Zinc supplementation in Bangladeshi women and infants:

Effects on pregnancy outcome, infant growth, morbidity and immune response.

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Stellingen

1. Suppletie met een enkel nutriënt in gebieden waar meerdere nutriënttekorten naast elkaar bestaan, zal niet leiden tot verbeteringen in geboortegewicht of zuigelingengroei (*Dit proefschrift*).

2. Vergeleken met suppletie tijdens de zuigelingenperiode, is suppletie met zink tijdens de zwangerschap een effectievere manier om de gezondheid van zuigelingen jonger dan 6 maanden te verbeteren (*Dit proefschrift*).

3. Een verbetering van de status van vrouwen in de maatschappij is de beste lange-termijn strategie voor een verlaging van de kindersterfte. (*UNICEF, 1999*).

4. De effecten van het langdurig geven van borstvoeding op de voedingstoestand van moeders in populaties waar de meeste moeders ondervoed zijn verdienen meer aandacht.

5. Een laag geboortegewicht is geen oorzaak maar een indicatie voor postnatale gezondheidsrisico's.

6. De gevolgen van luchtvervuiling op de voedings- en gezondheidstoestand van kinderen in stedelijke gebieden in ontwikkelingslanden wordt onderschat.

7. Het huidige Nederlandse ontwikkelingssamenwerkingsbeleid om hulpontvangende landen te selecteren op basis van criteria voor armoede en goed bestuur zal in de praktijk niet werken aangezien in de meeste landen deze twee zaken negatief met elkaar gecorreleerd zijn.

8. Het verdient aanbeveling om enige praktijkervaring op te doen met zwangerschap en lactatie alvorens een onderzoek te ontwerpen bij zwangere en lacterende vrouwen.

Stellingen behorende bij het proefschrift getiteld: *"Zinc supplementation in Bangladeshi women and infants: Effects on pregnancy outcome, infant growth, morbidity and immune response"*.
Saskia Josepha Maria Osendarp

Wageningen, 19 juni 2001

Propositions

1. Supplementation with a single nutrient will not improve birthweight or infant growth in areas where multiple nutrient-deficiencies are prevalent (*This dissertation*).

2. Compared to infant supplementation, is zinc supplementation during pregnancy a more effective way to improve health in infants below 6 months of age (*This dissertation*).

3. Improving the status of women in the society is the best long-term strategy for improvement of child survival (*UNICEF, 1999*).

4. The impact of prolonged breast feeding on maternal nutritional status in populations where most mothers are malnourished deserves more attention.

5. Low birthweight is not a cause but rather an indicator of risk for postnatal morbidity and mortality.

6. The effect of air-pollution on health and nutritional status of children in urban communities in developing countries is underestimated.

7. The present Dutch foreign policy to prioritize aid-receiving countries based on criteria for poverty and good governance, can not apply this in reality since in most countries these two issues are negatively correlated.

8. Personal experience with pregnancy and lactation before designing a study in pregnant and lactating women is preferred.

Propositions belonging to the dissertation entitled: "*Zinc supplementation in Bangladeshi women and infants: Effects on pregnancy outcome, infant growth, morbidity and immune response*".
Saskia Josepha Maria Osendarp

Wageningen, 19 June 2001

To the women of Bangladesh

Abstract

**Zinc supplementation in Bangladeshi women and infants:
Effects on pregnancy outcome, infant growth, morbidity and immune response.**

Ph.D. thesis by Saskia Osendarp, Division of Human Nutrition and Epidemiology, Wageningen University, the Netherlands. June 19, 2001.

Zinc plays an essential role during periods of rapid growth and development, and is therefore important during the periods of gestation, fetal life, and early infancy. In this thesis two intervention studies are described to evaluate the effect of zinc supplementation during the last six months of pregnancy or between 1 and 6 months of age in infancy on pregnancy outcome, infant growth, morbidity from infectious diseases and immune response to childhood vaccines during the first six months of life. The study was performed in the urban slum areas of Dhaka, Bangladesh; an area where low birth weight (LBW) is prevalent and zinc deficiency is assumed to be common.

The results showed that supplementation with 30 mg elemental zinc/day during the last two trimesters of pregnancy, did not improve intra-uterine or postnatal growth but resulted in a reduced morbidity from diarrheal diseases and impetigo in the infants during the first six months of life, particularly in infants born with LBW. These effects were most likely due to improved cellular immunity in these infants as indicated by a higher proportion of positive responses to the tuberculin skin test at 6 months of age. Supplementation of infants with 5 mg elemental zinc/day from 4 to 24 weeks of age improved linear and ponderal growth and reduced morbidity from Acute Lower Respiratory Infections (ALRI) but only in a small proportion of infants that were zinc deficient at 4 weeks of age. In most infants however, supplementation with zinc did not have any effect on growth or morbidity. There was no clear effect of zinc supplementation on infant's immune response. Zinc improved the antibody response to certain specific Pneumococcal serotypes whereas a very small but negative effect of zinc on the response to the tuberculin skin test was observed.

In conclusion, the findings of this study suggest that child health and survival programs in less developed countries should consider the inclusion of zinc in antenatal multiple micronutrient supplements especially in regions where LBW is prevalent. More research is required to enable formulation of policy directions regarding the use of zinc supplements in infants during the first six months of life.

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Effects on pregnancy outcome, infant growth, morbidity and immune response.

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1

General introduction

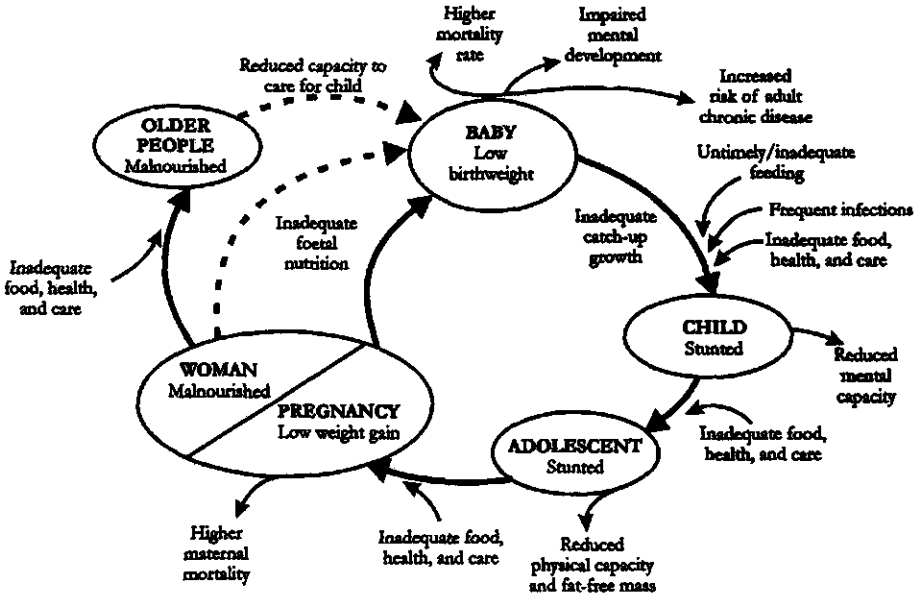
Despite impressive achievements in the area of nutrition during the last decade, undernutrition remains a major public health problem in many parts of the world with South Asia, and Bangladesh in particular, being especially affected. It is estimated that 55% of all Bangladeshi children under five years of age are moderately or severely stunted while 56% are underweight.[1] The prevalence rate of low birth weight (LBW; < 2500 g) is estimated at 40 to 50% of all live births, the highest in the world. As is the case in most developing countries, the vast majority of these LBW cases are due to intrauterine growth retardation (IUGR).

Infants born with IUGR have extreme rates of morbidity and mortality from infectious disease and malnutrition and are estimated to account for approximately one third of all deaths occurring in the first year of life.[2] These children are also more likely to experience abnormal cognitive development, neurologic impairment, and poor school performance.[3] There is increasing evidence that retarded fetal growth is associated with increased risks for chronic diseases in adulthood.[4] Barker's fetal origin of disease hypothesis proposes that nutritional deprivation during fetal life may result into an adaptation of the body to limited resources. As a consequence, the body will be more prone to obesity and related chronic diseases such as diabetes, hypertension and heart disease later in life during periods of relative affluence.[5]

The great majority of LBW is believed to be directly due to abnormalities that extend throughout the life cycle and is, therefore, considered an intergenerational problem. LBW infants grow up to be undernourished and stunted children and adolescents and, ultimately, undernourished women of childbearing age and undernourished pregnant women (figure 1). The life cycle approach currently adopted by the United Nations Sub-Committee on Nutrition (ACC/SCN)[6] has led to the recognition of maternal malnutrition as the most important determinant of childhood malnutrition.[7] In particular, decreased maternal height and below normal prepregnancy weight and pregnancy weight gain, are among the strongest predictors of delivery of a LBW infant.[8]

Undernutrition was long thought to be due to inadequate and insufficient dietary protein and energy intake. During the last decades however, it has been recognized that the problem of undernutrition is more complex and usually involves concurrent deficiencies of micronutrients as well. Since then, research and public health interventions were directed to deficiencies of the three most prominent micronutrients: iodine, iron and vitamin A. This recognition has led to great progress in the control of these deficiencies world-wide.[6]

It was only in 1997 that the ACC/SCN officially recognized the importance of other micronutrient deficiencies and in particular acknowledged that zinc deficiency may be widely prevalent in many developing countries and may have broad, adverse consequences on public health.[9]



Source: Prepared by Nina Seres for the ACC/SCN-appointed Commission on the Nutrition Challenges of the 21st Century.

Figure 1. Women's nutrition throughout the life cycle

ZINC STATUS AND ZINC DEFICIENCY

Zinc has been known as an essential trace element since the 1930s.[10] In the 1960s in Iran, Prasad et al.[11] for the first time identified zinc deficiency as the underlying cause for stunting and delayed sexual maturation. More recently, zinc deficiency has not only been associated with reduced growth and development, but also with impaired immunity and increased morbidity from infectious diseases.[12,13] As a constituent of over 200 metallo-enzymes in the human body, zinc is known to play an important molecular role in processes of gene replication, activation and repression, as well as DNA transcription and translation and protein synthesis.[14] The physiological role of zinc during periods of rapid growth and development emphasizes its importance during periods of gestation, fetal life, and early infancy.

