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Inclusion of zinc in therapeutic regimen can mitigate and/or obviate use of antimicrobials in neonatal calf diarrhea

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Abstract
The wide range of avoidable complications including the usage of high cost antimicrobials, poor owner compliance and immuno compromised state of the diarrheic calves owing to gastrointestinal loss of zinc that arise out of the current trends of neonatal calf diarrhea management enjoins for development and/or adoption of novel therapeutic options that ensure better clinical cure. One newer, sustainable and affordable alternative may be the incorporation of zinc in the therapeutic regimen. This may hasten recovery; reduce morbidity and mortality rates associated with NCD. WHO has long back recommended Zinc supplementation to decrease the duration and severity of infant diarrhea. Yet the same hasn't been fully evaluated in animal health. The aim of this study was to evaluate *in-vitro* antimicrobial activity of zinc salts by agar diffusion method against *E. coli* isolated from diarrheic calves (aged < 28 days), to assess their effect on the colony count and to check therapeutic effectiveness of zinc gluconate either alone or in combination with Sulfamethoxazole & Trimethoprim (Bactrim) in neonatal diarrheic calves. The results indicated the potential inhibitory effect of zinc oxide (ZnO) on the growth of *E. coli;* zinc chloride ($ZnCl_2$) showing no effect while as zinc sulphate ($ZnSO_4$) favoured the bacterial growth. All zinc-containing formulations tested (ZnO, zinc gluconate and zinc acetate) showed the reduction in fecal colony count; more pronounced and equivalent to ceftriaxone was observed with ZnO. Zinc gluconate administration to neonatal diarrheic calves showed promising therapeutic potential in terms of reduction in the number of days taken for clinical recovery.

Keywords: zinc, antimicrobial activity, neonatal calf diarrhea

1. Introduction
Diarrhea in calves younger than 4 weeks of age, known as neonatal calf diarrhea (NCD), is a major cause of calf mortality and economic loss owing to increased treatment expenses and decreased lifetime productivity in calf producing units. NCD accounts for approximately 75% of the mortality of dairy calves less than three weeks of age [1]. Generally, NCD results from complex interaction of the environment, infectious agents and the calf itself [2]. Infectious acute diarrhea in calves is most commonly associated with Enterotoxigenic *E. coli*, *Cryptosporidium parvum*, *Salmonella dublin*, *Salmonella typhimurium*, *Rotavirus*, *Coronavirus* or a combination of these pathogens [3] (De La Fuente *et al*., 1998). *E. coli* K99+ is known as the most common pathogen identified in scouring calves less than two months of age [4] (Acha *et al*., 2004).
Zinc, an intracellular signalling molecule, acts as an immunomodulator (enhances cell mediated immunity), an oxidant (decreases ROS generation) and anti-inflammatory agent (decreases generation of inflammatory cytokines and adhesion molecules). Owing to these unique properties it offers significant therapeutic benefits in several human and animal diseases [5].
There are reports on the considerable antibacterial activity of oxides of Ca, Mg and Zn [6], which is attributed to the generation of ROS on the surface of these oxides. The advantage of using these inorganic oxides as antimicrobial agents is that they contain environmentally safe elements essential for human health and exhibit strong activity even when administered in meager amounts. The non-judicious and indiscriminate uses of antimicrobial agents have led to the development of antimicrobial resistance in pathogens and thus pose a serious global public health concern. To overcome such problems extensive research is being done with various natural and inorganic substances vis-à-vis antimicrobial activity.

Among them zinc has shown strong inhibitory and antibacterial effects [7].

The aim of the study was to evaluate the *in-vitro* antimicrobial activity of zinc against *E. coli* so that its adjunct therapeutic potential could be tested by on-field trial in clinical cases of neonatal calf diarrhea.

