Cobalamin Status Modifies the Effect of Zinc Supplementation on the Incidence of Prolonged Diarrhea in 6- to 30-Month-Old North Indian Children[1–3]

Mari S. Manger,[4,5] Tor A. Strand,[5–7]* Sunita Taneja,[8,13] Helga Refsum,[9,10] Per M. Ueland,[4,11] Ottar Nygård,[4,11] Jørn Schneede,[12] Halvor Sommerfelt,[5,7] and Nita Bhandari[8,13]

[4]Institute of Medicine and [5]Centre for International Health, University of Bergen, Bergen N-5020, Norway; [6]Medical Microbiology, Innlandet Hospital Trust, Lillehammer N-2629, Norway; [7]Division of Infectious Disease Control, Norwegian Institute of Public Health, Oslo N-0403, Norway; [8]All India Institute of Medical Sciences, New Delhi 110029, India; [9]Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, N-0316 Oslo, Norway; [10]Department of Physiology, Anatomy and Genetics, University of Oxford, OX1 3QX, Oxford, UK; [11]Haukeland University Hospital, N-5020 Bergen, Norway; and [12]Department of Clinical Pharmacology, University of Umeå, SE-901 85 Umeå, Sweden

Abstract

The observed effect of zinc supplementation on diarrheal morbidity varies between trials and there is a need to identify subgroups most likely to benefit from improved zinc nutriture. In a randomized, double-blind trial in 2296 children in New Delhi, India, we assessed whether baseline cobalamin or folate status modified the effect of zinc supplementation on the incidence of prolonged ($\geq$7 d duration) and acute diarrhea. Children aged 6–30 mo received zinc or placebo daily for 4 mo. We measured plasma concentrations of folate, cobalamin, total homocysteine (tHcy), and methylmalonic acid (MMA) at enrollment and assessed the efficacy of zinc supplementation in subgroups based on these variables. The efficacy of zinc on reducing the risk of prolonged diarrhea was higher in those with plasma cobalamin concentrations below the 25th percentile and in those with tHcy and MMA concentrations above the 75th percentile. The OR (95% CI) for children below and above the 25th percentile for cobalamin were 0.53 (0.35–0.78) and 0.90 (0.73–1.11), respectively (P-interaction = 0.015). There were similar differences for the OR when comparing efficacy in those above and below the 75th percentile for tHcy and MMA (P-interaction = 0.045 and 0.188, respectively). Baseline folate status did not modify the effect of zinc on prolonged diarrhea. Neither cobalamin nor folate status influenced the effect of zinc on acute diarrhea. Children with poor cobalamin status benefited more from zinc supplementation for the prevention of prolonged diarrhea. J. Nutr. 141: 1108–1113, 2011.

Introduction

Prophylactic zinc supplementation in children <5 y of age in developing countries may reduce both the incidence and duration of diarrhea (1,2). However, the observed effect varies between trials. This heterogeneity cannot be explained by the dosage of zinc, the dose interval (2), the combination of zinc with other micronutrients, or the duration of supplementation (1). The results from a pooled analysis show that the effect of zinc supplementation on the incidence of diarrhea was negatively associated with baseline height and weight and positively associated with baseline serum ferritin concentrations (1). Therefore, different responses in different populations may in part be related to general nutritional status.

Reports from India and other low-middle income countries with mainly vegetarian populations show that deficiency of cobalamin is common (3). We previously reported poor cobalamin and folate status in 6- to 30-mo-old North Indian children (4). Intakes and bioavailability of cobalamin, folate, and zinc are interdependent. Many folate-rich foods are also high in phytate (5), the most potent inhibitor of zinc absorption (6). Cobalamin and highly bioavailable zinc are found in similar food sources such as liver, meat, and seafood (5). Breast milk continues to be an important source of both zinc and cobalamin after 6 mo of age (7,8), but the cobalamin content is strongly affected by maternal cobalamin status (9).

