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Effect of Zinc Supplementation on Respiratory Tract Infections in Children With Cystic Fibrosis

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Summary. Zinc (Zn) has significant anti-oxidant and anti-inflammatory activity. Zn deficiency can occur in subsets of patients with cystic fibrosis (CF) especially those with malabsorption and impaired growth. Although supplemental Zn has significantly reduced infections in various disorders, its efficacy has not been thoroughly investigated in CF. We performed a double blind placebo controlled pilot study to investigate the effect of daily 30 mg elemental Zn for 1 year on the rate of respiratory tract infections (RTIs), use of antibiotics and plasma cytokines in 26 children with CF (ages 7–18 years). Plasma Zn, Cu, inflammatory cytokines and ex vivo generation of IL-2 were measured at baseline and at the end of the study. The number of days of oral antibiotics was lower in Zn treated patients compared to placebo ($P = 0.05$). However, compared to placebo, the effect of Zn was greater in patients who exhibited low plasma Zn at baseline ($P = 0.02$) than those who had plasma Zn levels identical to normal subjects ($P = 0.55$). Zn supplementation was marginally effective in reducing percentage increase in plasma IL-6 and IL-8 while increasing the percentage change in ex vivo generation of IL-2 in isolated mononuclear cell. In conclusion, oral intake of 30 mg/day of Zn reduced the number of days of oral antibiotics used to treat RTIs in children with CF. A higher daily Zn dose may be needed to decrease RTIs and modify immune responses. **Pediatr Pulmonol. 2008; 43:281–287.**

Key words: cystic fibrosis; respiratory tract infections; zinc; cytokines.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by genetic mutations that code for cyclic AMP-activated chloride channel, the CF transmembrane regulator (CFTR).[1–3] The disease affects many organ systems, particularly the respiratory and gastrointestinal tracts. Due to pancreatic insufficiency, malabsorption of essential fatty acids, fat-soluble vitamins, and various trace elements, including zinc (Zn), can occur in subjects with CF.[4,5] The airways in patients with CF are uniquely susceptible to chronic infections with various bacterial agents, especially Gram negative organisms such as *pseudomonas aeroginosa*.[6] This infection causes the release of inflammatory cytokines, proteolytic enzymes, and oxygen radicals which leads to bronchiectasis, increased dead space, hypoxia, and hypercapnea.[7,8]

Approximately 90% of patients die of respiratory failure as a result of chronic bacterial infections, Treatment of CF lung disease is aimed at reducing airway obstruction, treating recurrent infections, improving airway clearance, and maintaining optimal nutritional status.[9–11]

Zn is an integral part of the structure and function of many biological enzymes and a regulator of ion transporters relevant to pulmonary function and disease in CF and other pulmonary disorders.[11–13] Zn is also known to exhibit powerful anti-oxidant activity in several organ systems including the lungs.[11,13] For example, Zn deficient rats developed significant lung toxicity 1 day after exposure to high oxygen concentrations and Zn replenishment prevented the hyperoxia lung damage in previously Zn deficient animals.[14]

Zn supplementation to non-CF infants and pre-school children resulted in reduced incidence of acute lower respiratory infections.[15] Zn supplementation lead to the reduction of plasma concentration of inflammatory cytokines, and oxidative stress markers in the absence of

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infection,[16] and decreased the duration of colds in healthy young adults.[17] It also lowered the incidence of infection and inflammatory cytokines levels in healthy elderly subjects.[18]

Using plasma and red cell Zn concentrations to reflect Zn status in CF has produced conflicting results as not all CF patients appear to exhibit low Zn status.[19] Administration of supplemental Zn to CF patients has also been non-conclusive for in one study, Zn therapy did not result in improvement of clinical status, growth velocity or pulmonary function.[20] Another study showed an association between low Zn status and impaired IL-2 activity, NK activity and thymulin activity in CF subjects, but there was no investigation as whether these parameters would be corrected following Zn supplementation.[21]

With these premises we initiated a pilot study to determine whether Zn supplementation administered to CF patients during a year long double blinded placebo controlled trial would reduce the requirement for oral antibiotics necessary during periods of active infection. We also assessed whether changes in selected plasma inflammatory cytokines and the ex vivo generation of IL-2 would be altered by oral Zn therapy. And lastly, we investigated the hypothesis that a response to Zn supplementation would be different in subjects with low plasma Zn levels compared to those expressing normal plasma Zn levels.