2. Materials and Methods

The research material consisted of *E. coli* isolated from neonatal diarrheic calves presented to Teaching Veterinary Clinical Complex, FVSc & AH (SKUAST-Kashmir), and identified cases by on-field visits to local villages (Shuhama, Alusteng, Buserbugh) and a near-by dairy farm against which the inhibitory effect of zinc salts/suspension was tested. The antimicrobial activity of zinc was evaluated by taking the varying concentration of different salts of zinc like zinc oxide (ZnO), zinc chloride ($ZnCl_2$) and zinc sulphate ($ZnSO_4$) on Muller-Hinton agar plates inoculated with *E. coli* culture- by agar well diffusion method [8]. The zone of inhibition (if any) was compared to a panel of antibiotics like cefotaxime, Ceftriaxone etc. using same bacterial culture. The concentration of zinc salts and antimicrobials is shown (Table 2 & 4). Further, the antimicrobial potential of different salts of zinc was evaluated by observing the reduction in a number of colonies of *E. coli* on MacConkey agar. Different concentrations of ZnO (2.50 mg and 5 mg), and $ZnSO_4$ (8.80 mg and 17.60 mg) were dissolved in autoclaved triple distilled water and poured on MacConkey agar plates; Zinc gluconate {Zn 20 suspension, marketed by Wallace pharmaceutical Ltd., 1 ml equivalent to 4 mg elemental zinc} was poured on MacConkey agar plates and also mixed with the media to observe the effect of pouring and/or mixing on the fecal colony count by using 10^{-4} dilution of *E. coli* suspension. Similar technique was followed for Zinc acetate {Zinconia, marketed by Zuventus Healthcare Ltd., 1 ml equivalent to 10 mg elemental zinc}.These were compared against Taxim (10 mg) and autoclaved distilled water as control (Table 3). Later the same experiment was repeated by taking DMSO as a solvent for ZnO. Therapeutic efficacy of Zinc as mentioned (Table 1) was evaluated in neonatal diarrheic calves (aged < 28 days, of either sex, by single-blinded randomized on-field trial) by considering the number of days taken for resolution of clinical signs (Clinical cure i.e. return of normal rectal temperature, Fecal consistency score of (FCS) 1 and Attitude score (ATS) of 1). The data were analyzed using SPSS (version16).

Table 1: Evaluation of therapeutic potential of zinc

Group	Status of calves (n=6)	Intervention (till resolution of clinical signs)
A	Healthy (Negative control)	**Nil**
B	Diarrheic	ORS + Zinc (Zinc gluconate syrup) orally @ 2 mg/Kg body wt. once daily.
C	Diarrheic	ORS + Zinc (Zinc gluconate syrup) orally @ 4 mg/Kg body wt. once daily.
D	Diarrheic	ORS + Sulphamethaxazole & Trimethoprim (Bactrim tab) orally @ 20 mg/kg body wt. 24 hourly.
E	Diarrheic	ORS + Sulphamethaxazole & Trimethoprim (Bactrim tab) orally @ 20 mg/kg body wt. 24 hourly. + Zinc (Zinc gluconate) orally @ 2 mg/Kg body wt. once daily.
F	Diarrheic	ORS + Sulphamethaxazole & Trimethoprim (Bactrim tab) orally @ 20 mg/kg body wt. 24 hourly. + Zinc (Zinc gluconate syrup) orally @ 4 mg/Kg body wt. once daily.

3. Results and Discussion

The dose-dependent increase in the zone of inhibition shown by ZnO is comparable to that produced by different antimicrobials. $ZnCl_2$ did not show any inhibitory activity. However, dose-dependent increase in growth was observed with $ZnSO_4$; even it masked the antimicrobial effect of Ceftriaxone. The effect of Zinc salts on fecal colony count revealed a dose-dependent decrease in colony count with respect to ZnO only. The additive effect of $ZnSO_4$ on the growth of *E. coli* needs further studies. Further, Zinc acetate/ Zinc gluconate showed the decrease in colony count either when poured on and/or mixed with the media. Zinc acetate showed better results compared to Zinc gluconate. The findings indicate that zinc has considerable antimicrobial activity against *E. coli* and its usage can mitigate and/or obviate the use of antibiotics in human/animal recipients. This can be attributed to prolonging of lag phase of growth cycle and generation time of microorganisms [9]. In a study by Sodeberg *et al*. [10] gram positive bacteria were the most susceptible to zinc ion compared to gram negative that usually were not inhibited even at the highest concentration (1024 µl/ml). A study carried out by Yousef and Daniel [7] reported 14 mm and 24 mm zone of inhibition for zinc oxide and nano-zinc oxide respectively against *E. coli* using agar diffusion technique. The antimicrobial effect of ZnO and nano-ZnO was attributed to reactive oxygen species (like OH^-, H_2O_2 and O^{2-}) released on the surface of ZnO which cause bacterial killing. In addition the combination of ZnO with Amoxycillin or Chloramphenicol produced 20 mm zone of inhibition against *E. coli.* Nano-ZnO inhibited the growth and multiplication of variety of microorganisms tested including the *E. coli*. Crane *et al.* (2007) also documented the direct inhibitory effect of zinc on enteropathogenic *E. coli* causing childhood diarrhea.

In the clinical trial zinc gluconate was preferred over zinc acetate based on the better results indicated by earlier studies with respect to reduction in median duration of illness [11]. The mean duration of illness was significantly reduced ($P<0.05$) by zinc gluconate either alone or in combination as compared to antimicrobial alone (Table 5).