Studies that jointly examine zinc, folate, and cobalamin status in humans are limited. It is known that zinc affects cobalamin and folate metabolism in several ways. Both cobalamin-dependent methionine synthase and intestinal brush border folate conjugase are zinc-dependent enzymes (10,11). Methionine synthase catalyzes the methyl group transfer from methyltetrahydrofolate to homocysteine to form methionine and tetrahydrofolate. Folate conjugase catalyzes the hydrolysis of polyglutamate folate forms to absorbable monoglutamyl species (12). In addition, several facets of the immune system are affected by zinc, folate, and cobalamin deficiencies. All play crucial roles in DNA synthesis (13,14) and thereby cell proliferation, including that of cells in the intestinal linings and those mediating immunity (13–15). Previous studies have not examined specific roles for folate and cobalamin status in the etiology of diarrheal illness.

Using data from an already completed trial among North Indian children from which we found that daily zinc supplementation reduced the odds of any diarrheal episode by 12% (16), we aimed to explain some of the heterogeneity between the above-mentioned studies and thereby identify subgroups most likely to benefit from improved zinc nutriture. In the current analysis, we assessed whether cobalamin and folate status modified the effect of zinc supplementation on the risk of acute and prolonged diarrhea. In addition to plasma cobalamin and folate, we also measured their metabolic markers, plasma total homocysteine (tHcy) and methylmalonic acid (MMA). Although tHcy is a functional indicator of both cobalamin and folate status, cobalamin is the main determinant of tHcy in infancy (17), and we previously demonstrated that plasma cobalamin and not folate determined tHcy concentrations in our study (4).

Participants and Methods

Study setting. This is a secondary analysis of an already completed, randomized, controlled trial that measured the efficacy of zinc supplementation on the incidence of diarrhea in North Indian children (16). Daily zinc supplementation reduced the odds for any diarrheal episode by 12% and the beneficial impact of zinc increased with progressively longer illness duration.

The trial took place in the urban slum of Dakshinpuri in New Delhi, India, comprising 15,000 dwellings and 75,000 inhabitants. Enrollment commenced on February 15, 1998. All children aged 6–30 mo in the community were identified through a survey (n = 3802). Children were excluded if consent was not given (17.8%), if they were likely to move out of the study area within the next 4 mo (15%), or if they needed urgent admission to hospital on the enrollment day (0.5%) or had received massive dose of vitamin A [100,000 IU (30 mg) for infants and 200,000 IU (60 mg) for older children] within the last 2 mo (n = 1.5%). The follow-up of the last child was completed on September 30, 2000. The trial was approved by the All India Institute of Medical Sciences ethics committee. Informed written consent was obtained from community leaders and parents. Signatures or thumb impressions were obtained on consent forms and a copy left with the family.

Randomization and blinding. The eligible children (n = 2482) were randomized to receive zinc or placebo syrup according to a randomization scheme using blocks of 8. Zinc or placebo syrups were prepared and packaged in unbreakable bottles by GK Pharma, which also labeled bottles with a unique child identification number according to the randomization list. Six bottles (1 for each month and 2 extra) for each child were produced and labeled before enrollment. The supplies for each child were kept separately in labeled plastic bags. The zinc and placebo syrups were identical in appearance and packaging and were similar in taste.

Intervention. Doses of 10 mg /d elemental zinc as zinc gluconate were given to infants (6–12 mo old) and doses of 20 mg/d (twice the recommended daily allowances) were given to older children (12–30 mo

old). A study attendant administered the zinc or placebo syrups daily for 4 mo except on Sundays and public holidays, when the mother administered it. One bottle was kept in the child's home and replaced monthly.

Outcomes and definitions. A study physician interviewed the caretaker, examined the child, and collected a venous blood specimen from the child at enrollment. Weight and length were measured using Seca Salter scales and locally manufactured length boards that read to the nearest 0.1 kg and 0.1 cm, respectively. Fieldworkers visited each child every 7 d for 4 mo. At each visit, information was obtained about the number and consistency of stools in the previous 7 d. If the child had diarrhea and vomiting, dehydration was assessed. Two packets of oral rehydration salts were given when a child had diarrhea. Clinical services were available at the study clinic. Children who spontaneously visited the clinic or sick children referred to the clinic by field workers were treated according to WHO guidelines (18).