MATERIALS AND METHODS

Subjects and Controls

For this double blind placebo controlled study, a total of 26 children with CF exhibiting mild to moderate lung disease groups A and B were recruited from the Pediatric Pulmonary Division, Carman and Ann Adams Department of Pediatrics, Children's Hospital of Michigan (CHM), and the Pediatric Pulmonary Division, DeVos Children's Hospital, Grand Rapids, Michigan. Subject's ages ranged from 7 to 18 years old. Exclusion criteria for the study included presence of acute severe infection at the time of enrollment, renal disease, severe hepatic disease, gall bladder disease, sickle cell disease (SCD), use of oral immunosuppressive drugs (steroids and non-steroid anti-inflammatory drugs), diuretics and Zn supplements. The study was approved by the Human Investigative Committees in both institutions. Informed and signed consent was obtained from parents or legal guardians.

Study Groups

Without respect to prior Zn status, CF subjects were randomly assigned to Group A ($n = 13$) received a daily dose of 30 mg of elemental Zn as Zn gluconate (15 mg/capsule) and CF subjects assigned to Group B ($n = 13$) received placebo for 12 consecutive months. Zn gluconate and placebo preparations were capsules generously provided by Labcatal, Montrouge, France for use in this study. Both groups were given identical daily Zn-free and mineral-free multivitamin supplements for the same period (Vitamax® from SK Labs, Chesterfield, MO) supplied by the CF services Pharmacy. Patients in groups A and B were followed every 3 months and as needed in their CF clinics for routine CF care. At each visit, the patient's interval medical history, height and weight, number of hospitalizations, the use of oral and intravenous antibiotics, pulmonary function test, and physical examination findings were recorded.

Respiratory tract infections (RTIs) was defined when the patient developed more than four of the following signs or symptoms: change in sputum; new or increased hemoptysis; increased cough; increased dyspnea; malaise, fatigue, or lethargy; temperature above 38°C; anorexia or weight loss; sinus pain or tenderness; change in nasal or sinus discharge; change in physical examination of the chest; decrease in pulmonary function from a previously recorded value; or radiographic changes indicative of pulmonary infection.

Choice and duration of oral or parenteral antibiotics used for treatment of RTIs were left to the discretion of the children's treating physicians.

Ten milliliter of heparinized whole blood was collected at baseline and at the end of the study for determination of plasma Zn, Cu and the inflammatory cytokines, TNF-α IL-1β IL-6 IL-8, soluble IL-1 receptor antagonist (sIL-1RA) and soluble TNF-α receptor 1 (sTNFR1). Peripheral blood mononuclear cells (PBMC) were isolated for ex vivo generation of IL-2.[18,22] Sterile procedures were carried out on all samples to ensure no contaminants would interfere with the ELISA assays or ex vivo generation of IL-2. Plasma samples were centrifuged at 1,500*g* for 20 min at room temperature to remove cellular components before being stored in 1 ml aliquots at −20°C for ELISA assays or being admixed with trace metal free Ultrex II Nitric Acid (J.T. Baker Chemicals, Phillipsburg, NJ) and digested for Zn and copper analysis by flameless atomic absorption (AA).[18,22]

Laboratory Measurements

Aliquots of plasma samples were stored at −20°C until assayed for cytokines to ensure that both pre- and post-samples could be determined using the same high-sensitivity ELISA kits available from R&D Systems (Minneapolis, MN). Plasma Zn and Cu levels were determined by flameless AA spectrophotometry with a Zeeman background corrector (AA220Z Varian, Sugarland, TX).[18,22]

PBMCs were isolated by discontinuous density gradient using Histopaque 1077 (Sigma–Aldrich, St. Louis, MO)

according to the manufacturer's protocol. Isolated PMNC were resuspended in incubation media consisting of RPMI-1640 supplemented with 10% fetal bovine serum, 1.5 gm/L sodium bicarbonate, 0.1% gentamicin, 2 mM L-glutamine and 50 μM β-mercaptoethanol. Cells were stimulated for 48 hr with 10 μg/ml PHA-p (Sigma, St. Louis, MO) at 37°C under an atmosphere of 85% air/5% CO_2. At the termination of the experiment, supernatants were harvested and assayed for IL-2 by ELISA (Quantikine ELISA, R&D Systems).

