ORIGINAL ARTICLE

EFFICACY OF ZINC AS AN ANTIBACTERIAL AGENT AGAINST ENTERIC BACTERIAL PATHOGENS

Umaira Faiz, Tariq Butt*, Luqman Satti, Wajid Hussain**, Faisal Hanif****

Department of Microbiology, Akhtar Saeed Medical College, Lahore, *Department of Clinical Laboratory, Fauji Foundation Hospital, Rawalpindi, **Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi

Background: Diarrhoea is a serious threat all over the world with great economic implications especially evident in the developing world. This study was aimed at determining *in vitro* efficacy of Zinc (Zn) against common enteric bacterial pathogens. **Method:** A total of 100 bacterial enteric pathogens: *Salmonellae* (n=16), enteropathogenic *Escherichia coli* (EPEC) (n=26), *Shigellae* (n=28) and *Vibrio cholerae* (n=30) were isolated from diarrhoeal stool specimens at Department of Microbiology, Armed Forces Institute of Pathology Rawalpindi during Aapril 2009 to Jan 2010. These isolates were tested against various concentrations of Zn supplemented in Mueller Hinton (MH) agar using a multipoint inoculator. A minimum inhibitory concentration of active Zn in $ZnSO_4.7H_2O$ ranging from 0.03 mg/ml to 1 mg/ml was used. **Results:** Zn completely inhibited the growth of all the tested pathogens and most of them were inhibited at a concentration of 0.06 mg/ml to 0.5 mg/ml of Zn. **Conclusions:** Zinc has an excellent antibacterial activity against enteric bacterial pathogens common in our setup which may provide basis for treatment of diarrhoea. Clinical study based on these findings is recommended.

Keywords: Diarrhoea, zinc, antibacterial, Enteric Pathogens, Cholera, Salmonella, E. coli, Shigella

INTRODUCTION

Diarrhoea is a serious threat all over the world with great economic implications especially evident in the developing world.[1] It is responsible for about 3.1 million deaths per year ranking second among all the causes of deaths due to infectious diseases worldwide.[2] The viruses account for major diarrhoeal infections in the developed nations, whereas, in the low income countries like Pakistan, bacterial agents such as *Shigellae, Salmonellae,* Enterotoxigenic *E. coli* (ETEC) and *Campylobacter* are the most common causes of infectious diarrhoea.[3]

There is data available that supports the effectiveness of zinc in the treatment of acute diarrhoea and its prophylaxis.[4] Several randomised hospital and community based trials have time and again established the efficacy of zinc in the treatment of acute and persistent diarrhoea in children less than 5 years of age.[5,6] It is also demonstrated in various efficacy trials that zinc not only reduces the diarrhoeal duration and its severity but also the likelihood of prolonged episodes.[7] In a trial carried out in International Centre for Diarrhoeal Diseases and Research (ICDDR) Bangladesh, children receiving daily zinc treatment for each diarrhoeal episode had a shorter duration of illness and less possibility of repeat episode of diarrhoea. This led to substantial reduction (50%) of non-injury mortality with simultaneous reduction in the use of antibiotics.[8]

The current study has been designed to determine the efficacy of zinc against enteric bacterial pathogens frequently isolated in patients suffering from diarrhoea in our set up.

MATERIAL AND METHODS

A total of one hundred enteric bacterial stool isolates from patients suffering from diarrhoea using non-probability convenient sampling. Bacterial isolates from stool samples irrespective of age and sex except for enteropathogenic *Escherichia coli* (EPEC) which were taken only from children of under 3 years of age having symptoms of diarrhoea/dysentery irrespective of signs of fever and abdominal cramps. The patients either did not use antibiotics or had omitted antibiotics more than 48 hours before collection of specimen. However, bacterial isolates from diarrhoeal stools from patients already received antimicrobials/omitted antibiotics within the last 48 hours, and all stool samples revealing adult parasites, larvae, ova or cysts were excluded from the study.

Enteric pathogens including *Salmonellae,* EPEC, *Shigellae* and *Vibrio cholerae* were isolated from diarrhoeal stool specimens submitted to the microbiology laboratory of Armed Forces Institute of Pathology (AFIP) Rawalpindi. The isolates were identified using biochemical and serological methods, according to the standard procedures.[9] Cultures were maintained in tryptic soy broth with 20% glycerol and stored at -70 ºC. Subsequently, the cultures were thawed and streaked on blood agar followed by incubation at 37 ºC for 18–24 hours. Isolated colonies were re-identified by biochemical reactions for confirmation of purity.