Diarrhea was defined as passage of ≥3 loose or watery stools in a 24-h period and recovery as the first day of a 72-h period when the child had no diarrhea. Acute diarrhea was defined as episodes lasting <7 d. Prolonged diarrhea was defined as episodes lasting ≥7 d.

Blood collection and biochemical analyses. Nonfasting venous blood specimens (≈5 mL) were collected in heparinized polypropylene tubes (Sarstedt) between 0900 and 1200 h. The samples were centrifuged at $447 \times g$ for 10 min at room temperature and the plasma was divided and stored in polypropylene vials (Eppendorf) at −20°C until analyzed.

Plasma cobalamin (n = 2261) and plasma folate (n = 2296) concentrations were measured by using microbiological assays with the use of a chloramphenicol-resistant strain of *Lactobasillus casei* and a colistin sulfate-resistant strain of *Lactobasillus leichmannii*, respectively (19,20). Both assays were adapted to a microtiter plate format and carried out by a robotic workstation (21). Plasma MMA (n = 2270) and tHcy (n = 2271) were analyzed with a modified GC-MS method based on ethylchloroformate derivatization (22). Plasma zinc was analyzed as described elsewhere (16).

Standardization and quality control of data collection. A study manual describing standard operating procedures was used during training and throughout the study. Training, retraining, and standardization exercises and supervision were conducted to achieve agreement within and between study personnel for questionnaire filling, assessment of dehydration, and weight and length measurements. Retraining exercises were conducted every 3 mo (16).

Data management and statistical analyses. The forms for the study were designed with FoxPro for Windows (Microsoft) and range and consistency checks incorporated. Double data entry by 2 data clerks followed by validation was completed within 48 h. Anthropometric measures were expressed as Z-scores, which were generated using the WHO Child Growth Standards (23).

All children for whom baseline venous blood specimens were analyzed for at least 1 parameter of folate and cobalamin status (n = 2296) were included in the analyses for the present study. The predefined subgrouping variables were categories below or equal to or above the 25th percentile in this dataset for cobalamin and folate, and above or equal to or below the 75th percentile for tHcy and MMA (24). We used percentile cutoffs for low or deficient status, because there are no established reference ranges for plasma MMA and tHcy concentrations in infants because of the uncertainties relating to commonly used cobalamin cutoffs for infants and young children (17), and in order to have a universal approach to determining low status for all 4 markers. Continuous variables were reported as means or medians as appropriate and categorical variables as proportions.

The 4-mo intervention was divided into 17 child-periods of 7 d each. For a child-period to be included in the analysis, we required information for ≥4 d of the given 7-d period. The occurrence of a new episode of diarrhea in a child-period was modeled as a binomial dependent variable and group allocation (zinc or placebo) as the independent variable. We calculated the effect of zinc supplementation as well as the interaction

between zinc supplementation and cobalamin or folate status (effect modification) on diarrhea using generalized estimating equations. Such models account for the correlation of multiple observation periods in the same child. We used a logit link function, binomial variance, and exchangeable correlation structure. Following assessment of separate baseline tables for each of the cobalamin and folate status subgroups, potential confounders of the effect of zinc on diarrhea were added to the model one at a time. These variables were weight-for-age, height-for-age, and weight-for-height Z-scores; sex; age; breastfeeding status (breastfed or not breastfed at inclusion;, years of schooling of mother and father; maternal literacy; household income; family type (nuclear or multigenerational); family size; and baseline plasma zinc. None of the covariates assessed changed the subgroup-specific estimates > 10% and the models were adjusted for only age and breastfeeding. Statistical analysis was performed with Stata, version 10 (StataCorp).

Results

Trial profile. The trial profile including the number of blood specimens analyzed for plasma cobalamin, folate, tHcy, and MMA was previously published (4). Briefly, of the 2482 children randomized in the main trial, baseline venous blood specimens were analyzed for 1 or more of the above variables for 2296 children (corresponding to 91.1% for cobalamin, 92.5% for folate, and 91.5% for tHcy and MMA of the number of children randomized to receive zinc or placebo).

