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ORIGINAL ARTICLE

Effect of high dose oral zinc in mice with severe infection with Streptococcus pneumoniae

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Abstract
Zinc is important for normal function of the immune system and inflammation increases the demand for zinc. We hypothesized that high doses of zinc given during acute pneumococcal illness would alter the severity of infection. 24 six-week-old BALB/c mice were anaesthetized and infected intranasally with Streptococcus pneumoniae. Zinc intake was controlled by administering zinc through an intragastric tube. One group was given normal doses (5 μg/d) and the other group high doses of zinc (225 μg/d). We counted the number of bacteria from venous blood at 24 and 48 h, and from heart puncture and nasal wash at 72 h after intranasal challenge. Mice given excess zinc had 2.65 μmol/l, i.e. 25% higher ($p = 0.05$) mean plasma zinc concentration compared to those given normal amounts. 75% of mice in both groups developed pneumococcal bacteraemia. There were no differences in the numbers of S. pneumoniae colony forming units (CFUs) in blood or nasal wash between the groups. Thus, high doses of zinc did not alter the severity of systemic pneumococcal infection in mice.

Introduction

Adequate zinc intake is essential for animal and human health [1]. It plays a crucial role in reproduction, regeneration, cell division, wound healing and in the normal functioning of the immune system [2]. Zinc is essential for normal development and function of cells mediating both specific and non-specific immunity. Severe zinc deficiency is clinically characterized by skin lesions, brittle hair, growth impairment, severely depressed immune function and frequent infections. Even marginal zinc deficiency causes depressed immunity and increased susceptibility to infections [2].

Previous studies have shown that the acute phase response (APR) is accompanied by a decrease in serum zinc concentrations of 10–69% because of hepatic sequestration [3,4]. The magnitude of the change in plasma zinc concentration is related to the severity and stage of infection [3,5]. Prophylactic zinc administration in humans protects individuals from respiratory infections and diarrhoea [6–10] and clinical trials have shown that therapeutic zinc is efficacious and effective in children with acute and persistent diarrhoea [11–14]. Furthermore, a clinical trial in Bangladeshi children demonstrated that therapeutic zinc accelerated recovery from pneumonia [15]. However, a recent study on therapeutic zinc during childhood pneumonia showed no overall effect but that zinc administration could be harmful when given in the hot season [16]. The authors speculated that these diverging results could be due to differences in microbiological aetiology. Moreover, the effect of zinc on childhood infections does not seem to be limited to those who are malnourished or zinc deficient [7]. However, some studies of

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severely malnourished patients or patients on total parenteral nutrition have suggested that giving excess zinc during an infection might be harmful [17,18].

A study in mice infected with Streptococcus pneumoniae showed that zinc deficient mice had a higher risk of severe infection and death than zinc replete mice [19]. We hypothesized that this dramatic consequence of zinc depletion was due to increased zinc demand during the massive inflammation caused by pneumococcal infection, and that zinc administration during pneumoccoccal infection could reduce infection severity.

The aim of this study was to assess whether high doses of zinc given to zinc replete mice during an infection with S. pneumoniae could alter the severity of the infection.

Materials and methods

Animals

24 female BALB/cJBomTac mice (Taconic M&B 8680 Ry, Denmark) obtained at 5 weeks of age were allowed to acclimatize for 5 d before the start of the experiment. The mice were matched for weight and randomized into 2 experimental groups. The local experimental animal board under the Norwegian Ministry of Agriculture approved the study protocol, and the experiment was in conformity with the laws and regulations controlling experiments with live animals in Norway.

Housing

During the experiment the mice were kept in cages with stainless steel floors. The cages were washed with sterile deionized water before 3 animals were introduced into each cage.

Feeding

The mice were fed a diet containing 35.75 μg of elementary zinc (Zn) per gram (Pelleted Rat and mouse No. 1 maintenance, SDS, England, Product code: 801151). Then, from 8 h prior to challenge until sacrifice, the mice were given fodder that contained <5 μg Zn/g (C1040, Zinkarme Diät, Altromin GMBH, Germany) ad libitum. Additional zinc gluconate (Zink Gluconate GLUCONAL ZN-P, Purac, The Netherlands, Product code: 7543100, UN NR N/A) was given in 200-μl deionized water through an intragastric feeding tube (stainless steel needle with silicone tip, Fuchigami, Japan/ Scanbur BK, Norway, 75-4202), as a daily dose for the 3 d until sacrifice. The first dose was given 1 h after challenge. One group of 12 mice received 5 μg zinc per d (35 μg zinc gluconate), while the other group of 12 mice was given 225 μg zinc (1575 μg zinc gluconate) in addition to the fodder. The mice had free access to deionized water throughout the experiment.