Zinc Sulphate ($ZnSO_4.7H_2O$) manufactured by Merck was used in the study. The MIC of active Zn in $ZnSO_4.7H_2O$ ranging from 0.03 mg/ml to 1 mg/ml was used. The molecular weight of zinc Sulphate ($ZnSO_4.7H_2O$) is 287.5. So 4.42 mg of zinc Sulphate

contains 1 mg of Zn. A sterile stock solution of Zinc Sulphate was prepared by dissolving 1,768 mg of $ZnSO_4.7H_2O$ in 20 ml of sterile distilled water (equivalent to 20 mg zinc/ml). The solution was sterilised using a millipore filter (Millipore Co). When 1 ml of this stock solution was mixed with 19 ml of MH agar, the final concentration of Zn was 1 mg/ml in the MH agar. Further, stock solution was diluted to obtain final concentration of 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 1.25 mg/ml, and so on. One ml from each dilution was added in 19 ml of MH agar at the time of pouring plates to achieve a final concentration of 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.06 mg/ml and 0.03 mg/ml respectively. Plates without $ZnSO_4$ were also prepared using 1 ml sterile distilled water instead of $ZnSO_4$ solution. To determine that the activity against micro-organisms was due to Zn and not due to SO_4, we demonstrated the inactivity of SO_4 part by using Na_2SO_4 instead of $ZnSO_4$.

Enteric pathogens: *Salmonellae*, *Shigellae,* EPEC and *V. cholerae* were isolated and identified before and maintained at -70 ºC. Subsequently, the cultures were thawed and streaked on blood agar followed by incubation at 37 ºC for 18–24 hours. Isolated colonies were re-identified by biochemical reactions for confirmation of purity and these overnight bacterial cultures in tryptic soy broth were adjusted to 0.5 McFarland turbidity standards by adding normal saline. Bacterial suspensions were inoculated on plates containing abovementioned Zn concentrations using a multipoint inoculator (Denley Instruments Ltd.). Approximately 20 µL of each of bacterial suspension was dispensed in the wells of multipoint inoculator and 1 µL of the suspension was inoculated on the Zn Sulphate agar plate. Zinc Sulphate free control plates were also inoculated before and after inoculating Zinc Sulphate plates along with Na_2SO_4 control plates. The plates were incubated at 35–37 ºC for 18–24 hours.

The lowest concentration of Zinc Sulphate that completely inhibits visible growth will be recorded as MIC. A single colony or a faint haze left by the initial inoculums was not recorded as growth.

The data was analysed using SPSS-17. Frequencies and percentages of the susceptible bacterial pathogens to zinc were determined. Mean±SD was calculated for normally distributed, or Median±IQR for non-normally distributed quantitative variables. The *p*-value was calculated using Chi-square test to determine the significant differences of Zinc Sulphate MIC among the different isolates.

RESULTS

A total of 100 enteric bacterial pathogens were included in the study. Different enteric pathogens included in the study were *Salmonella* sp. (n=16), *Shigella* sp. (n=28), *Vibrio cholerae* (n=30) and Enteropathogenic *Escherichia coli* (EPEC) (n=26). Among *Salmonellae,* different species used were, *S. typhi* (n=5), *S. paratyphi* A (n=2), *S. paratyphi* B (n=2), *S. typhimurium* (n=2), *S. enteritiditis* (n=4) and *S. infantum* (n=1). All the *Shigellae* (n=28) used were serologically identified as *Shigella flexneri. Vibrio cholerae* serogroup O1, biotype ElTor (n=30) isolated, belonged to serotype Ogawa (n=26) and serotype Inaba (n=4). Age of the patients ranges from less than a year to 60 years with a median age of 20.00±28.5 years (Median±IQR). All the EPEC (n=26) were isolated from the diarrhoeal stool samples of children <3 years of age. The male to female ratio for *Salmonellae* was 1.3:1, for *Shigellae* 1.6:1, for *Vibrios* 3.3:1, and for EPEC it was 1:1 (p=0.2059).