Baseline characteristics. The children assigned to the zinc or placebo groups, for which baseline data on folate, cobalamin, tHcy, or MMA were obtained and who had ≥4 d information in each period, were comparable for baseline characteristics such as age, breastfeeding status, socio-economic status, anthropometric indices, and plasma concentrations of zinc, ferritin, folate, cobalamin, tHcy, and MMA (**Table 1**).

Effect of zinc supplementation on prolonged and acute diarrhea by subgroups of cobalamin and folate status. We investigated the effect of zinc on incident episodes of prolonged and acute diarrhea in children with low (<25th percentile; $n = 566$) and normal (≥25th percentile; $n = 1695$) plasma cobalamin, low ($n = 577$) and normal ($n = 1719$) plasma folate, high (>75th percentile; $n = 568$) and normal (≤75th percentile; $n = 1703$) plasma tHcy, and high ($n = 561$) and normal ($n = 1709$) plasma MMA.

There were 45 periods with prolonged diarrhea in children with low plasma cobalamin concentrations receiving zinc ($n = 267$) and 102 periods in the placebo group [$n = 299$; OR = 0.53 (95% CI = 0.35–0.78); adjusted for age and breastfeeding]. In children with normal plasma cobalamin concentrations, the corresponding numbers of prolonged diarrhea periods were 220 and 249 for the 865 and 830 children receiving zinc and placebo, respectively [OR = 0.90 (95% CI 0.73–1.11)]. Cobalamin status modified the effect of zinc on incident episodes of prolonged diarrhea (*P*-interaction = 0.015).

There were 547 periods with acute diarrhea in the zinc group and 654 periods in the placebo group in children with low plasma cobalamin concentrations [OR = 0.97 (95% CI = 0.84–1.13)]. In children with normal plasma cobalamin concentrations, the corresponding figures were 1605 and 1716, respectively [OR = 0.91 (95% CI = 0.83–1.00); *P*-interaction = 0.536]. Similar OR were observed for subgroups of plasma concentrations of tHcy or MMA above or below the respective 75th percentiles (**Fig. 1**). Zinc supplementation decreased the risk of prolonged but not acute diarrhea in children with elevated concentrations of these biomarkers.

TABLE 1 Baseline characteristics of 6- to 30- mo-old North Indian children by intervention group for which baseline data on folate, cobalamin, tHcy, or MMA were available and who had valid observation periods for analyses[1,2]

Characteristic	Zinc, $n = 1144$	Placebo, $n = 1152$
Age at enrollment, *mo*	15.7 ± 7.6	14.9 ± 7.2
Male	574 [50.2]	631 [54.8]
Breastfed	786 [68.7]	802 [69.6]
Literate mother	756 [66.1]	730 [63.4]
Annual family income, *rupees*	36,000 [24,000, 54,000]	36,000 [24,000, 55,500]
Weight-for-age Z score[3]	−1.82 ± 1.06	−1.82 ± 1.14
Weight-for-length Z score[3]	−1.18 ± 1.01	−1.15 ± 1.07
Length-for-age Z score[3]	−1.77 ± 1.21	−1.82 ± 1.21
Plasma zinc, *μmol/L*	9.3 ± 2.4	9.4 ± 2.0
Plasma ferritin,[4] *μg/L*	7.0 [5.0, 14.0]	7.0 [5.0, 14.0]
Plasma folate, *nmol/L*	10.7 [6.6, 19.7]	10.8 [6.3, 20.4]
Plasma cobalamin, *pmol/L*	205 [143, 298]	205 [136, 301]
Plasma tHcy, *μmol/L*	10.7 [8.3, 14.8]	11.1 [8.4, 15.1]
Plasma MMA, *μmol/L*	0.63 [0.37, 1.25]	0.67 [0.37, 1.34]

[1] Values are mean ± SD, median [IQR], or *n* (%).
[2] A child had to contribute ≥4 d to a given 7-d period to be included in the analyses.
[3] WHO 2005 standards (23); height was used for ages 24–30 mo; the impact of zinc supplementation on growth reported in (46); Z-scores based on 1978 reference data for zinc and placebo groups were −2.00 ± 0.97 and −1.97 ± 1.03 for weight-for-age, −1.19 ± 0.90 and −1.19 ± 0.90 for weight-for-length, and −1.66 ± 1.09 and −1.69 ± 1.09 for length-for-age.
[4] *n* = 665 (zinc) and 695 (placebo).