Characterization of Zn− Versus Zn+ Subjects

Zn adequate CF subjects (Zn+) were defined as those subjects with ≥90 μg/dl plasma Zn as determined by flameless AA at baseline. Subjects exhibiting inadequate Zn status (Zn−) had plasma Zn levels ≤89 μg/dl or two standard deviations below our normal database of 110 ± 10 μg Zn/dl.[18,22] Although all subjects exhibited plasma Zn levels within the clinically accepted "normal" range (70–120 μg/dl),[23] results from a number of our research studies have demonstrated that subjects exhibiting low Zn levels (<90 μg/dl) are more susceptible to infections and tend to have higher levels of plasma oxidative stress molecules and inflammatory cytokines even in the absence of outward infections.[18,22]

Statistical Analyses

Baseline data comparison between Zn adequate and Zn inadequate CF patients were determined by the Student's *t*-test. Data representing differences between pre- and post-values were analyzed using the Student's *t*-test for plasma cytokines Zn and Cu and ex vivo generation of IL-2, as well as for weight, height, hemoglobin, and FEV_1. Correlation between compliance and days of oral antibiotic usage was determined by regression analysis using Pearson r (GraphPad Instat Ver. 3.05 for Windows95/NT, San Diego, CA).

RESULTS

Overall, there were no statistical differences in the baseline parameters examined between Zn adequate and inadequate CF children with the exception of plasma Zn status (Table 1). Zn adequate CF patients also tended to have higher levels of the sIL-1RA, IL-1 β, and IL-6 and although the differences did not reach statistical significance (Table 1).

Zn supplementation administered to 12 CF patients (one subject dropped) resulted in a significant ($P = 0.05$) reduction in the number of days of oral antibiotics use per year compared to the 13 patients on placebo. However, the response to Zn supplementation was different for the Zn inadequate versus Zn adequate CF patients. Average days of antibiotic use for the Zn adequate CF subjects given Zn were not different from those given the placebo ($P = 0.55$; Table 2). This is in contrast to the response from the Zn inadequate CF subjects in which the effect of Zn supplementation was significantly greater ($P = 0.025$) compared to those given placebo (Table 2).

Compliance of Zn adequate CF subjects given Zn was less than placebo treated Zn adequate subjects (Table 2).

TABLE 1—Baseline Characteristics of Subject Population

Parameter	Zinc adequate subjects (n = 13)	Zinc inadequate subjects (n = 13)	*P*-value
Age (year)	12.0 ± 3.4 (7–17)[1]	11.8 ± 3.6 (8–18)	0.8854
Weight (kg)	37.58 ± 13.6 (23–67)	42.67 ± 13.0 (21–59.7)	0.3391
Height (cm)	146.53 ± 17.12 (124–178)	147.87 ± 14.44 (123–167)	0.8310
Hbg (g/dl)	13.66 ± 0.85 (12.2–15.2)	13.66 ± 0.68 (12.5–15.0)	0.9999
Pl.[2] Zn (μg/dl)	101 ± 9.8 (90–125)	82 ± 4.9 (73–89)	<0.0001
Pl. Cu (μg/dl)	106 ± 20 (80–150)	113 ± 20 (66–142)	0.3811
FVC-P[3]	83.85 ± 12.1 (65–98)	87.0 ± 10.8 (70–104)	0.4908
FEV_1P[4]	67.5 ± 17.7 (41–100)	76.77 ± 15.3 (54–98)	0.1660
FEF_{25-75} P[5]	48.69 ± 29.47 (10–109)	65.38 ± 32.43 (12–109)	0.1829
Gen. IL-2[6]	411 ± 529 (25–1590)	382 ± 695 (3–2370)	0.9058
Pl. sIL-1ra (pg/ml)	1501 ± 1880 (149–6900)	927 ± 938 (131–2995)	0.3384
Pl. sTNF-R1 (pg/ml)	1368 ± 887 (348–3017)	1179 ± 463 (716–2060)	0.5054
Pl. IL-1β (pg/ml)	2.48 ± 2.66 (0.35–7.1)	1.47 ± 1.08 (0.43–3.6)	0.2240
Pl. TNF-α (pg/ml)	2.84 ± 2.13 (1.5–9.3)	2.30 ± 1.05 (1.15–4.6)	0.4236
Pl. IL-6 (pg/ml)	6.18 ± 5.71 (1.8–18.6)	3.82 ± 4.99 (0.52–17.2)	0.2734
Pl. IL-8 (pg/ml)	440 ± 864 (0–2858)	581 ± 1427 (0–5552)	0.7639