Although zinc deficiency is thought to be wide-spread in most developing countries, there is no reliable information on the magnitude of zinc deficiency mainly because of the existing controversy on reliable and feasible indicators to assess body zinc status. Till date, plasma and serum zinc are still considered to be the best available indicators for assessment of zinc status on a group level.[15] However, it represents only 0.1% of the total body zinc.[16] Furthermore, plasma/serum levels are known to vary by 15-20% within individuals throughout the day[17] and are affected by physiological conditions such as infections.[15] In populations where the habitual dietary intake of zinc is low, plasma and serum

zinc levels may reflect a possible adaptation to these low intakes and are therefore not an adequate measure of marginal zinc status.[18] Inverse relationships between dietary zinc supply and serum zinc status have been observed in several studies,[16] probably because of reduced excretion of endogenous zinc.[19]

More recently stable isotope techniques were developed[20] which yield more reliable estimates of an individual's zinc status. These techniques however, are still relatively expensive, difficult to perform, and require frequent drawing of blood which make them less feasible for use in larger cohort studies especially when infants or young children are involved.

It has been suggested that an effect on growth after zinc supplementation may be used as a proxy for zinc deficiency in a population.[12] However, recent studies in Mexican[21], Jamaican[22] and Ugandan children[23] were not able to demonstrate a growth effect after zinc supplementation, indicating that zinc might not be the primary limiting nutrient for growth in populations with multiple nutrient deficiencies.[24] Therefore, growth performance after supplementation has limitations as an indicator of zinc deficiency in such populations.[25]

ZINC IN THE DIET

Zinc is available in a wide variety of foods but its bioavailability may vary depending on the source of origin as well as other components of the diet. The primary inhibitor of zinc absorption is phytic acid, which is present in significant amounts in staple foods like cereals, corn and rice. Inositol hexaphosphates and pentaphosphates are the phytate forms that exert these negative effects, whereas the lower phosphates have no or little effect on zinc absorption.[26] The amount of protein in a meal has a positive effect on zinc absorption and animal proteins in particular have been shown to counteract the inhibitory effect of phytate on zinc absorption.[27] In contrast, high concentrations of calcium in the diet may exacerbate the inhibitory effect of phytate although the calcium content of most plant-based diets in less developed countries is considered to be too low to influence the effects of phytate.[28] Based on these considerations, a model has been developed for categorizing diets according to the potential high (i.e., >50%), moderate (i.e., 30%) or low (i.e. < 15%) availability of zinc.[16] This model takes into account the dietary content of animal or fish protein, daily intakes of calcium and daily molar ratios of phytate to zinc. In developing countries, where most dietary zinc originates from cereals rich in phytate, the bioavailability of zinc is thought to be only 10-20%, based on a phytate:zinc molar ratio exceeding 15.[16] The current dietary recommendations take this into account and provide estimates of required basal and normative intakes for diets with different levels of bio-availability.[29] The basal requirement is the amount needed to prevent clinically detectable signs of

functional impairment whereas the normative requirements reflect the amount needed to maintain tissue stores or reserve capacity.

Table 1. Zinc and phytate daily intakes in selected regions in the world[1]

	W Europe	USA & Canada	SE Asia	Sub-Saharan Africa	South Asia	World
Number of countries	20	2	10	46	6	178
Population (millions)	457	305	504	581	1297	5882
Zinc (mg/d)	12.4 ± 1.3	12.2 ± 0.5	9.0 ± 0.9	9.3 ± 2.0	7.6 ± 0.6	10.0 ± 2.0
Phytate (mg/d)	1596 ± 391	1542 ± 58	2248 ± 586	2530 ± 645	2068 ± 263	2045 ± 504
Phytate: Zinc ratio	13.2 ± 4.8	12.5 ± 0.1	24.5 ± 4.6	26.9 ± 3.7	26.9 ± 1.7	21.3 ± 6.0
Absorbable zinc (mg/d)[2]	3.2 ± 1.2	2.9 ± 0.1	1.1 ± 0.2	1.0 ± 0.2	0.8 ± 0.1	1.5 ± 0.9
Estimated % population at risk of low zinc intake[3]	8.0 ± 17.1	0.9 ± 0.2	71.2 ± 14.2	68.0 ± 25.9	95.4 ± 2.1	48.9 ± 36.8

[1] Values in mean ± SD

[2] Amount of zinc available for absorption estimated as 50% if phytate:zinc molar ratio <5, 35% if phytate:zinc molar ratio = 5-15, and 15% if phytate:zinc ratio >15.[16]

[3] Risk of low intake was based on theoretical mean requirements for respective national population distributions, assuming a 25% coefficient of variation in zinc intake.

Adapted from: Brown KM, Wuehler SE, Peerson JM. The importance of zinc in human nutrition and estimation of the global prevalence of zinc deficiency. Food and Nutrition Bulletin (supplement; in Press)[30] (Reproduced with permission).

Assumptions on levels of zinc deficiency have been made based on comparisons of observed dietary zinc intake with recommended dietary intakes for zinc. In an attempt to estimate the global prevalence of zinc deficiency, national food balance sheets compiled by the Food and Agriculture Organization of the United Nations (FAO) were used. Absorbable zinc in the food supplies were calculated based on the phytate:zinc molar ratio's and risk of low intake was based on theoretical mean requirements for respective national population distributions.[30] The estimated mean per capita amount of absorbable zinc in the national food supplies and proportion of the population at risk of low intake are shown in table 1. This table indicates that zinc deficiency is likely to be a major public health problem, particularly in South Asia with an estimated 95% of the population at risk of low dietary zinc intake.

ZINC DEFICIENCY DURING PREGNANCY

Despite the difficulties described in measuring zinc status validly, it is estimated that about 82% of all pregnant women worldwide are likely to suffer from zinc deficiency.[31] During the last two trimesters of pregnancy, a net intake of 3 mg elemental zinc/day is recommended which translates into an intake of 15 mg zinc/day assuming a bioavailability of 20%.[16] Median dietary zinc intakes of 8-14 mg/day have been reported from pregnant women in developed countries[31] while intakes of 6.2-7.0 mg/day were observed in studies in Malawi[32] and Brazil.[33] These figures are well below the recommended intakes for pregnant women and support the hypothesis that zinc deficiency is widely prevalent in pregnancy, especially among women from less developed countries.

The possible mechanisms and pathways of maternal zinc deficiency and adverse health effects to the mother and fetus have been previously reviewed.[31] Maternal zinc status during pregnancy may have a direct effect on fetal growth and infant birth weight. Moderate maternal zinc deficiency has also been related to complications during labor and delivery that may in turn affect pregnancy outcome. It is further possible that maternal zinc status during pregnancy influences infant growth and morbidity beyond the neonatal period through its effect on intra-uterine growth and development of the immune system[34] and possibly through an interaction with vitamin A metabolism.[35] This theoretical framework has been supported by the results of many cross-sectional studies suggesting that low dietary zinc intake or low maternal plasma zinc are associated with an increased risk of LBW and preterm delivery.[36-38]
Low plasma zinc has also been reported to correlate with pregnancy complications such as prolonged labor, hypertension, postpartum hemorrhage, spontaneous abortion, and congenital malformation.[39] Despite these associations, the evidence from zinc supplementation trials in pregnant women to improve pregnancy outcome has been less convincing.

Till date, most randomized controlled trials have been performed in developed countries and the results have been described and summarized.[40,41] Nine of these trials are presented in table 2. The studies differed substantially in number of participants, duration of supplementation and dosages used while their results have been inconclusive. A clinically and statistically significant increase of 126 g in birth weight and 0.4 cm in head circumference after zinc supplementation was observed in the United States among African-American women, in which only women with low plasma zinc concentrations at enrollment were included.[49]

Based on the results of this study it was hypothesized that in less developed countries where diets low in zinc are likely to result in zinc deficiency and where the prevalence of LBW is high, the positive effects of maternal zinc supplementation on pregnancy outcome would even be larger.

Table 2. Double blind, randomized-controlled intervention trials on the effect of maternal zinc supplementation on pregnancy outcome in developed countries.

Reference	N	Duration[1]	mg Zn/ day	Growth Effect	Other Responses
Hambidge et al. (1983) USA [42]	46	9-7 mo	15	None	None
Hunt et al. (1984) USA [43]	213	from < 27 wk gestation	20	Not measured	Decreased number of low serum zinc
Hunt et al. (1985) USA [44]	138	last 3 mo.	20	No sign. effect on birth weight	None
Cherry et al. (1989) USA [45]	556	last 3 mo.	30	Longer gestational length (only in underweight)	Reduced rates prematurity
Mahomed et al. (1989) UK [46]	494	last 4 mo.	20	No sign. effect on birth weight	None
Simmer et al. (1991) UK [47]	56		22.5	Lower incidence IUGR	Labor induced+C-section less often.
Jameson et al. (1993) Sweden [48]	1231		15-90 (depen-ding serum Zn)	Fewer preterm (= <33 wk)	Reduced perinatal death + spont. abortions
Goldenberg et al. (1995) USA [49]	580, low plasma - Zn	from >19 wk gestation	25	Higher birthweight & larger head circumference	None
Jonsson et al. (1996) Denmark [50]	1206	from <20 wk gestation	25	None	None

[1] Duration of supplementation

Unfortunately, at the time of the onset of the study described in this thesis, very few trials had been performed in these settings (table 3). Garg et al.[52] showed a significant increase in birth weight after zinc supplementation in India, whereas a study carried out in South Africa[51] did not show any effect. However, both studies suffered from methodological flaws as they did not use a double-blind design and lacked a placebo group.

Table 3. Intervention trials on the effect of zinc supplementation during pregnancy on pregnancy outcome performed in less developed countries.