Folate status did not modify the effect of zinc supplementation on either prolonged or acute diarrhea (*P*-interaction = 0.932 and 0.736, respectively.).

Discussion

This secondary analysis indicates that baseline cobalamin status modified the efficacy of zinc in reducing the incidence of prolonged diarrhea. In this population, children with low plasma cobalamin concentrations (<25th percentile) benefited more from zinc supplementation than did children with cobalamin values ≥ 25th percentile. Assessment of cobalamin status using plasma cobalamin or the cobalamin markers, tHcy and MMA, gave essentially the same results.

Zinc supplementation substantially reduced the incidence of prolonged diarrhea in this study [OR = 0.77 (95% CI = 0.65–0.91); data not shown] compared with a smaller reduction in incidence for acute diarrhea [OR = 0.92 (95% CI 0.85–1.00)] (16). Indeed, the greatest effect of preventive and therapeutic zinc supplementation has been seen on diarrhea of longer duration (16,25), for which poor nutritional status is a risk factor (26,27). In this trial, however, being stunted or wasted or having low plasma zinc concentrations did not modify the effect of zinc (16). Nevertheless, it is possible that poor cobalamin status is a marker of general undernutrition in this population.

There are many parallels between zinc and cobalamin nutriture and function. Animal source foods are good sources of both micronutrients. However, foods consumed by the children in our study, such as roti (a flat bread) and fruit (28), do not contain any cobalamin and only low amounts of absorbable zinc (5). Cow or buffalo milk is one of the few frequently consumed food products containing both zinc and cobalamin
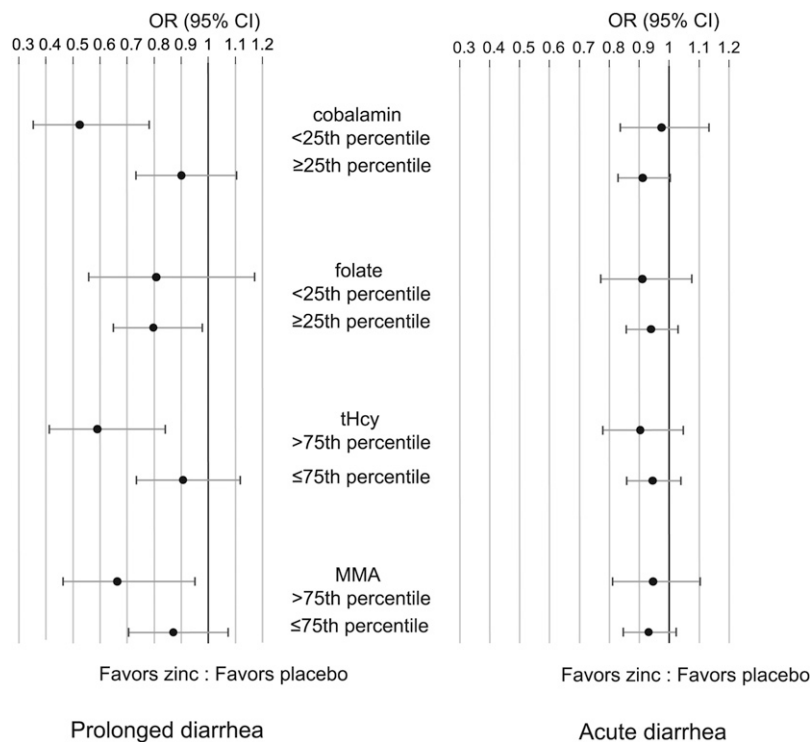
FIGURE 1 Effect of zinc supplementation prolonged and acute diarrhea in subgroups of baseline plasma cobalamin, folate, tHcy, and MMA concentrations among n = 2296 6- to 30-mo-old North Indian children. The 25th percentiles for plasma cobalamin and folate were 141 pmol/L and 6.4 nmol/L and the 75th percentiles for tHcy and MMA were 14.9 and 1.29 μmol/L, respectively. Values are OR (95% CI) adjusted for age and breastfeeding. For prolonged diarrhea, the P-interactions for zinc supplementation with cobalamin, folate, tHcy, and MMA were 0.015, 0.932, 0.045, and 0.188, respectively. The P-values for acute diarrhea were >0.5.