The MIC of active Zn in $ZnSO_4$ ranging from 0.03 mg/ml (30 mg/L) to 1 mg/ml (1,000 mg/L) was used based on the results of the pilot studies conducted before taking up the project. MIC_{90} of $ZnSO_4$ was 0.5 mg/L and MIC_{100} was 1.0 mg/L. Almost all the isolates of *Shigellae* (MIC 0.2314±0.0445), *Salmonellae* (MIC 0.2500±0.000) and EPEC (MIC 0.2208±0.0699) showed an MIC of >0.125 mg/ml ≤0.25 mg/ml whereas *V. cholerae* had the higher MIC of Zinc Sulphate (MIC 0.4667±0.0864) as compared to all other species. (Table-1, Figure-1). Most of the isolates were inhibited at a concentration of 0.06 mg/ml to 0.5 mg/ml of Zn, out of which 61% failed to grow at a concentration of 0.25 mg/ml. All of the isolates of *Salmonellae* were completely inhibited at 0.25 mg/ml. Four percent of EPEC isolates showed an MIC of >0.03 mg/ml <0.06 mg/ml and 5% of *S. flexneri* were inhibited at a concentration of 0.125 mg/ml.

MIC of Zinc Sulphate against all different isolates from male and female did not differ significantly (p=0.9769) (Table-2).

Table-1: MIC of Zinc Sulphate (mg/L) of the tested isolates (n=100)

ISOLATES	Number of different isolates revealing MIC of Zinc Sulphate (mg/L							Mean MIC	SD
	0.03	0.06	0.125	0.25	0.5	1.0	Total		
***Shigella* Species**	0	0	5	23	28	28	28	0.2314	0.0445
EPEC	0	4	0	22	26	26	26	0.2208	0.0699
Vibrio cholera	0	0	0	4	26	30	30	0.4667	0.0864
***Salmonella* species**	0	0	0	16	16	16	16	0.2500	00000

Table-2: Diarrheogenic bacterial isolates from male (n=62) and female (n=38) with mean of MIC of Zinc Sulphate against them

Isolates	Males	MIC of $ZnSO_4$	Females	MIC of $ZnSO_4$	p-value
***Shigellae* (n=28)**	17	0.2427±0.0303	11	0.2096±0.0631	0.9769
EPEC (n=26)	13	0.2354±0.0527	13	0.2208±0.0714	
***Vibrio cholerae* (n=30)**	23	0.4674±0.0861	7	0.4643±0.0945	
***Salmonellae* (n=16)**	9	0.25±0.0000	7	0.25±0.0000	
Total	62		38		

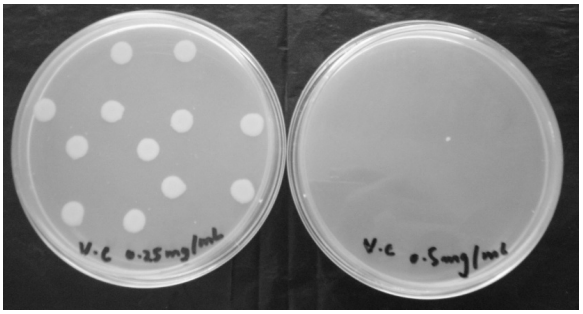

Figure-1: MH agar plates with incorporated Zinc Sulphate concentration of 0.25 and 0.5 mg/ml.
White plaques are the growth of *Vibrio cholerae* (VC), which failed to grow at a concentration of 0.25mg/ml of Zinc Sulphate

DISCUSSION

Infectious diarrhoeal diseases are a leading cause of morbidity and mortality worldwide. According to World Health Organization mortality country fact sheet 2006, diarrhoeal diseases rank third among the top ten causes of death in Pakistan.[10]

WHO/UNICEF recommends a dose of 10 mg of zinc daily for children less than 6 months of age and 20 mg zinc per day for children between 6–59 months of age for a period of 10–14 days for management of childhood diarrhoea.[11] Zinc is being efficiently used in various forms like Zinc Sulphate, Zinc Gluconate and Zinc Acetate.[12] After sufficient verification of effectiveness of zinc as a treatment for childhood diarrhoea, ICDDR Bangladesh launched a programme Scaling Up Zinc for Young Children (SUZY) to make zinc available as treatment to the masses in the form of dispersible, 20 mg Zinc Sulphate tablet.[13]