[1]Values represent Mean ± SD (range).
[2]Pl. represents plasma values.
[3]FVC-P = forced vital capacity expressed as percent predicted.
[4]FEV_1P = forced expiratory volume in 1 sec, expressed as a percentage of predicted.
[5]FEF_{25-75} P = forced expiratory flow between 75 and 25 of FVC expressed as percent predicted.
[6]IL-2 generated ex vivo.

TABLE 2— Number of Episodes Requiring Antibiotics, Number of Days of Oral Antibiotic Usage and Compliance in Zinc and Placebo Supplemented Subjects With Cystic Fibrosis

	Zinc adequate subjects			Zinc inadequate subjects		
Parameter	Placebo (n = 7)	Zinc (n = 5)	*P*-value	Placebo (n = 6)	Zinc (n = 7)	*P*-value
No. of episodes req. i.v. AB[1]	0.57 ± 0.53[2]	0.40 ± 0.55	0.559	0.17 ± 0.41	0.14 ± 0.37	0.915
No. of episodes req. oral AB[3]	3.28 ± 2.9	2.6 ± 0.89	0.574	3.16 ± 1.6	1.57 ± 0.97	0.049
%Compliance[4]	92.8 ± 7.6	66.7 ± 21.1	0.012	81.4 ± 18.8	70.3 ± 37.7	0.566
Avg. days/year Req. oral AB[5]	49.1 ± 36.6	37.4 ± 6.0	0.55	47.5 ± 24.3	20.5 ± 12.8	0.025

[1]Average number of episodes/year requiring intravenous (i.v.) antibiotics.
[2]Values represent Mean ± S.D.
[3]Average number of episodes/year requiring oral antibiotics.
[4]Average percent compliance.
[5]Average days/year requiring oral antibiotic use.

However, overall compliance of the two groups of Zn treated subjects was similar. Moreover, Pearson correlation coefficient indicated no correlation between compliance and number of days of oral antibiotic usage in all Zn supplemented subjects ($r^2 = 0.025$). There were no adverse events identified for any of the subjects in this study from oral or i.v. antibiotic use, Zn or placebo supplementation or any other parameter associated with this study.

Analysis of pre- versus post-values for each parameter resulted in non-significant percent change compared to baseline in selected parameters between the Zn supplemented and placebo supplemented group (Table 3). This was more likely due to the small number of patients and the large standard deviation within each group consisting of both Zn inadequate and and Zn adequate CF patients. In Zn treated CF patients, Zn supplementation was associated with less increase in plasma IL6 and IL-8, and a greater increase in plasma sIL-1ra and the ex vivo generation of IL-2 (Table 3). This may indicate an anti-inflammatory role of Zn in this group of patients.