Challenge

Each mouse was given a suspension containing 3.5×10^7 S. pneumoniae, strain PLN-A2 [20], intranasally in 30 μl PBS under isoflurane anaesthesia (Forene, Abbot Scandinavian AB, Sweden. MTnr 6993(NO) Lotnr 57143VA). Pilot studies showed that this strain produces invasive infection in two-thirds of BALB/c mice aged 6–8 weeks. PLN bacteria were grown in Todd Hewith broth with 0.5% yeast extract to mid-logarithmic growth, and frozen at -70°C in 1-ml aliquots with 100 μl of 8% sterile glycerol. The aliquots were thawed immediately prior to challenge, and there was no substantial difference in concentration of viable bacteria between thawing and challenge, indicating that there was no increase or loss of bacteria during the procedure. Bacteria were confirmed to be S. pneumoniae by colony morphology and optochin sensitivity.

Blood sampling

The mice were bled from the hind leg vein 24 and 48 h after challenge, and by cardiac puncture 72 h after challenge. From the leg vein we collected 20 μl of blood into heparinized tubes (Heparin Leo, 100 IE/ ml, Leo Pharma AS, Norway, MTnr NO 6449) and diluted in 80 μl of PBS in a 1.5-ml micro tube (Sarstedt, Germany No./REF 72.690). Subsequently, 75 μl of the suspension was used for bacterial quantification immediately after sampling.

Clinical monitoring of mice

The mice were observed at least once every 8 h throughout the experiment. In order to assess the clinical status of the mice, we looked for bristle hair, abnormal behaviour and symptoms related to the central nervous system.

Sacrifice

72 h after challenge, the mice were anaesthetized in a CO_2 chamber and 0.5–1 ml of blood was obtained by cardiac puncture while they were unconscious but while the heart was still beating. Cardiac puncture failed in 3 mice, i.e. too little blood was obtained to perform plasma zinc analysis. These 3 mice

belonged to the group that was fed normal amounts of zinc. 20 μl of blood was taken for bacterial quantification. The trachea was opened just distal to the larynx, and 100 μl of sterile PBS was flushed through the nose (Vasofix certo, 24 G/ 3/4″, 0.7×19 mm, REF 4269071, using a 1-ml syringe (Omnifix, Ref 4616022V, Lot 4A12048, B. Braun Melsungen, Germany)), and collected in a 1.5-ml micro tube.

Bacterial quantification

The numbers of viable bacteria from the infection batch, nose washes, and blood were determined by quantitative culture of serial dilutions on blood agar with 5 μl of gentamicin per ml. Duplicate plating on blood agar was performed from the infection batch and blood from cardiac puncture, to check for contamination. The agar plates were incubated at 37°C in a microaerophilic environment.

Zinc analysis

The plasma specimens were analysed for zinc using ICP-AES (IRIS/AP) (Thermo Jarell-Ash, Franklin, MA, USA) at the Laboratory of Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway. Spectrascan Element Standard for Atomic Spectroscopy (Teknolab A/S, Norway) was used as the reference. All samples were analysed in duplicate and the mean concentration calculated. The zinc analyses were repeated if the single results deviated more than 5% from their common mean.

Statistical analysis

The colony forming units (CFUs) per ml blood are presented as geometric means. Comparisons of CFUs and zinc levels between the 2 groups were carried out using the Mann-Whitney *U*-test.

Results

Mice that were given excess zinc had 2.64 μmol/l, 25% higher ($p=0.05$), plasma zinc concentrations than those given normal amounts of zinc (Table I).

None of the mice died before the observation period was complete. We recovered bacteria from the blood of 75% of the mice in both groups, most of them during the first 24 h post-infection.

The number of CFUs per ml blood ranged from 0 to 1,272,600, 0 to 541,800, and 0 to 3,774,000 on d 1, 2, and 3 after challenge, respectively. There were no significant differences in the CFU counts, or proportions of mice that were bacteraemic, between the 2 groups on any d (Table I), nor was there any significant difference in the number of CFUs per ml nasal wash between the groups ($p=0.19$).

Discussion

Clinical trials have shown that prophylactic zinc supplementation protects against respiratory infections [9,10]. We carried out this experiment to study if zinc has an effect given during pneumonia and severe bacterial infection. There are to our knowledge currently 4 published papers on the therapeutic efficacy of zinc given during pneumonia [15,16,21,22]. Only the paper by Brooks et al. [15] showed an overall beneficial effect of zinc given during pneumonia. No studies have demonstrated an effect of zinc given during severe bacterial illness or pneumonia on mortality.