Reference	n	Duration[1]	mg Zn/ day	Pregnancy outcome	Other Responses
Ross et al. (1985) South Africa* [51]	127	before 20th week	30-90 mg Zn gluconate (4 cell trial with food supplements)	No effect on birth weight or length of gestation	Not investigated
Garg et al. (1993) India** [52]	168	different duration	45 (as zinc sulphate)	Higher birth weight and longer gestational age	Higher Apgar score in zinc group

[1] = Duration of supplementation
* = Not designed as zinc supplementation trial; **= No double-blind design

ZINC DEFICIENCY DURING INFANCY

In developed countries zinc deficiency is thought to be relatively rare among term breast-fed infants during the first months of life[53]. This is due to large concentrations of highly bioavailable zinc in early breastmilk and utilization of hepatic metallothionein as a source of zinc during the first six months of life.[54] Breastmilk zinc concentrations are quite high in the first weeks postpartum, averaging > 3ml/L but then decline rapidly over the early months postpartum, resulting in a longitudinal decline in dietary zinc intake. Zinc requirements also decrease as growth velocity declines during the first months of life, with requirements falling from approximately 8 mg elemental zinc/day for male infants at 1 month of age to 5 mg elemental zinc/day by 5 months. Zinc intakes from breastmilk are therefore thought to be adequate until approximately 6 months of age.[55] However, the situation might be different among infants in less developed countries where inadequate milk supplies, poor substitution of nutrients and increased losses of zinc during infections all contribute to an increased requirement. Infants born with low birth weight may be especially at risk for developing zinc deficiency because of their relatively faster growth rate, smaller liver stores and because they are at-risk for reduced intake of breastmilk. In addition, the bioavailability of preterm breast-milk zinc may not be adequate for LBW preterm infants.[56]

Zinc deficiency in infants and children has been associated with reduced growth, impaired immunity, increased prevalence and incidence of infectious diseases and delayed cognitive and motor development.[12,13,57,58] Evidence for a causal relationship has been provided by randomized controlled intervention trials in infants above 6 months of age and children in both industrialized and developing countries showing improved immune functions,[59,60] reduced morbidity[13] and a small

but significant effect on growth[12] after zinc supplementation. The effects on morbidity are now thought to be more attributable to a correction of the zinc deficiency status that causes an impaired immunity and intestinal mucosal damage[61] rather than to a pharmacologic effect of zinc.[62] The effect of zinc on growth may be due to a direct role of zinc in protein synthesis and gene expression, but there may also be a secondary impact of zinc on growth resulting from reductions in morbidity and increased appetite. Predictors for a positive effect of zinc on growth in children are low height-for-age and low plasma zinc values on enrollment.[12] A study on zinc supplementation among 6-12 month old infants in Ethiopia observed more pronounced effects on weight and length growth in stunted compared to non-stunted infants after zinc supplementation.[63]

Only limited and inconclusive information is available on the effect of zinc supplementation in young infants, when interventions may be especially effective due to high requirements in this age group. Beneficial effects on growth and morbidity were observed after zinc supplementation among low birth weight (LBW) and small for gestational age (SGA) infants in Brazil[64] and Chile[65] respectively. However, in India no effect of zinc supplementation on diarrheal morbidity was observed in children between 6-11 months of age whereas strong beneficial effects were observed among older children.[66] The varying responses to zinc supplementation in very young infants may be due to different prevalences of zinc deficiency among younger age groups caused by varying rates of IUGR, breast-feeding practices and early morbidity.

OBJECTIVES OF THE STUDY.

As outlined above zinc deficiency has a marked effect on maternal and infant health. Zinc supplementation of pregnant women and infants might help to improve the health status of women and children especially in areas where zinc deficiency is common. Unfortunately, very few randomized-controlled trials have been performed in these settings. Therefore, two studies were performed in an urban, poor area of Bangladesh and the results are described in this thesis. The objective of the first study was to evaluate the effect of zinc supplementation during the last six months of pregnancy on pregnancy outcome, infant growth, morbidity from infectious diseases and immune response to childhood vaccines during the first six months of life. The second study aimed to evaluate the effect of zinc supplementation between 1 and 6 months of life on infant growth, morbidity from infectious diseases and immune response to childhood vaccines. The studies were performed in a population at high risk of growth faltering and increased morbidity in an area where LBW is highly prevalent and zinc deficiency is assumed to be common.

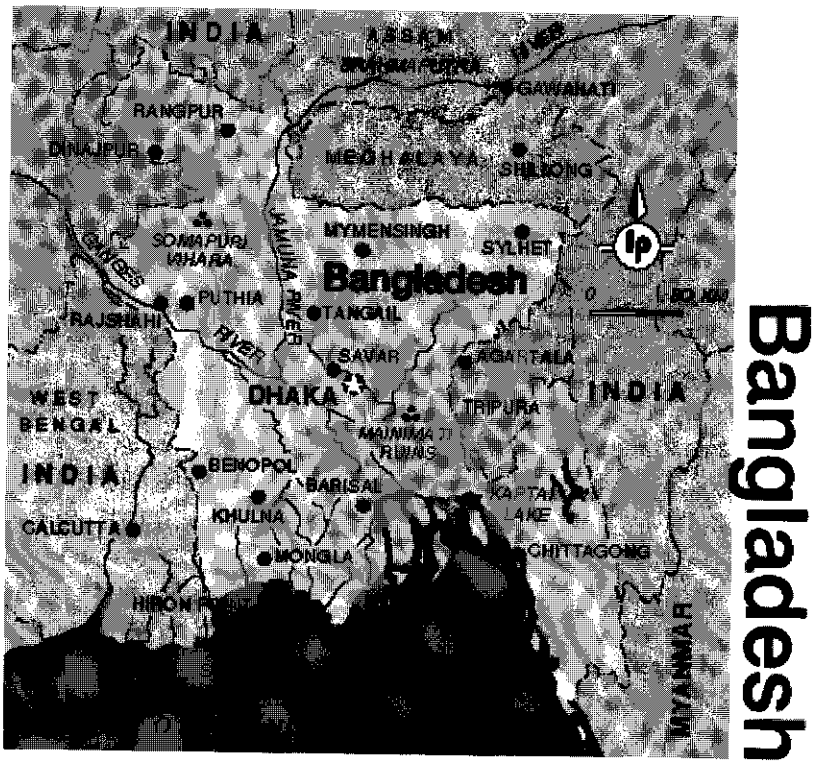


Figure 2. Bangladesh in South Asia

DESCRIPTION OF THE STUDY SITE.

The study was conducted between March 1996 and January 1998 in three selected areas of Dhaka, Bangladesh (figure 2). With a per capita GNP of US$273 and a population of an estimated 124 million,[67] Bangladesh is still one of the poorest and most densely populated countries in the world. The average life expectancy in Bangladesh in 1998 was 61 years.[67] During the last decades the success of public health interventions contributed to improvements in child survival. However the infant- and under-five-mortality rates remain high with 79 and 106 per thousand live births respectively in 1998.[1] Even though the majority of its population still lives in rural areas, the process of urbanisation seems irreversible with 20% of the population currently living in cities which is expected to grow to about 40% in 2020.[68] With an estimated population of 10 million,[67] the nation's capital Dhaka is rapidly becoming one of the most populated cities in the world. Most of the urban population, an estimated 85%,[68] live in temporary or makeshift housing in the ever expanding city's slum areas. The population

described in this study came from a representative sample of households from Dhaka's slum population.[69] The slum areas are characterised by high population density, poor housing, multi-family water sources and latrines, poor sewerage and drainage facilities and irregular garbage collection. The population is relatively young and mostly illiterate.[69] The 1996 infant mortality rate in the slum areas was estimated to be 138 per 1000 live births,[70] substantially higher than the national average. Malnutrition is known to be widely prevalent and prevalences of malnutrition among children of the urban slums are even higher than among children of the rural poor.[71]

OUTLINE OF THE THESIS

Chapter 2 describes the dietary intake during pregnancy and the relationship with pregnancy outcome in women from the urban slums of Dhaka. No data were available on dietary zinc intake during pregnancy in this population and the assumption that zinc deficiency was highly prevalent was primarily based on reports of a low intake of foods from animal origin in urban slums.[72] To enable a more precise description of the dietary intake during pregnancy in the urban slum population, dietary intake assessments were collected during each month of pregnancy.

In *Chapter 3* the effects are evaluated of a supplementation with 30 mg elemental zinc per day or a daily placebo during the last two trimesters of pregnancy on infant birth weight and gestational age and maternal weight gain during pregnancy. The effects on infant growth and morbidity during the first six months of life are presented in *Chapter 4* while *Chapter 5* describes the effect of maternal zinc supplementation on infant's immune response to childhood vaccines during the first six months of life.

Subsequently, an additional cohort of infants was supplemented with daily 5 mg elemental zinc or placebo from 4 to 24 weeks of age and the effects on growth and morbidity during the first six months of life are evaluated in *Chapter 6*. *Chapter 7* describes the effects of infant supplementation on infant's immune response to childhood vaccines and in particular to the recently developed pneumococcal conjugate vaccine.

Finally, in *Chapter 8* the main findings of this dissertation are discussed and the effects of the two interventions are compared. Possible implications for public health programs and future research are discussed.

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2

Effects of energy and zinc intake during pregnancy on pregnancy outcome in women from urban slums in Bangladesh.

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Abstract

Background: Little is known about the association between energy and zinc intake and pregnancy outcome and the effect of maternal zinc supplementation on dietary intake.
Objective: To evaluate energy and zinc intakes during the last two trimesters of pregnancy, the relation with pregnancy outcome and the effect of zinc supplementation on dietary intake in Bangladeshi women from Dhaka slums.
Design: A total of 224 women had five monthly dietary intake assessments by means of 24h-recalls. The women were randomly allocated to 30 mg daily elemental zinc or placebo from 12 to 16 weeks gestation until delivery. Anthropometric measurements were performed monthly. Birth weight, length and gestational ages were measured within 72h and infants were followed until 6 months for growth measurements.
Results: Median daily intakes of energy (6065 kJ/d) and zinc (6.1 mg/d) at 4 months gestation were low although intakes increased between 4 and 8 months of pregnancy by 11% for energy and 23% for zinc. An increased risk (OR:2.22) for premature delivery was observed among women with energy intakes below the median. There was no relationship with birth weight or post-partum nutritional status despite the positive associations between energy intake and maternal weight- and mid- upperarm circumference (MUAC)-gains. Dietary zinc intakes were not related to pregnancy outcome. Supplementation with zinc did not have an impact on dietary intake.
Conclusions: These women were at-high risk for inadequate energy and zinc intakes. Larger energy- but not zinc- intakes were related to greater maternal weight gains and reduced risk of premature deliveries.

INTRODUCTION

Maternal pre-pregnancy nutritional status and maternal weight gain during pregnancy are among the most important predictors of pregnancy outcome and subsequent infant health and survival.[1] Energy balance during pregnancy is affected by complex and highly interrelated biological, social and cultural factors,[2] including dietary energy and nutrient intake. Several studies however, failed to observe associations between dietary intake and/or dietary supplementation during pregnancy and pregnancy outcome.[3] It has been suggested that the effect of prenatal dietary supplementation is dependent on maternal nutritional status, such that supplementation would benefit the infant of moderately malnourished women and benefit the mother in severely malnourished women. In well-nourished women supplementation would have no effects.[4,5]

Women in developing countries are known to consume only two-thirds of the recommended daily intake of energy while actual energy and nutrient intakes for pregnant women tend to be only slightly higher than those of non-pregnant women,[6] despite the large estimated energy requirements of about 1.2 MJ/day during pregnancy.[7] There have been reports from developing countries, including India and Bangladesh, that women may restrict their food intake during pregnancy deliberately in order to have smaller infants.[8,9]

Evidence is accumulating that intake of several micro-nutrients, including zinc, might also determine pregnancy outcome independent of energy intake.[10,11] Low dietary zinc intakes during pregnancy have been associated with preterm deliveries[10] and lower birth weights[11] in the USA and Egypt respectively. Zinc might, at least in children, also affect the intake of other nutrients through an effect on appetite.[12,13] The results of prenatal zinc supplementation trials on pregnancy outcome however, have been inconclusive.[14,15] We previously reported that zinc supplementation during pregnancy had no effect on infant's birth weight or gestational age.[16]

In Bangladesh, poor maternal nutritional status[17] and low pregnancy weight gains[18] contribute to extremely high incidences of low birth weight (LBW; < 2500g) and subsequent childhood malnutrition. In this report we evaluate the association between dietary energy and zinc intake during the last 2 months of pregnancy, and maternal nutritional status, weight gain and infant's nutritional status. We also evaluate the effect of zinc supplementation on dietary intake during pregnancy, as part of a prospective study on the effect of zinc supplementation among pregnant women from an urban slum area in Dhaka, Bangladesh .[16]

SUBJECTS AND METHODS

Study design and population.