TABLE 3— Percentage Increase (Decrease) in Selected Parameters Obtained From Zinc and Placebo Supplemented Cystic Fibrosis Subjects

Parameter	Placebo Tx (n = 13)	Zinc Tx (n = 12)	*P*-value[1]
Pl[2]. Zn (μg/dl)	8.6 ± 22[3]	6.1 ± 9.4	0.73
Pl. sIL-1ra (pg/ml)	1.9 ± 141	11.4 ± 101	0.84
Pl. sTNFR1 (pg/ml)	9.7 ± 56	8.1 ± 27	0.93
Pl. IL-1β (pg/ml)	86 ± 304	78 ± 188	0.94
Pl. TNF-α (pg/ml)	26 ± 93	32 ± 87	0.85
Pl. IL-6 (pg/ml)	173 ± 374	106 ± 192	0.57
Pl. IL-8 (pg/ml)	481 ± 1507	122 ± 337	0.41
Gen[4]. IL-2 (pg/ml)	55 ± 274	185 ± 275	0.24

[1]*P* values for difference between placebo and zinc supplemented group.
[2]Pl. represents plasma.
[3]Mean ± SD percent change from baseline.
[4]Ex vivo generation of IL-2 in PHA-p stimulated mononuclear cells.

DISCUSSION

A number of investigators have reported conflicting relationships between Zn status and malabsorption, impaired growth, or pulmonary function in CF patients.[5,19,21,24,25–28] Few studies found no correlation between Zn levels and nutritional or pulmonary status[19,25] Other reports found abnormal Zn status in certain patients with CF especially those with moderate to severe growth retardation and severe pulmonary disease.[26–28] Low plasma Zn in CF was also associated with low plasma IL-2 levels, and an impairment of NK cell activity and low thymulin activity.[21] Thymulin activity, necessary for the development of IL-2 producing T lymphocytes, was dependent upon the presence of the Zn molecule being present in the thymulin peptide structure.[29] An increase in the number of CD3+ lymphocytes positive for IL-2 after stimulation has been reported in CF but was not correlated with Zn status.[30] In this present study, isolated PMNC from Zn supplemented CF patients had increased ability of generating IL-2 over the time compared to those from subjects administered placebo.

While some studies showed an association between Zn deficiency, growth retardation and malnutrition in a subset of patients with CF, our group of CF subjects did not appear to be ''Zn deficient'' but had plasma Zn levels still within clinically acceptable normal range.[23] Based on our long term studies in trace metal research, we have found that subjects with plasma levels of <90 μg/dl are more likely to suffer adverse reactions to immunological challenges such as cold, flu and infections than those subjects with plasma Zn levels above this range. In this study, Zn inadequate CF subjects exhibited lower baseline levels of plasma sIL-1ra, sTNFR1, IL-1β, IL-6, and IL-8 than their Zn adequate counterpart. One hypothesis may be that in CF subjects with low plasma Zn levels, maintainence of immunological surveillance is decreased in the absence of acute exacerbation. In CF subjects with inadequate Zn status, Zn therapy was associated with

significantly fewer days of oral antibiotics and insignificant change in plasma inflammatory cytokines pre- to post-therapy than their Zn adequate counterpart. However, the small number of subjects and large SD for selected parameters are not optimal for determination of a greater beneficial effect for Zn inadequate subjects compared to their Zn adequate counterpart. Changes in plasma Zn levels, weight, height, FEV1, and Hgb in CF receiving Zn were not different from the placebo treated CF group. This suggests that the dose of Zn (30 mg/day) may not have been sufficient to affect growth rate or other parameters of lung function due to possible suboptimal absorption of Zn from the gastrointestinal tract. Slightly higher dose of Zn (no greater than 45 mg) may produce a more favorable response on anthropometric, and lung function measures. In patients with SCD in which loss of Zn due to red cell destruction and hyperzincuria leaves approximately 70% of SCD subject with mild to moderate Zn deficiency, supplemental Zn in dosages of 50–75 mg/day are required to reduce incidence of infection and the presence of endogenous plasma inflammatory cytokines.[31] Supplemental Zn at 45 mg/day has been shown to reduce the incidence of infection and reduce plasma inflammatory cytokines in the elderly population even in the absence of active infection[18] and to reduce the presence of oxidative stress markers in young adults without adverse effects or changes in copper levels.[16] Various studies in developing countries that used relatively high doses of Zn, ranging from 88 to 150 mg per week, in pre-school children for up to 15 months have reported significant reduction respiratory and gastrointestinal infections with out adverse effects.[32,33]