Although the exact mechanism of action of zinc is still unknown, mucosal protective role, enhanced cell mediated immunity and modification of intra-luminal electrolyte secretion and absorption mechanisms have been proposed [11]. Glower *et al*. [12] reported that calves treated with Zinc oxide had 1.4 times higher rate of clinical cure compared to calves in the placebo group. Fernandes *et al*. [13] reported that clinical symptoms abated earliest in diarrheic calves treated with sulfadiazine-trimethoprim followed by amoxicillin trihydrate, whereas recurrence of diarrhea in two cases was observed in calves treated with ciprofloxacin-tinidazole combination. Wegmuller *et al*. [14] observed high but equivalent zinc absorption in young adult human recipients from zinc citrate and zinc gluconate, and less absorption from more insoluble zinc oxide. Equal effectiveness of zinc from zinc citrate or zinc gluconate in the treatment of diarrhea was also noticed.

Table 2: Zone of inhibition shown by zinc salts

Zinc salt	Amount taken (mg)	Concentration of elemental Zinc (mg)	Zone of inhibition (mm)
Zinc oxide	2.50	2.00	12
	5.00	4.00	14
	12.50	10.00	16
	18.75	15.00	14
	25.00	20.00	18
	31.25	25.00	20
Zinc chloride	4.18	2.00	No zone of inhibition
	8.36	4.00	No zone of inhibition
Zinc sulphate	8.80	2.00	No zone of inhibition. Instead dose dependent increase in growth was observed. Effect of Ceftriaxone (CTR) was marked as evident from lack of clear cut zone of inhibition around CTR.
	17.60	4.00	
	44.00	10.00	
	66.00	15.00	

Table 3: Effect of zinc salts on fecal colony count

Zinc salt	Amount taken (mg)	Concentration of elemental zinc (mg)	Coliform count with autoclaved distilled water	Coliform count with DMSO as solvent
Zinc oxide	2.5	2.0	3.3×10^6	2.1×10^6
	5.0	4.0	No colony	No colony
Zinc sulphate	8.8	2.0	1.71×10^7	-
	17.6	4.0	1.43×10^7	-
Zinc gluconate	1ml (pour on)	2.0	5.20×10^6	-
	1ml (mix)	2.0	1.48×10^7	-
Zinc acetate	1 ml (pour on)	2.0	4.8×10^6	-
	1ml (mix)	2.0	1.3×10^6	-
Autoclaved distilled water	1 ml	-	1.71×10^7	
Taxim (Ceftriaxone)	10	-	No colony	

Table 4: Zone of inhibition shown by antimicrobial agents

S. No	Antibiotic	Concentration	Zone of inhibition (mm)	Result
1	Doxycycline	30 mcg	25 mm	Sensitive
2	Trimethoprim	5 mcg	16 mm	Sensitive
3	Co-Trimoxazole	25 mcg	16 mm	Sensitive
5	Enrofloxacin	10 mcg	25 mm	Sensitive
6	Azithromycin	15 mcg	25 mm	Sensitive
7	Norfloxacin	10 mcg	22 mm	Sensitive
8	Erythromycin	15 mcg	20 mm	Sensitive
9	Ofloxacin	5 mcg	16 mm	Sensitive
10	Ampicillin	10 mcg	17 mm	Sensitive
11	Streptomycin	10 mcg	15 mm	Sensitive
12	Cefotaxime	30 mcg	24 mm	Sensitive
13	Ceftriaxone	10 mcg	24 mm	Sensitive
14	Cefuroxime	30 mcg	22 mm	Sensitive
15	Cefpodoxime	10 mcg	10 mm	Sensitive
16	Cefadroxil	30 mcg	12 mm	-

Table 5: Effect of zinc on duration of illness

Group	Status of calves (n=6)	Number of days taken for recovery
A	Healthy (Negative control)	**Nil**
B	Diarrheic	2-3 days ($2.17^a \pm 0.17$)
C	Diarrheic	2-6 days ($3.00^a \pm 0.63$)
D	Diarrheic	4-6 days ($5.00^b \pm 0.37$)
E	Diarrheic	3 days ($3.00^a \pm 0.00$)
F	Diarrheic	2-3 days ($2.83^a \pm 0.17$)

4. Conclusions

The experimental observations reveal the potential *in-vitro* antimicrobial activity of ZnO, and Zn acetate/gluconate. Zinc (especially zinc oxide) has potent antibacterial activity against enteric pathogens. Inclusion of zinc in therapeutic regimen either alone or in combination with antimicrobials reduces duration and severity of diarrhea in neonatal calves.

The effectiveness of zinc gluconate in neonatal calf diarrhea is promising, advocating the need for its incorporation in therapeutic regimens against all- causal diarrheic conditions of varying clinical severity.

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