The study was conducted between March 1996 and October 1997 as part of a community-based, double-blind randomized controlled trial, to evaluate the effect of zinc supplementation during pregnancy on birth weight, infant growth and morbidity from infectious diseases.[16,19] The study was approved by the Ethical Review Committee of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). Details on the study population, intervention and data collection procedure have been reported elsewhere.[16]

A total of 559 pregnant women from selected areas of Dhaka city slums were enrolled in the study between 12 and 16 weeks gestation. Women were stratified by parity and randomly assigned to two treatment groups, to receive either 30 mg elemental zinc per day (twice the RDA, as zinc acetate) or a cellulose placebo. Supplementation continued until delivery and compliance with tablet consumption was 86% as assessed by counting remaining tablets in bubble packs during weekly compliance visits. Maternal weight, height and mid upper arm circumference (MUAC) and maternal dietary intake were measured monthly until 8 months gestation during home visits and serum zinc and blood hemoglobin were assessed at 4 and 7 months gestation.[16] Gestational age assessment, birth-weight measurements and infant anthropometric measurements were performed by trained physicians within 72 h of birth. Infants were followed until 6 months of age for monthly anthropometric measurements.[19]

Dietary intake

Information on a woman's dietary intake was collected every four weeks from 4 to 8 months gestation by a 24-h recall using standardized methods.[20] The interviews were conducted by trained health workers between March and October 1996 corresponding with the spring and monsoon season in Bangladesh. This time-period usually reflects a period of pre-harvest food scarcity.[21] Women were asked to recall all foods and beverages consumed during the previous 24 h, including snacks. Women were asked to provide information on the ingredients of composite dishes as much as possible. Separate questions were asked on the use of vitamin and mineral supplements and on the amount of oil used for cooking in the household. To estimate the amount of oil consumed by the woman, the total amount of oil consumed in the household was divided by the number of persons that had been eaten in the household during the previous day. Adults were counted as one person and children as ½ person to compensate for the smaller amounts consumed by children. Portion sizes were estimated by using standard household measures. The quantities in grams equivalent for the uncooked foods were estimated by repeated measurements of 10 independent samples for each household measure and each food.

A computerized food-composition table was developed especially for this study on the basis of nutrient values taken primarily from the Hyderabad table for Indian foods,[22] and completed with values from other sources.[23-25] To estimate the composition of common recipes and composite dishes, separate interviews and cooking sessions were performed in a representative sample of 38 women and average composition of recipes were calculated. The table contained energy, protein, carbohydrate and fat concentrations for 295 common foods and recipes. Iron and zinc concentrations were available for 92 and 65% of the foods respectively while only 38% of the foods had values for phytate. All dietary data forms were coded by trained nurses following standardized procedures. The conversion of foods into nutrients was done using the package KOMEET, developed at Wageningen University, Wageningen, The Netherlands (Bware, 1997).

Anthropometry

The weights of the women were measured by trained field workers to the nearest 0.1 kg on an electronic bathroom weighing scale (model 770; Seca, Hamburg, Germany), and height was measured to the nearest 0.1 cm with a locally-made height board. Midupper arm circumference (MUAC) was measured to the nearest millimeter with numeral-insertion tapes.

Infants were weighed at home within 72 h of birth by physicians using a portable beam-balance scale (model 725; Seca) to the nearest 10 g and then monthly by trained field workers until six months of age. The scales were regularly calibrated against standard weights. Infants' length and head, arm, and chest

circumferences were measured to the nearest millimeter. Gestational age was calculated by using the last date of menstrual period (LMP) as recalled on enrollment. The Capurro method[26] was used for 72 infants (18%) for whom LMPs were considered invalid (ie, <26 or >46 completed weeks or a difference between the LMP and the results of the Capurro method of >4 wk). Underweight (low weight-for-age), stunting (low length-for-age) and wasting (low weight-for-length) were defined as standard deviation scores (Z-scores) of < -2.00 compared with the sex-specific US NCHS reference charts using EPI Info software (version 6.1, 1994, CDC, Atlanta, GA, USA).[27] All anthropometric measurements were done in duplicate. Intra- and interobserver variation were regularly assessed and found to be acceptable, with CVs <2% for all anthropometric measures.

Blood analysis

Nonfasting venous blood was obtained during morning hours for serum zinc determination by using trace-mineral-free plastic syringes, stainless steel needles, and trace-mineral-free plastic tubes. Serum was separated at a maximum of 6 h after collection and stored at -20°C until analyzed. Before analysis, the serum samples were diluted (1:12) with 0.03% polyoxyethylene 23 lauryl ether and 10 mmol HNO3/L. Zinc concentration was measured by using flame atomic absorption spectrophotometry.[28] A standard curve was established using a commercial zinc reference (BDH Laboratory Supplies, London, UK) in concentrations of 0.1, 0.25, 0.5 and 1.0 mg/l. Commercial serum with known concentrations of zinc were used as a measure of quality control. The coefficient of variation of the measurements was always < 5%.

Hemoglobin was measured by using a commercial kit (Sigma Diagnostics, St Louis). According to general practice in Bangladesh, iron supplements (200 mg ferrous sulfate plus 200 µg folate/d) were provided by the study team to women with a hemoglobin concentration <90 g/L at 4 mo (n = 14; 2.5%) and 7 mo (n = 40; 8.5%) of gestation. The women were specifically instructed not to consume iron tablets together with the zinc supplements to avoid potential competition for intestinal absorption.[29]

Data analysis and statistical methods

Basal metabolic rates (BMR) were predicted based on women's self-reported age and baseline weight, and were multiplied with a factor 1.64 for moderate activity levels to predict woman's pre-pregnancy energy requirements.[7] Pregnancy energy requirements were increased by 1.2 MJ/d to compensate for the additional energy required during pregnancy. Subsequently, the average daily energy intakes of the women were compared to the corresponding recommended intakes. Energy-adjusted nutrient intakes were computed as the residuals from the regression model with total energy intake as the independent variable and absolute nutrient

intake as the dependent variable.[30] The average daily nutrient intakes for protein, fat and iron were compared to the Indian recommended dietary intakes during pregnancy.[22] Dietary zinc intakes with or without the supplemental zinc were compared to the WHO recommended basal and normative requirements during pregnancy, assuming a low bio-availability of the dietary zinc.[31] The basal requirement is the amount needed to prevent clinically detectable signs of functional impairment whereas the normative requirements reflect the amount needed to maintain tissue stores or reserve capacity. The percentage of women at-risk for inadequate intakes was calculated using the probability approach.[20]

An analysis of variance (ANOVA) for repeated measures was used to examine within-women trends in energy and nutrient intakes during pregnancy. Differences between energy and nutrient intakes at different months in pregnancy were then tested with the Wilcoxon signed rank-test for non-normally distributed data (SPSS7.5 FOR WINDOWS; SPSS Inc, Chicago). Differences in dietary intake between women supplemented with zinc or placebo were compared by ANOVA for repeated measures. To examine the association between dietary intake during pregnancy and pregnancy outcome we first calculated nonparametric Spearman correlation coefficients. Linear regression models were then made with the variable for pregnancy outcome as dependent and the nutrient of interest as independent variable. Potential confounders were identified by means of a multiple step-wise regression and added to the main regression models. Logistic regression models were used to evaluate the association between low caloric and zinc intakes during pregnancy and preterm delivery or low birth weight (LBW; < 2500g) after controlling for confounding factors. P-values of <0.05 were considered statistically significant.

RESULTS

Of the 559 women (269 zinc, 290 placebo) enrolled in the study at 4 months gestation a total of 113 (20.2%) were lost-to-follow-up before delivery, primarily (n=60) due to out-migration during the course of the study.[16] Reasons and numbers of lost-to-follow-up were not different for women of zinc or placebo groups. Of the 446 women who completed the follow-up until delivery, 224 (115 zinc, 109 placebo) had complete dietary intake data on all five measurement occasions during pregnancy. Baseline demographic and nutritional status characteristics of the 224 women with complete dietary intake data and the 335 excluded from the dietary intake analysis were not different (table 1). There were no significant differences in energy intake or in any of the nutrient intakes. The women with incomplete dietary data had a non-significant higher carbohydrate intake (270 vs. 247 g/day), and as a consequence a lower fat and carbohydrate intake per MJ energy intake than women with complete dietary intake data (table 1).