According to various clinical studies the possible mechanisms by virtue of which it reduces the duration of diarrhoea includes enhanced absorption of water and electrolytes by the intestine, increased regeneration of intestinal epithelium, high levels of enterocyte brush border enzymes and improvement of immune response which helps in clearing the pathogens from gut during diarrhoea.[4]

A novel surveillance is on the rise, establishing that zinc also acts as a drug[14], and it has an *in vitro* antibacterial effect on various bacteria[15] besides having anti-diarrhoeal activity.[4] Zn also binds to the membranes of the micro-organisms, consequently prolonging the lag phase of the growth cycle and increasing the generation time of the organisms so that it takes each organism more time to complete the cell division.[16] Zn is also found to block the secretory effect of cholera toxin and the *E. coli* heat labile enterotoxin which, act by cyclic adenosine monophosphate.[17] Very few studies have so far been conducted on the antibacterial effect of various salts of zinc on enteric pathogens.

In the present study EPEC were isolated only from the diarrhoeal stool samples of children <3 years of age as it is among the most important pathogens causing infantile diarrhoea in the developing countries.[18] All *Shigellae* isolated during our study were identified as *Shigella flexneri.* It is the most prevalent species in the developing countries accounting for 58.0% of cases of diarrhoea in Pakistan.[3,19–21]

Our results revealed that Zn completely inhibited growth of all tested enteric bacterial pathogens. In Indonesia an *in vitro* study was conducted by Surjawidjaja *et al*[22] to determine the inhibitory effect of Zinc Sulphate against enteric bacteria. All enteric pathogens tested were inhibited by Zinc Sulphate, as seen in our study. The study was different from our study in certain ways; first, they determined the antibacterial activity of Zinc Sulphate while we calculated the amount of active zinc from Zinc Sulphate to achieve the required MIC range of 0.03 mg/ml to 1.0 mg/ml. We also determined the inactivity of Sulphate part in Zinc Sulphate and made control plates by dissolving a calculated amount of Na_2SO_4 in MH agar. It confirmed that antibacterial activity against micro-organism was only due to Zn and Sulphate part was totally inactive. Furthermore, they demonstrated the bactericidal effect of Zinc Sulphate on enteric pathogens acquired from their local clinics, whereas, we tested the indigenous enteric pathogens isolated in our setup.

Another study determined the *in vitro* antibacterial activity of Zinc Sulphate against *Shigella* spp. with different concentrations. The results showed that all the isolates were readily inhibited by Zinc Sulphate, again comparable with our results.[23] Crane *et al* documented the direct inhibitory effect of Zn on certain common pathogens in childhood diarrhoea like EPEC.[24]

The current study provides an impetus that zinc has potent bactericidal activity against enteric pathogens besides having anti-diarrhoeal effect which will definitely be helpful in controlling the gratuitous use of antibiotics during diarrhoea. There is no other treatment that has proved as effective as zinc in reducing

the duration of acute diarrhoea in children especially in the developing world.

CONCLUSION

Zinc has an excellent antibacterial activity against enteric bacterial pathogens common in our setup which may provide basis for treatment of diarrhoea. Clinical study based on these findings is recommended.