Clinical trials have shown that therapeutic zinc is efficacious and effective in children with acute and persistent diarrhoea [12–14]. Routine zinc administration to children in developing countries appears to be more effective in preventing severe illness than mild illnesses [14,23]. Furthermore, there are indications that zinc given during diarrhoea is more

Table I. Streptococcus pneumoniae recovered in blood at 24, 48 and 72 h after challenge in mice given normal doses ($n=12$) or excess doses ($n=12$) of zinc (Zn). Presented as percentage of mice with pneumococcal bacteraemia, median of colony forming units (CFU) pr ml blood with interquartile range (IQR), and geometric mean of CFUs.

		Normal Zn	Excess Zn	
24 h	Mice with bacteraemia	58%	67%	
	Median CFU (IQR)	1318 (0, 19220)	11747 (0, 41115)	$p=0.47$
	Geometric mean	1914	4159	
48 h	Mice with bacteraemia	75%	75%	
	Median CFU (IQR)	8060 (1860, 27425)	4805 (155, 120576)	$p>0.99$
	Geometric mean	6109	5649	
72 h	Mice with bacteraemia	67%	67%	
	Median CFU (IQR)	14183 (0, 280040)	121000 (0, 907200)	$p=0.58$
	Geometric mean	15101	30620	
72 h	Plasma Zn μmol/l	13.68	16.32	$p=0.05$

Mean plasma zinc concentration at sacrifice.

efficacious in those with severe inflammation (unpublished findings).

The inflammatory cytokines TNF, IL-1 and IL-6 are released in serum, bronchoalveolar lavage (BAL) and lungs within 72 h after intranasal challenge of S. pneumoniae in mice [24]. Inflammation is associated with dramatic changes in plasma micronutrient concentrations [1,2,4]. Changes in the micronutrient environment may be the host's attempt to optimize immune function and to deprive invading organisms of essential micronutrients for replication [25]. Braunschweig et al. found that preventing the serum zinc decline yielded an increased febrile response during a mild APR in adult humans on parenteral nutrition [18]. Doherty et al. reported a significantly higher mortality in severely malnourished children receiving approximately 5 times the recommended zinc intake compared to those who were given the recommended daily doses [17]. One recent clinical trial studying zinc supplementation given during pneumonia showed that Indian children who received zinc during pneumonia in the hot season had a significantly longer duration of the illness than those who did not receive zinc [16]. This subgroup effect was not seen in those that were enrolled outside the hot season. Thus, there is a possibility that giving zinc during certain infections can be harmful.

The amount of zinc fed in the present study was the minimum daily Zn requirement for mice [26]. The amount of zinc given to the excess zinc group was at least 10 times this level (225–242.5 ug/d). The differences in plasma zinc concentrations indicate that the intragastric feeding procedure yielded higher net zinc absorption in the mice that were given more zinc, although the 2 groups were given different doses for only 72 h. Nevertheless, the excess doses of zinc fed might have been too small and the duration too short to have an effect on immunity.

The mice were fed a diet with a very low zinc concentration, and additional zinc was administered through an intragastric tube. There is a possibility that the mice could have developed ulcers from the tube or the high zinc concentration that again could affect the outcome of the experiment. In one-third of the mice, however, the oesophagus was opened after sacrifice and examined for ulceration. No macroscopic damage was found.

The strain of S. pneumoniae (PLN-A2) used in this study may not have been capable of causing sufficiently severe infection in the mice. A previous study showed that for host immunity to be triggered, levels of PLN pneumococci in the blood must exceed 1 million per ml [27].

The standard deviation of the $\log_{10}$ CFU pr ml blood on d 1, 2, and 3 was 2.2, 2.0 and 2.3, respectively. In similar experiments with mice where bacterial counts from blood have been the outcome, the effects of the interventions have been between 2 and 2.5 $\log_{10}$ units (i.e 100–316-fold difference) [19,28,29]. If we assumed a 2 $\log_{10}$ unit difference between the groups, the power to detect this difference would be 60% on d 1, 69% on d 2, and 57% on d 3. The power to detect a 2.5 $\log_{10}$ unit difference would be 80%, 86%, and 76% on d 1, 2 and 3, respectively. The standard deviation of the $\log_{10}$ CFU from the nasal wash was 2.9 and the power to detect a 2 and 2.5 $\log_{10}$ reduction was 40% and 56%, respectively. 75% had bacteraemia; the required sample size to detect a 50% reduction in the proportion with bacteraemia would then be 40 mice per group with a power of 80%. Thus, this study was adequately powered to detect a meaningful difference in CFU from blood between the groups but not to detect a reduction in the proportion with bacteraemia or difference in bacterial counts from the nasal mucosa.

Bacteraemic mice showed clinical sign of illness such as bristled fur and abnormal behaviour. However, none of mice died and none became sufficiently ill for us to consider taking them out of the experiment. The power to detect differences in adverse events was low.

In conclusion, high doses of zinc given through an intragastric tube increased plasma zinc but did not alter the severity of pneumococcal infection.

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