Table 1. Baseline (12-16 weeks' gestation) characteristics of 559 pregnant Bangladeshi women with or without complete dietary intake data.[1]

	Women with incomplete dietary intake data (n=335)	Women with complete dietary intake data (n=224)
Age at enrollment (yr)	23.5 (5.6)[2]	22.8 (5.4)
Nulliparous (%)	24.5	23.7
Socioeconomic status (%)[3]		
Poor	38.8	39.3
Very poor	42.1	36.6
Years of completed education	1.3 (2.4)	1.5 (2.3)
Family income (tk/month)[4]	3068 (1520)	3235 (1817)
Weight (kg)	41.8 (6.5)	41.9 (6.1)
Height (cm)	148.7 (5.7)	148.6 (4.8)
BMI (kg/m^2)	18.8 (2.4)	18.9 (2.4)
MUAC (mm)	225 (25)	227 (23)
Hemoglobin (g/L)	115 (13)	116 (13)
Serum zinc (μmol/L)	15.3 (4.5)	15.6 (4.3)
Energy intake (kJ/d)	6291 (4543-8521)[5]	6065 (4297-7727)
Fat intake (g/d)		
Crude	31.3 (19.2-43.8)	31.1 (22.9-43.3)
Per MJ energy intake	5.0 (3.5-7.2)	5.7 (3.8-7.5)*
Carbohydrate intake (g/d)		
Crude	270 (185-397)	247 (164-397)
Per MJ energy intake	43.4 (38.3-47.3)	41.9 (37.2-45.4)*
Protein intake (g/d)		
Crude	40.4 (24.1-62.3)	38.9 (25.7-59.6)
Per MJ energy intake	6.2 (4.9-8.0)	6.7 (5.4-8.1)*
Dietary zinc intake (mg/d)		
Crude	6.8 (4.3-9.6)	6.1 (4.1-8.9)
Per MJ energy intake	1.0 (0.9-1.3)	1.0 (0.8-1.3)
Iron intake (mg/d)		
Crude	11.3 (5.8-21.2)	10.9 (6.2-21.4)
Per MJ energy intake	1.8 (1.1-2.9)	1.8 (1.1-2.9)

[1] BMI= Body Mass Index, MUAC= midupper arm circumference
[2] mean (SD)
[3] Based on an index of household assets[15]
[4] One Bangladesh Taka (Tk) ≈ US $ 0.02
[5] Median (1st-3rd quartile)
* Significantly different from women with incomplete dietary intake data, P < 0.05 (Mann-Whitney U-test)

During pregnancy the 224 women that were included in the analyses gained an average of 5.0 kg (SD 2.2) between 4 and 8 months gestation while the mean increase in mid upper arm circumference (MUAC) was 4.6 cm (SD 11.1). Mean serum zinc and hemoglobin concentrations at 7 months gestation were 15.6 μmol/l (SD 0.29) and 10.8 g/dL (SD1.3) respectively.

A total of 209 singleton infants were born to these women. The mean birth weight was 2.53 kg (SD 0.40) and 43% of the infants were classified as low birth weight (LBW, < 2500 g). Mean birth length was 46.7 cm (SD 2.2) and the mean gestational age was 38.7 weeks (SD 2.2). A total of 193 infant-mother pairs completed the follow-up until 6 months post-partum. Mean maternal weight at one

Table 2. Energy and nutrient intakes of 224 poor urban Bangladeshi women during pregnancy.

	Per day				
	4 months gestation	5 months gestation	6 months gestation	7 months gestation	8 months gestation
Energy (kJ/d)	6065[1] (4297-7727)	6456* (4981-8309)	6478 (4972-8055)	65608* (4856-8631)	6751*# (5067-8667)
Protein (g/d)	38.9 (25.7-59.6)	43.9 (32.2-61.4)	40.6 (30.1-57.5)	41.7 (28.8-61.2)	44.9* (32.9-61.8)
Fat (g/d)	31.1 (22.9-43.3)	31.8 (20.8-45.8)	33.2 (23.6-44.8)	35.4*¶ (26.0-46.9)	35.6* (24.8-47.8)
Carbohydrate (g/d)	247 (164-342)	282* (202-365)	279 (200-359)	273* (193-369)	280* (207-389)
Iron (mg/d)	10.9 (6.2-21.4)	11.5 (7.1-22.0)	11.1 (6.6-18.8)	9.5¶ (6.0-17.1)	10.0 (6.5-18.5)
Zinc (mg/d)	6.1 (4.1-8.9)	7.1* (5.4-9.6)	7.3* (5.0-9.8)	7.4* (4.9-10.6)	7.5* (5.2-10.0)
Phytate (mg/d)	238 (146-341)	278* (198-394)	279* (193-371)	274* (172-355)	276* (179-381)

[1] Median (1st-3rd quartile)
* Significantly different from value at 4 mo gestation, P<0.05 (Wilcoxon signed-rank test)
¶ Significantly different from value at 5 mo gestation, P <0.05 (Wilcoxon signed rank test)
# Significantly different from value at 6 mo gestation, P < 0.05 (Wilcoxon signed rank test)

and six months post-partum were 42.9 kg (SD 6.0) and 41.9 kg (SD 5.6) respectively.

Dietary intake during pregnancy

Median energy and nutrient intakes during pregnancy are presented in table 2. Intakes of energy, fat, carbohydrate, zinc and phytate were significantly different within women between 4 and 8 months gestation. However, median energy and zinc intakes during the second half of pregnancy (at 5, 6, 7, and 8 months gestation) were not different within women.

Energy (6677 kJ/d *vs.* 6065 kJ/d, respectively) and zinc intakes (7.5 mg/d *vs.* 6.1 mg/d, respectively) during the second half of pregnancy were significantly higher compared to intakes at 4 months gestation ($P < 0.001$; Wilcoxon signed rank-test). Significantly higher intakes of energy, fat, carbohydrate, zinc and phytate were observed at 5, 7 and 8 months gestation compared to the median intakes at 4 months gestation (table 2). The intake of protein at 8 months gestation was also significantly higher than the protein intakes at 4 months gestation. There were no differences within women over time for energy-adjusted intakes of protein, fat and carbohydrates (table 3). Energy-adjusted zinc intakes, excluding the supplement were significantly higher in the second half of pregnancy (at 5, 6, 7 and 8 months gestation) compared to 4 months gestation (table 3).

Table 3. Nutrient intakes per MJ energy intake of 224 poor urban Bangladeshi women during pregnancy.

	Per MJ				
	4 months gestation	5 months gestation	6 months gestation	7 months gestation	8 months gestation
Protein (g/MJ)	6.7[1] (5.4-8.1)	6.7 (5.4-8.4)	6.4 (5.3-7.9)	6.5 (5.1-8.3)	6.5 (5.4-8.4)
Fat (g/MJ)	5.7 (3.8-7.5)	5.3 (3.5-6.8)	5.2 (3.7-7.5)	5.6 (4.2-7.7)	5.4 (3.8-7.3)
Carbohydrate (g/MJ)	42 (37-45)	43 (39-47)	43 (38-46)	42 (37-46)	42 (39-46)
Iron (mg/MJ)	1.8 (1.1-2.9)	1.9 (1.2-3.0)	1.7 (1.1-2.6)	1.4*¶# (1.0-2.3)	1.4*¶ (1.0-2.4)
Zinc (mg/MJ)	1.0 (0.8-1.3)	1.1* (0.9-1.3)	1.1* (0.9-1.3)	1.1* (0.9-1.4)	1.1* (0.9-1.4)
Phytate (mg/MJ)	40 (32-47)	43* (38-49)	43*¶ (35-48)	41¶ (34-46)	41¶ (33-48)

[1] Median (1st-3rd quartile)
* Significantly different from value at 4 mo gestation, P <0.05 (Wilcoxon signed-rank test)
¶ Significantly different from value at 5 mo gestation, P < 0.05 (Wilcoxon signed-rank test)
# Significantly different from value at 6 mo gestation, P < 0.05 (Wilcoxon signed-rank test)

Energy-adjusted dietary iron intakes were significantly lower at the end of pregnancy (7 and 8 months gestation) compared to early in pregnancy (4 and 5 months gestation).

Overall during pregnancy, carbohydrates supplied 72% of the daily energy intake, whereas fat and protein contributed 20% and 12% respectively. Most (61%) of the energy intake came from cereals and only 10% came from animal products. Cereals and pulses contributed to 72% of the daily zinc intake.

Overall during pregnancy, 88.8% of the women (n=199) had energy intakes below the recommended energy intakes for their age and bodyweights (table 4). The percentage of women estimated to be at risk for inadequate dietary zinc intake based on normative requirements was 98.2% while 94.2% were at risk for inadequate iron intake. If the supplement of 30 mg elemental zinc/d was added to the daily dietary zinc intake, an estimated 54.9% of the population was at risk for inadequate intakes (8.3% in the zinc supplemented and 97.9% in the placebo supplemented group). A total of 41% of the population was estimated to be at risk for inadequate intake of protein while the intake of fat was apparently more adequate with an average estimate of 16% at risk for inadequate intakes. There were no differences in percentages of women at risk of inadequate intakes for energy and nutrients between the second and third trimester of pregnancy.

Table 4. Percentages (%) of the population of pregnant urban Bangladeshi women estimated at-risk of inadequate intakes.

	All women (n=224)		Zinc group (n=118)		Placebo group (n=109)	
	Second trimester	Third Trimester	Second trimester	Third trimester	Second trimester	Third trimester
Energy[1]	87.1	79.0	89.6	82.6	84.4	75.2
Protein[2]	46.3	43.6	50.5	45.0	41.9	42.2
Fat[2]	14.7	13.8	13.4	14.7	16.1	12.9
Iron[2]	92.1	92.1	92.8	93.3	91.4	91.1
Zinc[3]						
-basal requirements	78.6	90.0	81.2	92.0	76.2	88.2
-normative requirements	94.5	97.4	95.1	97.8	93.9	97.0
Total zinc[4]						
-basal requirements	42.5	46.2	6.1	0	76.2	88.2
-normative requirements	52.8	50.8	8.2	0	93.9	97.0

[1] Proportion below recommended intakes for age and kg bodyweight[6]
[2] Estimates at-risk calculated with probability approach[19] using the recommended intakes for Indians[21]
[3] Estimates at-risk calculated with probability approach[19] using the basal and normal requirements by trimester according to WHO[30]
[4] Total zinc = dietary zinc plus supplemental zinc

The effects of zinc supplementation on dietary intake

There were no significant differences between women supplemented with zinc or placebo for energy intake (figure 1) during pregnancy or for crude and energy-adjusted protein, fat, carbohydrate and iron intakes during pregnancy (ANOVA for repeated measurements, data not shown). Dietary zinc intakes excluding the supplemental zinc at any measurement occasion were also not different between women from zinc or placebo group (figure 2). There were no differences between treatment groups for percentage of women at risk of inadequate intakes for energy and nutrients (table 4). When the supplemental zinc was added to the dietary zinc intakes, the proportion of women at risk for inadequate zinc intakes decreased dramatically in the zinc group (table 4). With the additional 30 mg zinc per day all women were able to meet the requirements for zinc during the third trimester of pregnancy whereas the vast majority (92.5%) was able to meet the requirements during the second trimester as well.

Dietary energy and zinc intake and pregnancy outcome

A higher mean energy intake during pregnancy was associated with higher maternal weight gains (β=0.16, P=0.02) and MUAC gains (β=0.20, P=0.003) during gestation. Higher mean energy intake during pregnancy was also associated with higher serum zinc concentrations as measured at 7 months gestation (β=0.13, P=0.05). These associations remained statistically significant after controlling for woman's age, the household's socio-economic status and family income, baseline maternal weight, and baseline hemoglobin and serum zinc concentrations.