Several studies reported increased plasma and bronchoalveolar lavage fluid (BALF) levels of various cytokines such as IL-8 in patients with CF even in newly diagnosed infants with and with out documented airway infections.[10,34–39] One report showed increased IL-8 in plasma and BALF and increased in sera IL-8 was associated with age in CF subjects as compared to serum IL-8 from normal children which decreases with age.[35] Along with IL-8, increased TNF-α is also found in BALF samples from CF subjects and similar to IL-8, is inversely correlated to lung function FEV1.[34] BALF levels of both IL-8 and TNF-α tend to decrease with antibiotic therapy. Compared to normal controls, other dysregulations of immune function have been reported in CF subjects such as decreased plasma IL-10 in response to antigenic stimuli,[40,41] increased plasma adhesion molecules sICAM and sE-selectin,[42] and decreased IL-8 production in isolated T cells.[30] In other studies, results reflect data obtained from CF populations separated into groups of responders versus non-responders based on the cytokine studied. However, rarely has the Zn status of these patients been considered even though some subjects with CF are known to exhibit lower plasma Zn levels. Studies performed in non-CF adults and elderly demonstrate that plasma levels of inflammatory cytokines and oxidative stress markers are reduced following Zn therapy[16–18,31] and provide support for Zn as an anti-inflammatory agent in CF.

Results of our research in humans and in cell models indicate the effect of Zn in reducing inflammatory cytokines is at the mRNA level and involves inhibition of NF-κB activation.[43] HL-60 cells, (a promyelocytic cell line) rendered Zn deficient and then stimulated with LPS, produce significantly greater amounts of TNF-α, IL-1β and IL-8 than those cells that were Zn sufficient.[43] Activation of the NF-κB pathway by *P. aeroginosa* in subjects with CF and in CF animal models can lead to an overproduction of adhesion molecules, inflammatory cytokines (IL-1β, TNF-α, IL-6, and IL-8), and oxidative stress molecules, which in turn continue to provide stimulus for further NF-κB activation and the relentless vicious cycle.[44,45]

The most significant observation in this present study was the association of Zn supplementation with the reduction in the average days of oral antibiotic usage required to treat RTIs particularly in Zn inadequate subjects. It is possible that a more profound effect of Zn than inflammatory cytokine reduction was to modulate the airway environment. The effect of Zn on pulmonary function also include such events as inhibition of NADPH oxidase which generate extracellular superoxide anions[11] and the restoration of chloride secretion across the CF airway epithelia independent of changes in the CFTR as has been demonstrated in the CF mouse model.[13] In the CF mouse model, direct application of ATP and Zn was shown to trigger calcium entry via the P2X purinergic receptor channels leading to the activation of calcium-dependent chloride channels, circumventing the defective cyclic AMP dependent CFTR chloride channel.[13] Zn therapy can also modulate pulmonary sodium absorption via epithelial sodium channel (ENaC) directly and through the regulation of redox conditions.[46] The height of the airway surface liquid lining the apical side of the respiratory epithelium is tightly regulated and involves a balance between sodium absorption by ENaC and chloride secretion by the CFTR in normal children or by activation of the calcium-dependent chloride channel in CF with defective CFTR. Inducing ''near normal'' airway environmental conditions by Zn in CF would provide for suboptimal condition for infectious organisms.[47]

In summary, we have provided evidence that 30 mg/day supplemental Zn was associated with reduction in the number of days of oral antibiotics used to treat RTI and marginal reduction in the level of plasma inflammatory cytokines in children with CF. Our study has several limitations. The number of patients with CF was not large enough to enhance the potential differences between the treated groups. As a result, this study can be considered as

a pilot study providing preliminary data for power analysis for future studies designed with larger number of subjects. Slightly higher doses of Zn therapy may be needed to statistically change anthropometric measures, lung function, the level of plasma inflammatory cytokines, and attenuate the severity of pulmonary exacerbations in subjects with CF. Finally, the effectiveness of Zn therapy may be optimally determined by assessing cytokine levels in BALF or sputum samples which would more directly representative of inflammation in the compartmentalized CF airways. Further investigations are needed to understand the role of Zn in mediating pulmonary infection in CF subjects.

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