a control. Plates were incubated for 18–20 h at 37°C.

Inhibition Concentration

The inhibition concentration was recorded as the lowest concentration of zinc sulfate that completely inhibited growth, disregarding a single colony or faint haze caused by the inoculum.

Results

The results of enteric pathogen inhibition by zinc sulfate are shown in table 2. Twenty percent of *S. typhi* were inhibited by zinc sulfate at a concentration of 0.8 mg/ml, while other isolates except *Salmonella* group C were beginning to show inhibition at concentrations of ≥ 1.2 mg/ml. All *S. typhi* isolates were completely inhibited at 1.6 mg/ml. All *S. paratyphi* A failed to grow at a concentration of 1.2 mg/ml. Half of *Salmonella* group B were inhibited at 1.2 mg/ml and did not grow at concentrations ≥ 1.4 mg/ml. Fifty percent and 80% of *Salmonella* group C were inhibited at concentrations of 1.4 and 1.6 mg/ml, respectively, and were totally inhibited at 1.8 mg/ml. *Salmonella* group E did not grow at 2.0 mg/ml.

Complete inhibition was shown at 1.2 mg/ml for isolates of *V. cholerae* O1 and *Shigella flexneri*, whereas *Shigella sonnei* was inhibited at 1.6 mg/ml. *Enterobacter aerogenes* and *Enterobacter cloacae* were inhibited at 1.4 and 1.8 mg/ml, respectively, while *E. coli* was inhibited at 1.6 mg/ml. All enteric pathogens tested, except for 40% of *Salmonella* group E isolates, were completely inhibited at zinc sulfate concentrations ≥ 1.8 mg/ml.

Table 2. Inhibition concentration of zinc sulfate (0–2 mg/ml) for enteric pathogens

Organism	Number tested	Cumulative percentages of isolates inhibited by the following concentrations of zinc sulfate										
		0	0.2	0.4	0.6	0.8	1	1.2	1.4	1.6	1.8	2
<i>Shigella flexneri</i>	10	0	0	0	0	0	30	100				
<i>Shigella sonnei</i>	10	0	0	0	0	0	10	20	60	100		
<i>Salmonella paratyphi A</i>	10	0	0	0	0	0	40	100				
<i>Salmonella</i> group B	10	0	0	0	0	0	10	50	100			
<i>Salmonella</i> group C	10	0	0	0	0	0	0	0	50	80	100	
<i>Salmonella</i> group D	10	0	0	0	0	0	50	70	90	100		
<i>Salmonella</i> group E	10	0	0	0	0	0	30	60	60	60	60	100
<i>Salmonella typhi</i>	10	0	0	0	0	20	70	80	90	100		
<i>Enterobacter aerogenes</i>	10	0	0	0	0	0	40	40	100			
<i>Enterobacter cloacae</i>	10	0	0	0	0	0	50	80	80	100		
<i>Escherichia coli</i>	10	0	0	0	0	0	30	80	80	100		
<i>Vibrio cholerae</i> O1	10	0	0	0	0	0	20	100				
<i>Vibrio cholerae</i> non-O1	10	0	0	0	0	0	50	100				

Discussion

Zinc supplementation has been associated with clinical reduction in duration and severity of diarrhea in infants and young children [5–7]. It also reduced the risk of continued diarrhea, measured as decreased frequency of prolonged diarrhea and the number of days with watery stools. The probable mechanisms for the antidiarrheal effect of zinc sulfate supplementation have included improved absorption of water and electrolytes by the intestine, regeneration of intestinal epithelium and the restoration of its function, increased levels of enterocyte brush border enzymes and enhanced immunological mechanisms for clearance of infection [8–10].

Our in vitro study shows that zinc sulfate at concentrations between 1.2 and 1.8 mg/ml inhibited growth in enteric pathogens commonly associated with diarrheal disease. Zinc sulfate in the form of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ was used in this study because this agent has been widely administered for supplementation in diarrheal disease and has proven to be effective in reducing the frequency and severity of diarrhea episodes. Except for 40% of *Salmonella* group E which was inhibited by zinc sulfate at a concentration of 1.8 mg/ml, most other enteric pathogens were readily inhibited at concentrations between 1.4 and 1.6 mg/ml (table 2). The dosage of zinc supplementation used in India as reported by Sazawal et al. [11] was 20 mg daily which is equal to 88 mg zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$). Taking into account that only 5–26% of zinc sulfate will be absorbed [12], 74–95% or 65.1–83.6 mg of the initial

dose (88 mg) given to the patients will actually remain in the gut unabsorbed. This amount will be distributed in the entire gut, and some factors, namely relative gut motility, water intake and retention as well as the number of zinc tablets taken by patients, may alter the relative concentration of the unabsorbed zinc sulfate and may cause the local concentration of zinc sulfate in a given site of the gut to be much lower. However, this small amount of zinc sulfate may still be effective to inhibit the enteric pathogens. Thus, zinc may not only have a physiological therapeutic effect, but an antimicrobial one as well, suggesting that the improvement in severity and the decreased risk of prolonged diarrhea attacks may be due to these dual effects of zinc sulfate.

Conclusion

Zinc has an antimicrobial effect on enteric pathogens and may contribute to the treatment of acute diarrhea.

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