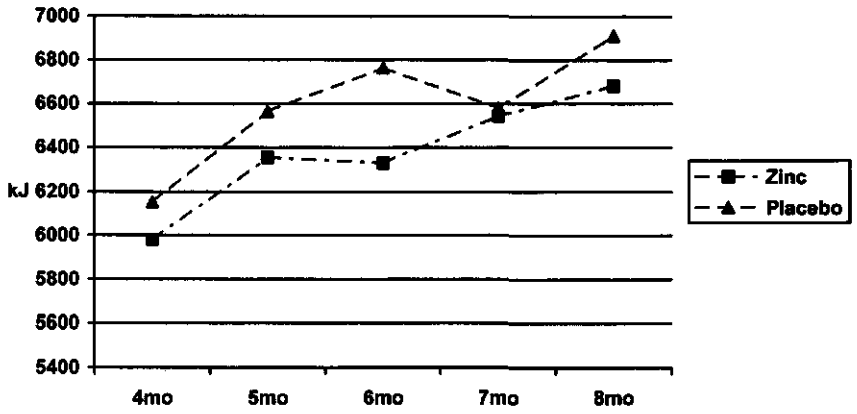

Figure 1. Median energy intake (kJ/d) over pregnancy for women in the zinc (n=115) and placebo group (n=109).

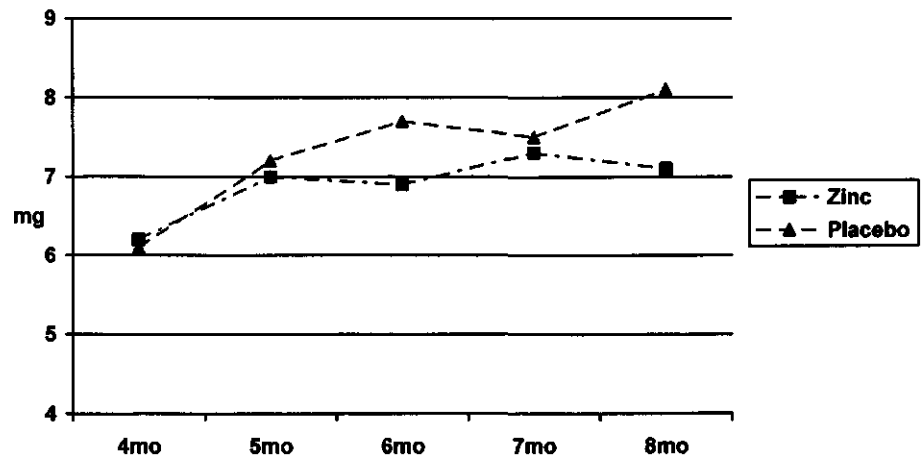

Figure 2. Median dietary zinc intakes (mg/d) excluding the zinc supplement over pregnancy for women in the zinc (n=115) and placebo group (n=109).

Energy intakes during pregnancy were also associated with higher hemoglobin concentrations at 7 months gestation. However, this association was not statistically significant anymore after controlling for the above-mentioned confounding factors (β=0.03, P=0.54).

Higher mean dietary zinc intakes adjusted for total energy intake during the third trimester of pregnancy were associated with lower maternal weight gains (β= -0.15, P=0.02). Higher crude dietary zinc intakes during the third trimester of pregnancy were associated with larger increases in MUAC during pregnancy (β=0.15, P=0.02) and with higher serum zinc concentrations at 7 months gestation (β=0.15, P=0.03). The association between crude dietary zinc intake and hemoglobin concentrations at 7 months gestation was not statistically significant anymore (β=0.04, P=0.42), after controlling for the confounding factors.

Women with low energy intakes during pregnancy (< 6569 kJ/d=median) had an increased risk for preterm delivery (OR: 2.22; [95%CI: 1.05-4.67]) even after controlling for confounding factors compared to women with higher energy intakes. The risk for delivering a LBW infant (< 2500g) was not different between women with lower or higher energy intakes (table 5). Dietary zinc intakes during pregnancy (excluding supplement) were not associated with infant birth weight or prematurity (table 5). Energy and dietary zinc intakes during pregnancy were not associated with infant or maternal nutritional status post-partum as measured at one or six months of age.

Table 5. Association of low dietary energy and zinc intakes during pregnancy with low birth weight (LBW) and preterm delivery.

	LBW OR (95% CI)[1]	Preterm delivery OR (95%)
Energy intake[2]		
Above median (n=113)	1.00	1.00
Lower than median (n=111)	1.53 (0.88-2.65)	2.22 (1.05-4.67)*
Dietary zinc intake[3]		
Above median (n=114)	1.00	1.00
Lower than median (n=110)	1.01 (0.58-1.74)	1.28 (0.64-2.58)

[1] OR= Odd Ratio (95% Confidence Interval) adjusted for baseline weight, baseline height and socio-economic status.
[2] Energy intake in kJ/d; median=6569 kJ/d
[3] Dietary zinc intake (excluding supplement) in mg/d; median=7.3 mg/d

DISCUSSION

The incidence of Low Birth Weight (LBW) in Bangladesh is reported to be the highest in the world with 40-50% of all live births classified as LBW in both urban[32] and rural[33] Bangladesh. We previously reported an incidence of 43% LBW and 17% premature deliveries among a population of pregnant women from urban Dhaka

slums.[16] The results of the current study in the same cohort of women, indicate that women's energy intakes during pregnancy may have contributed to the number of premature deliveries but had no impact on birth weight in this community. An increased risk for premature delivery was observed among women with energy intakes below the median compared to women with higher energy intakes. There was no relationship between energy intake during pregnancy and infant birth weight, length or post-partum nutritional status even though positive associations between energy intake and maternal weight and MUAC gains were observed. Dietary zinc intake during pregnancy was not related to pregnancy outcome independent of energy intake, except for a negative association between maternal pregnancy weight gain and energy-adjusted zinc intakes. Supplementation with daily 30 mg elemental zinc did not alter these associations nor did it have an impact on dietary intake during the last 2 trimesters of pregnancy.

Dietary intake in our study was assessed by means of a 24h dietary recall at five different time locations during the last two trimesters of pregnancy. The limitations of the 24h recall method in estimating an individual's dietary intake are well known although it is considered to be a reasonable estimator of usual group's intakes.[30] In a study among pregnant women in Malawi, a repeated, interactive 24h dietary recall was found to slightly overestimate intakes of minerals including zinc and slightly underestimate intakes of energy, protein and fat as compared to intakes assessed by weighed records.[34] We checked for a possible underestimation of energy intake in our population by calculating the ratio of energy intake (EI) and estimated basal metabolic rate (BMR). It is assumed that a ratio EI/BMR below 1.27 is incompatible with long-term survival.[7,35] In our study on all measurement occasions, none of the women had energy intakes below this level. In addition, the final sample size of 224 women should have been sufficient to provide estimates on any single measurement occasion within 7% of the true intakes for energy and within 9% of the true intakes for zinc.[20] We therefore believe that the estimates as presented in our study were reasonably reflecting true group intakes.

In our population energy intakes during pregnancy were found to be extremely low (6065 kJ/d at 4 months gestation) but comparable to energy intakes observed in other studies from Bangladesh in pregnant[36] and lactating[37,38] women. The low energy intakes in our population are consistent with poor pregnancy outcomes and with the observation that 26% of these women had a decrease in MUAC during pregnancy, while 1.5% lost weight between 4 and 8 months gestation. The fact that energy intake during pregnancy was associated with maternal weight gain but not with infant birth weight might indicate that differences in energy intake in this population of extremely malnourished women, primarily are reflected in benefits to the mother. This is consistent with observations from East Java[4], and Guatemala,[5] where maternal energy supplementation was found to be

beneficial to the mother in severely malnourished women (≈ < 42 kg bodyweight) while it would benefit the infant on expense of the mother in moderately malnourished women (≈ 42-50 kg).

It is interesting to note that energy intake in our population was related to premature delivery, despite the lack of effect on mean gestational age or low birth weight. In an overview of clinical trials on balanced energy and protein supplementation a similar reduced risk for preterm delivery was found while there was no effect on mean gestational age.[3] The author contributed this inconsistency to problems in gestational age measurements. The fact that we did not observe an effect on LBW despite the effects on preterm deliveries, is most likely due to the fact that the LBW cases in this population were primarily (75%) term deliveries and caused by intra-uterine growth retardation (IUGR).

Although observations from India[8] and Bangladesh[9] reported the practice of "down-eating" during pregnancy, i.e., eating less on purpose in order to have smaller babies and presumably smoother deliveries, we did not found evidence for this practice in these urban Bangladeshi women. Instead, women in our study tended to increase their caloric intakes during pregnancy and energy intakes at 8 months of gestation (6751 KJ/d) were significantly higher compared to the intakes at 4 months (6065 KJ/d).

On average women were consuming only 75% of their required energy intakes although we do not know whether the proportion of women at-risk of low energy intakes might have been overestimated in our population. The fixed incremental requirements of 1.2 MJ/d during pregnancy, as recommended by the WHO[7] have been questioned recently.[39] BMR is known to increase over time during pregnancy while the costs for energy deposition also vary throughout gestation and therefore lower increments, varying by trimester of pregnancy have been recommended instead. Prentice et al.[39] further recommends the use of Physical Activity Levels (PAL) into calculations of energy requirements during pregnancy. In rural Bangladesh, PAL levels of > 1.75 were measured in lactating housewives while levels of > 2.10 were recorded for female, lactating tea-pluckers.[38] PALs of women in our study were not known but it could be argued that activity levels of these women, almost exclusively housewives from a poor, urban community, may be different from the traditional rural activity patterns. The energy required for household activities may be less in an urban environment where women usually don't have to walk long distances to collect water or fuel-woods and we therefore assumed a moderate PAL (1.64) for the women in our study.

The median dietary zinc intake in our population was 6.1 mg/d at 4 months gestation and 7.5 mg/d at 8 months gestation, lower than intakes observed in pregnant women from developed countries[10,40] but comparable to intakes observed in Malawian women.[41] Unfortunately, we were not able to calculate phytate:zinc molar ratio's because the phytate concentrations of many of the foods consumed

were not known. However, dietary zinc in our study was assumed to be poorly bioavailable since it came mainly from cereals that are known to be rich in phytate.[31]

Dietary zinc intake was not related to zinc status or pregnancy outcome. Dietary zinc intakes during pregnancy have been related to birth weight in some studies,[10,11] although others did not find evidence for such an association.[40] The poor nutritional status and low overall energy intake in our population may have prevented the detection of any association between individual nutrients and pregnancy outcome.

The negative association observed between dietary zinc intake adjusted for total caloric intake and pregnancy weight gain might be artificial. Positive associations between pregnancy weight gain, body weight and energy intake have been often observed in other studies,[1] and might have resulted in a negative association between pregnancy weight gain and energy-adjusted zinc intakes. On the other hand, zinc is known to compete for intestinal absorption with other essential trace elements like iron and copper[29] and a higher intake of zinc in the diet may have reduced the bioavailability of these other minerals, especially when consumed together in one meal.