and, although often quoted as a poor source of zinc, it may be one of the main sources in this setting. Although plasma zinc is an accepted marker of zinc status on a population level (29), it is affected by recent zinc intake (30) as well as by inflammation (31). Plasma cobalamin, on the other hand, provides information about cobalamin status over time (32). Given that the sources of vitamin B-12 and zinc are similar, it is plausible that children with low plasma cobalamin also had the poorest zinc status and thus exhibited a stronger response to zinc supplementation.

It is possible is that the etiology of diarrhea in children with poor cobalamin status was different from that in children with normal status. There is some evidence to suggest that zinc supplementation is not equally effective against all pathogens (33–37). However, we did not undertake microbiological stool examinations and can only speculate as to how our effect-modifying variable, cobalamin status, is associated with etiology. Alternatively, infection with some pathogens may lead to impaired absorption of food cobalamin (38,39). Further studies are needed to elucidate this possible link between cobalamin status and the effect of zinc supplementation on prolonged diarrhea.

In this study population, the median plasma cobalamin concentrations in the breast-fed children were on an average almost 50% lower than that of nonbreast-fed children of similar age (4). The cobalamin status of the mother affects the concentration of the vitamin in breast milk and will influence the cobalamin status of the child (40). We know from previous studies in similar North Indian populations that the majority of families were lacto-vegetarian or had limited access to animal source foods and that cobalamin deficiency is common among women of childbearing age (41,42). Breastfeeding protects against intestinal infections (43) and acute episodes of diarrhea becoming persistent, i.e. of ≥14 d duration (44). However, breastfeeding did not, as opposed to cobalamin status, modify the effect of zinc on the incidence of prolonged diarrhea (16).

A strength of the current study is that it is based on a large, representative sample of a well-defined, low-income community. It was embedded in one of the largest supplementation trials carried out to assess the preventive effect of zinc on diarrhea incidence. To our knowledge, no other such trial included data on folate and cobalamin status as well as their functional metabolic markers, tHcy and MMA. Limitations regarding the prevalence estimates of folate and cobalamin deficiency in this study population have been discussed earlier. Briefly, the use of a nonfasting blood specimen may have led to an underestimation of the prevalence of folate deficiency (4). In the current study, carrying out multiple subgroup analyses may have increased the risk of type I errors. However, we used predefined subgroups and outcomes in the analyses (45) and our subgroups were relatively large. The cutoffs we used for defining low plasma cobalamin and folate in this study may appear arbitrary. Cutoffs for children used in clinical practice vary from country to country and are partly method dependent. More importantly, there are no established reference limits for tHcy and MMA in small children. For these reasons, we based our cutoffs on percentiles for all 4 markers of cobalamin and folate status. None withstanding, a post hoc analysis of our data using 150 pmol/L for plasma cobalamin and 5 nmol/L for plasma folate, which we used in a previous publication as cutoffs for deficiency (4), did not alter our conclusions (data not shown). The zinc and placebo groups were well balanced in the original study (16), but subgrouping increases the likelihood of group imbalances. However, the baseline tables for each investigated subgroup showed only minor differences (magnitude of ~5%) for age and breastfeeding status (data not shown), for which the OR were adjusted. Finally, information about breastfeeding frequency and types and amounts of complementary foods eaten in this population would have been desirable for the interpretation of our findings. Unfortunately, these data were not available.

In conclusion, for prolonged diarrhea, our findings indicate that children with low plasma cobalamin concentrations benefited

more from zinc supplementation than did children with higher concentrations. Our results may explain some of the heterogeneity between studies of the effect of zinc supplementation on the occurrence of diarrhea.

Acknowledgments

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