REFERENCES

1. Black RE, Morris SS, Bryce J. Where and why are 10 million children dying every year? Lancet 2003;361:2226–34.
2. Sur D, Ramamurthy T, Deen J, Bhattacharya SK. Shigellosis: challenges & management issues. Indian J Med Res 2004;120:454–62.
3. Jafari F, Shokrzadeh L, Hamidian M, Ahrabi SS, Zali MR. Acute Diarrhoea due to Enteropathogenic Bacteria in Patients at Hospital in Tehran. Jpn J Infect Dis 2008;61:269–73.
4. Hoque KM, Binder HI. Zinc in the treatment of acute diarrhea: Current status and assessment. Gastroenterology 2006;130:2201–5.
5. Strand TA, Chandyo RK, Bahl R, Sharma PR, Adhikari RK, Bhandari N, *et al*. Effectiveness and Efficacy of Zinc for the Treatment of Acute Diarrhoea in Young Children. Pediatrics 2002;109:898–903.
6. Faruque AS, Mahalanabis D, Haque SS, Fuchs GJ, Habte D. Double-blind, randomized, controlled trial of zinc or vitamin A supplementation in young children with acute diarrhea. Acta Paediatr 1999;88:154–60.
7. Bhutta ZA, Bird SM, Black RE, Brown KH, Gardner JM, Hadayat A, *et al*. Zinc Investigators' Collaborative Group. Therapeutic effects of oral zinc in acute and persistent diarrhoea in children in developing countries: pooled analysis of randomised controlled trials. Am J Clin Nutr 2000;72:1516–22.
8. Baqui AH, Black RE, Arifeen EI, Yunus M, Chakra-borty J, Ahmad S, *et al*. Effect of zinc supplementation started during diarrhea on morbidity and mortality in Bangladeshi children: community randomized trial. BMJ 2002;325:1059.
9. Schreckenberger PC, Linquist D. Algorithms for identification of Aerobic Gram-Negative bacteria. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, (eds.). Manual of Clinical Microbiology, 9th ed, Washington DC: ASM Press, 2007: p.371.
10. WHO mortality country fact sheet 2006. Available from: http://www.who.int/whosis/mort/profiles/mort_emro_pak_pakistan.pdf
11. WHO/UNICEF Joint Statement. Clinical management of acute diarrhea. Geneva: World Health Organization, 2004. p.7.
12. Scrimgeour AG, Lukaski HC. Zinc and diarrheal disease: current status and future perspectives. Curr Opin Clin Nutr Metab Care 2008;11:711–7.
13. Larson CP, Hoque AM, Larson CP, Khan AM, Saha UR. Initiation of zinc treatment for acute childhood diarrhea and risk for vomiting or regurgitation: A randomized, double-blind, placebo-controlled trial. J Health Popul Nutr 2005;23:311–9.
14. Crane JK, Hoque KM. Zinc for Infectious Diarrhea in Developed Countries: Should We Be Sprinkling Our Own Lawns? Journal of Pediatric Gastroenterol Nut 2008;46:484–5.
15. Sawai J. Quantitative evaluation of antibacterial activities of metallic oxide powders (ZnO, MgO and CaO) by conductimetric assay. J Microbiol Methods 2003;54:177–82.
16. Selahattin A, Kadri G, Ramazan C. Effect of zinc on microbial growth. Tr J Med Sciences 1998;28:595–7.
17. Canani R, Ruotolo S. The Dawning of the "Zinc Era" in the Treatment of Pediatric Acute Gastroenteritis Worldwide? J Pediatr Gastroenterol Nutr 2006;42:253–5.
18. Chart H. Escherichia. In: Greenwood D, Slack RC, Peutherer JF, (eds.). Medical Microbiology A guide to Microbial Infections: Pathogenesis, Immunity, Laboratory diagnosis and Control, 16th ed, Philadelphia: Elsevier Inc; 2006. p.265–74.
19. Ranjbar R, Dallal MM, Pourshaffie MR. Epidemiology of shigellosis with special reference to hospital distribution of *Shigella* strains in Tehran. Iranian J Clin Infect Dis 2008;3:35–8.
20. Samal SK, Khunita HK, Nanda PK, Satapathay CS, Nayak SR, Sarangi AK, *et al*. Incidence of Bacterial Enteropathogens among Hospitalized Diarrhoea Patients from Orisa, India. Jpn J Infect Dis 2008;61;350–5.
21. Seidlein LV, Kim DR, Ali M, Lee H, Wang XY, Thiem VD, *et al*. A Multicentre Study of *Shigella* Diarrhoea in six Asian Countries: Disease Burden, Clinical Manifestations and Microbiology. PLoS Med. 2006;3:e353.
22. Surjawidaja JE, Hidayat A, Lesmana M. Growth inhibition of enteric pathogens by Zinc Sulphate: an *in vitro* study. Med Princ Prac 2004;13:286–9.
23. Iwalokun BA, Bakare S. Comparative sensitivity to Zinc Sulphate of *Shigell*a isolates recovered from Nigerian children with low and marginal plasma zinc concentrations. J Pediatr Infect Dis 2008;3:167–74.
24. Crane JK, Naeher TM, Shulgina I, Zhu C, Boedeker EC. Effect of zinc in Enteropathogenic *Escherichia coli* infection. Infect Immun 2007;75:5974–84.

Address for Correspondence:

Dr. Umaira Faiz, House No. 26, Ayub Colony, Khayaban-e-Tanvir, Chaklala Scheme-III, Rawalpindi, Pakistan. **Tel:** +92-51-5704399, **Cell:** +92-334-5291955

Email: umaira_faiz@hotmail.com