Zinc supplementation during pregnancy did not have an effect on dietary intake although in children zinc status or zinc supplementation has been associated with improved appetite[12] and reduced anorexia.[13] We are not aware of any reports in adults on the association between food intake and zinc. Dietary intake in adults is likely to be influenced by many different factors and, compared to young children, adult's food intake and/or taste acuity may be less responsive to zinc supplementation.

In conclusion, the overall low energy and zinc intakes in this population of pregnant urban Bangladeshi women indicate a population at risk for poor maternal nutritional status and poor pregnancy outcomes. Larger energy -but not zinc- intakes were related to greater maternal weight gains and a reduced risk of premature deliveries. Maternal zinc supplementation did not have an impact on dietary intake.

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3

A randomized, placebo-controlled trial of the effect of zinc supplementation during pregnancy on pregnancy outcome in Bangladeshi urban poor.

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ABSTRACT

Background: Maternal zinc supplementation has been suggested as a potential intervention to reduce the incidence of low birth weight in developing countries. To date, placebo-controlled trials have all been performed in industrialized countries and the results are inconsistent.

Objective: The objective of this study was to evaluate whether zinc supplementation in Bangladeshi urban poor during the last 2 trimesters of pregnancy was associated with pregnancy outcome.

Design: We conducted a double-blind, placebo-controlled trial in which 559 women from Dhaka slums, stratified by parity between 12 and 16 wk of gestation, were randomly assigned to receive 30 mg elemental Zn/d (n = 269) or placebo (n = 290). Supplementation continued until delivery. Serum zinc was estimated at baseline and at 7 mo of gestation. Dietary intake was assessed at baseline and anthropometric measurements were made monthly. Weight, length, and gestational ages of 410 singleton newborns were measured within 72 h of birth.

Results: At 7 mo of gestation, serum zinc concentrations tended to be higher in the zinc-supplemented group than in the placebo group (15.9 ± 4.4 compared with 15.2 ± 4.3 μmol/L). No significant effect of treatment was observed on infant birth weight (2513 ± 390 compared with 2554 ± 393 g; NS) or on gestational age, infant length, or head, chest, or midupper arm circumference. The incidence and distribution of low birth weight, prematurity, and smallness for gestational age also did not differ significantly after zinc supplementation.

Conclusions: Supplementation with 30 mg elemental Zn during the last 2 trimesters of pregnancy did not improve birth outcome in Bangladeshi urban poor. These results indicate that interventions with zinc supplementation alone are unlikely to reduce the incidence of low birth weight in Bangladesh.

INTRODUCTION

South Asia, and Bangladesh in particular, has among the highest incidences of low birth weight (LBW; <2500 g) in the world. An estimated 40-50% of all live births in Bangladesh are classified as LBW, 70-80% of which are the result of intrauterine growth retardation (IUGR).[1-3] Infants born with IUGR have higher mortality rates and are at higher risk of growth retardation, poor cognitive development, increased morbidity, and impaired immunity later in life than are their normal-birth-weight counterparts.[4,5] Effective interventions aimed at preventing LBW are therefore particularly important potential strategies for reducing childhood malnutrition and improving infant health. Recently, maternal zinc supplementation was suggested as one possible nutritional intervention during pregnancy to improve pregnancy outcome in developing countries.[6]

The results of cross-sectional studies suggest that low dietary zinc intake or low maternal plasma zinc are associated with an increased risk of LBW and preterm delivery.[7-9] Low plasma zinc has also been reported to correlate with

pregnancy complications such as prolonged labor, hypertension, postpartum hemorrhage, spontaneous abortion, and congenital malformation.[10] However, the results of zinc-supplementation trials in pregnant women to improve pregnancy outcome are not consistent,[11-18] possibly reflecting the use of insufficient sample sizes[11,13,16] or the fact that populations have varying risks of LBW and zinc deficiency.[19] A clinically and statistically significant effect on birth weight and head circumference was observed in one controlled intervention trial in which only women with low plasma zinc concentrations at enrollment were selected.[17] These results strengthened the hypothesis that zinc supplementation during pregnancy might be beneficial only in populations that are zinc deficient and at high risk of poor fetal growth.[19,20] Although women from developing countries are more likely to be zinc deficient and to have a greater risk of producing LBW infants, we are aware of only 2 published supplementation trials from developing countries.[21,22] One trial in India showed a significant increase in birth weight after zinc supplementation,[21] whereas the other study, which was carried out in South Africa, showed no effect.[22] However, neither of these studies used a double-blind design and both were lacking a true placebo group.

It is in this context that we performed a double-blind, placebo-controlled zinc-intervention trial among pregnant women from the urban slums of Dhaka, Bangladesh. These women belonged to a very poor and deprived part of the population in which LBW is highly prevalent and the poor quality of diets is likely to result in zinc deficiency.

SUBJECTS AND METHODS

Study population

Between March and June 1996, a total of 559 pregnant women from selected areas of Dhaka city slums were identified between 12 and 16 wk of gestation through an established pregnancy-identification system. Gestational age on enrollment was determined by the women's recalled date of their last menstrual period (LMP). Women were included in the study if they planned to remain at or near their residences in Dhaka for the delivery, did not have an established medical risk for reduced or excessive birth weight (eg, hypertension, renal disease, or diabetes), and provided informed consent. The study was approved by the Ethical Review Committee of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). The final sample size required to enable detection of a difference of 250 g in birth weight with 80% power and a type I error of 5%, assuming an SD of 500 g, was estimated to be 300 (150 per treatment group).

Study design

The study was double blind and women were stratified by parity and randomly assigned to receive either 30 mg elemental Zn/d as zinc acetate (n = 269) or a cellulose placebo (n = 290). Before randomization, information was collected on the socioeconomic status of the women's households and the reproductive history of the women. Categories for socioeconomic status were defined according to an index for urban populations that was developed on the basis of ownership of household durable goods.[23] The amount of zinc given was based on twice the recommended daily intake for zinc during the last 2 trimesters of pregnancy, assuming low or moderate bioavailability,[24,25] and was used previously in pregnant women without reports of adverse effects.[26] The zinc content of the zinc tablets (: 31.0 mg Zn/tablet; range: 28.6-32.6; n = 20) and placebo tablets (: 0.0 mg Zn/tablet; range: 0.0-0.1; n = 20) was verified in our laboratory and confirmed in a second independent laboratory. The placebo was a cellulose tablet indistinguishable from the zinc supplement in both appearance and taste. Health workers provided a 1-wk supply of zinc or placebo tablets (ACME Ltd, Dhaka) to the houses of the women weekly and instructed the women to consume one tablet daily between meals and not together with other vitamin or mineral supplements. Compliance with this regimen was assessed by counting the remaining tablets in each strip at the next visit. Unannounced compliance checks between regular visits were performed monthly in subsamples of 10% of the study participants. The women were prospectively followed up and supplementation continued until delivery. Randomization was achieved by computer-generated random-letter assignment, and the codes remained unknown to both investigators and participants until the study was completed. Serum zinc concentrations, hemoglobin concentrations, and blood pressure were assessed at baseline and again at 7 mo of gestation during visits to the ICDDR,B Clinical Research and Service Centre. Information on dietary intake was collected at baseline and anthropometric measurements were made monthly from baseline until 8 mo of gestation during home visits. Gestational age assessment, birth-weight measurements, and infant anthropometric measurements were performed by trained physicians within 72 h of birth.

Blood analyses and blood pressure

Nonfasting venous blood was obtained during morning hours for serum zinc determination by using trace-mineral-free plastic syringes, stainless steel needles, and trace-mineral-free plastic tubes. Serum was separated at a maximum of 6 h after collection and stored at -20°C until analyzed. Before analysis, the serum samples were diluted (1:12) with 0.03% polyoxyethylene 23 lauryl ether and 10 mmol HNO3/L. Zinc concentration was measured by using flame atomic absorption spectrophotometry.[27] For quality control, a commercial zinc reference (Utak Laboratories, Inc, Los Angeles) was used in concentrations of 0.1, 0.25, 0.5, and

1.0 mg/L. The CV of the analyses was always <5%. Hemoglobin was measured by using a commercial kit (Sigma Diagnostics, St Louis). According to general practice in Bangladesh, iron supplements (200 mg ferrous sulfate plus 200 µg folate/d) were provided by the study team to women with a hemoglobin concentration <90 g/L at 4 mo (n = 14; 2.5%) and 7 mo (n = 40; 8.5%) of gestation. The women were specifically instructed not to consume iron tablets together with the zinc supplements to avoid potential competition for intestinal absorption.[28] The blood pressure of the women was measured at 4 and 7 mo of gestation by registered nurses following standard procedures.

Anthropometry and dietary intake during pregnancy

The weights of the women were measured to the nearest 0.1 kg on an electronic bathroom weighing scale (model 770; Seca, Hamburg, Germany), and height was measured to the nearest 0.1 cm with a height stick. Midupper arm circumference (MUAC) was measured to the nearest millimeter with numeral-insertion tapes. Intra- and interobserver variation were regularly assessed and found to be acceptable, with CVs <1% for all anthropometric measures.

Dietary intake was assessed by a 24-h dietary recall and separate questions on the use of vitamin or mineral supplements. Portion sizes were estimated by using standard household measures quantified in grams by repeated measurements of 10 independent samples representative of the food concerned. A computerized food-composition table was developed especially for this study on the basis of nutrient values taken primarily from the Hyderabad table for Indian foods[29] and completed with values from other sources.[30-33] All anthropometric measurements and assessments of dietary intake were performed by trained fieldworkers who were closely supervised.

Pregnancy outcome

Newborns were weighed by physicians on a portable beam-balance scale (model 725; Seca) to the nearest 10 g during a home visit within 72 h of birth. The scales were regularly calibrated against standard weights. The period of 72 h was considered feasible and valid as a measure of birth weight in a field setting and was used in previous studies conducted at the ICDDR,B.[2] In our study, the average time between measurement and birth was found to be similar in both groups. Infants' length and head, arm, and chest circumferences were measured to the nearest millimeter. Gestational age was calculated by using the LMP as recalled on enrollment. The Capurro method[34] was used for 72 infants (18%) for whom LMPs were considered invalid (ie, <26 or >46 completed weeks or a difference between the LMP and the results of the Capurro method of >4 wk). Infants were classified as small for gestational age or appropriate for gestational age by using the 10th percentile of a US fetal growth curve as a cutoff.[35]

Statistical methods

Differences between the zinc-supplemented and placebo groups for the main outcome variables were tested by using Student's t test, a chi-square test, or the Wilcoxon signed-rank test (SPSS 7.5 FOR WINDOWS; SPSS Inc, Chicago). Multiple stepwise regression was performed to identify variables that contributed significantly to the variation in outcome variables and that required adjustment as covariates during multivariate analyses on the effect of supplementation.

Analysis of covariance (ANCOVA) was used to test for differences after adjustment for the effect of the covariates with the different outcome measures as dependent variables and treatment as the independent variable. For maternal weight and maternal MUAC during gestation, ANCOVA for repeated measurements was used. Some of the confounding variables had to be transformed into dummy variables[36] to enable the mathematical model for ANCOVA to be expressed as a model for multiple regression. For serum zinc, log-transformed values were used because the distribution approached normality only after log transformation. The distribution of other variables did not violate the assumption of normality. P values <0.05 were considered significant.

RESULTS

Of the 559 women enrolled, 113 (20.2%) were lost to follow-up before delivery [55 (20.4%) in the zinc-supplemented group and 58 (20.0%) in the placebo group; NS]. As anticipated for this highly mobile population and despite the restrictions at enrollment, most losses to follow-up (n = 60) were due to out-migration during the course of the study or to women leaving the area to deliver in their home villages. No differences in reasons for women being lost to follow-up were observed between the 2 groups.

A higher percentage of nulliparity was observed among the women who were lost to follow-up because nulliparous women were more likely to go back to their home villages for delivery. No differences in other relevant baseline characteristics were observed (Table 1).

Of the 446 women who completed the follow-up until delivery, 19 (12 in the zinc-supplemented and 7 in the placebo group; NS) delivered a stillborn infant or an infant that died shortly after birth. Four deliveries (2 in the zinc-supplemented and 2 in the placebo group; NS) took place outside Dhaka and 6 birth weights (3 in the zinc-supplemented and 3 in the placebo group; NS) were obtained >72 h after delivery and were excluded from further analyses. Another 7 women (3 in the zinc-supplemented and 4 in the placebo group; NS) delivered twins and were excluded from the birth-weight analyses. The final sample size for the analyses of birth weight and gestational age therefore consisted of 410 singleton infants.

Table 1. Maternal baseline characteristics[1].

	Women lost-to-follow-up (n=113)	Women who completed the follow-up (n=446)
Age at enrollment (yr)	23.6 ± 5.8[2]	23.1 ± 5.5
Nulli-parae	32.7%	22.0%[3]
Multi-parae only:		
- Previous SGA infant	9.1%	13.2%
- Previous preterm	13.0%	9.5%
- Previous miscarriage	30.9%	26.5%
Socio-economic status[4]:		
- Poor	41.6%	38.3%
- Very poor	41.6%	39.5%
Weight (kg)	41.6 ± 6.3	41.8 ± 6.3
Height (cm)	148.9 ± 5.9	148.6 ± 5.1
BMI (kg/m^2)	18.7 ± 2.3	18.9 ± 2.5
MUAC (mm)	224 ± 24	226 ± 24
Hemoglobin (g/L)	117 ± 13	114 ± 13
Serum zinc (μmol/L)	15.8 ± 4.6	15.3 ± 4.3

[1] SGA: Small for Gestational Age; MUAC: Mid Upper Arm Circumference
[2] means ± SD
[3] Differed significantly from lost-to-follow-up group ($P < 0.05$; Chi-square test)
[4] As based on an index of household assets[23]

Overall, 79% of the women came from households classified as "poor" or "very poor" on the basis of an index of the household assets.[23] Median dietary intakes of energy (6073 kJ/d) and zinc (6.5 mg/d) at baseline were not significantly different between the zinc-supplemented and placebo groups (Table 2). Carbohydrates supplied 67.8% of the daily energy intake, whereas fat and protein contributed 19.8% and 12.4%, respectively. Most (74%) of the daily zinc intake came from cereals (rice) and pulses. Consumption of iron and vitamin or mineral supplements was not common in this population, with only 11 women (2%) reporting intake of iron supplements and 45 (8%) reporting intake of other vitamin or mineral supplements during the previous 14 d at 4 mo of gestation.

Table 2. Maternal daily intake of energy and selected nutrients at baseline[1]

	Zinc supplemented women (n=214)	Placebo supplemented women (n=232)
Energy (kJ)	5881 (4386 - 7726)[a]	6252 (4357 - 8488)
Protein (g)	40 (24 - 58)	39 (26 - 69)
Fat (g)	31 (21 - 43)	32 (21 - 45)
Carbohydrate (g)	240 (179 - 349)	263 (168 - 379)
Iron (mg)	11.3 (6.0 - 19.5)	11.7 (6.0 - 23.4)
Zinc (mg)	6.3 (4.2 - 8.8)	6.4 (4.3 - 10.2)

[1] All values are expressed in median (1st quartile - 3rd quartile).
None of the differences was statistically significant

The mean compliance with supplementation as calculated by the percentage of days during follow-up that a woman reported having consumed a supplement was 86%, and the mean (±SD) duration of supplementation of the 446 women who completed the follow-up until delivery was 24.6 ± 3.5 wk. No differences in compliance with and duration of supplementation were observed between the 2 groups.

Pregnancy and maternal characteristics

At 7 mo of gestation, serum zinc concentrations tended to be higher in the zinc-supplemented group (15.9 ± 4.4 µmol/L) than in the placebo group (15.2 ± 4.3 µmol/L; P = 0.065; Student's t test) (Table 3). The zinc-supplemented women tended to have higher mean serum zinc concentrations at 7 mo of gestation than at 4 mo of gestation, whereas zinc concentrations in the placebo group tended to be lower at 7 mo of gestation than at 4 mo of gestation. The difference in response (1.08 µmol/L) was not significant. After 4 mo of supplementation (at 8 mo of gestation) there were no significant differences in mean maternal weight and MUAC between women in the zinc-supplemented and placebo groups who completed the follow-up (ANCOVA for repeated measurements). Mean weight gain between 4 and 8 mo of gestation was 4.9 ± 2.2 kg (4.7 ± 2.3 kg in the zinc-supplemented group compared with 5.1 ± 2.1 kg in the placebo group; NS, ANCOVA). The mean change in MUAC was 3 ± 11 mm (3 ± 12 mm in the zinc-supplemented group compared with 4 ± 11 mm in the placebo group; NS, ANCOVA). Mean blood pressure and hemoglobin concentrations at 7 mo of gestation were also not significantly different between groups.

Table 3. Maternal characteristics at baseline and at 7 or 8 mo of gestation[1]

	Zinc supplemented group (n=214)		Placebo group (n=232)	
	4 months gestation	7/8 months gestation	4 months gestation	7/8 months gestation
Weight (kg)	41.7 ± 6.2	46.4 ± 6.0	42.0 ± 6.5	47.1 ± 6.6
MUAC (mm)	226 ± 24	229 ± 23	226 ± 24	231 ± 22
Bloodpressure :				
Diastolic (mm Hg)	54.0 ± 7.8	53.8 ± 6.6	53.7 ± 7.1	54.6 ± 6.7
Systolic (mm Hg)	86.6 ± 11.1	84.4 ± 8.7	85.1 ± 10.1	84.7 ± 8.7
Hemoglobin (g/L)	114 ± 14	108 ± 13	115 11	108 ± 11
Serum zinc (µmol/L)	15.3 ± 4.1	15.9 ± 4.4	15.5 ± 4.4	15.2 ± 4.3

[1] Means ± SD. Values were controlled for maternal height, age and parity. There were no significant differences between zinc and placebo groups.
MUAC= Mid Upper Arm Circumference.

Infant characteristics

No significant effect of treatment was observed for weight, gestational age, length, head or chest circumference, or MUAC at birth in the 410 singleton infants with valid anthropometric measures. The incidence and distribution of LBW (<2500, <2000, and <1500 g), prematurity (<37 and <32 wk), and smallness for gestational age also did not differ significantly between infants of the zinc-supplemented and placebo groups (Table 4).

Infant birth weight was significantly correlated with maternal body mass index (BMI; in kg/m2) at baseline, socioeconomic status of the mother's household, and parity of the mother. We therefore examined the effect of zinc supplementation on women from different subgroups. Overall, nulliparous women (n = 92) delivered infants with a significantly lower birth weight than did multiparous women (n = 318) (2446 ± 325 and 2560 ± 405 g, respectively; P = 0.01). Likewise, women with a baseline BMI <18.5 (n = 194) had significantly smaller infants than did women with a higher initial BMI (n = 211) (2461 ± 400 and 2598 ± 375 g, respectively; P = 0.00), whereas infants from very poor households (n = 160) had significantly lower birth weights than did infants from middle-income households (n = 93) (2466 ± 392 and 2605 ± 419 g, respectively; P = 0.001). No treatment effects of zinc or placebo were observed for any of the outcome variables in women from the different subgroups. A total of 45 pregnancies (20 in the zinc-supplemented group and 25 in the placebo group; NS) resulted in miscarriage, stillbirth, or early neonatal death.

Table 4. Birth characteristics of singleton infants of the zinc-supplemented and placebo groups[1]

	Zinc supplemented group (n=194)	Placebo supplemented group (n=216)
Weight (g)	2513 ± 390[2]	2554 ± 393
Low Birth Weight:		
<2500g	45.9%	40.3%
<2000g	8.9%	8.8%
<1500g	1.0%	0.5%
Gestational age (wk)	38.8 ± 2.4	38.9 ± 2.1
Prematurity :		
<37 wk	17.5%	15.7%
<32 wk	1.0%	0.9%
Small-for-Gestational-Age :	74.7%	74.5%
Length (cm)	46.8 ± 2.3	47.0 ± 2.2
Head circumference (cm)	32.6 ± 1.4	32.7 ± 1.5
Chest circumference (cm)	30.2 ± 1.8	30.4 ± 1.9
MUAC (mm)	92 ± 8	91 ± 8

[1]Values were controlled for baseline maternal weight, parity and socio-economic status. There were no significant differences between groups. MUAC= Mid Upper Arm Circumference.
[2]Mean ± SD

DISCUSSION

In a recent systematic review of 5 published randomized, controlled maternal-zinc-supplementation trials,[20] it was concluded that, although current available data did not provide enough evidence for routine zinc supplementation during pregnancy, supplementation may be beneficial in zinc-deficient populations in which LBW is highly prevalent. We therefore performed a randomized, double-blind, placebo-controlled trial in a poor urban population of Dhaka. The prevalences of LBW (42.9%) and smallness for gestational age (74.6%) in our study population were very high, similar to data from other studies in rural[1,3] and urban[2] Bangladesh and higher than observed in other developing countries.[3] The median dietary intake of zinc was 6.5 mg/d, although an intake of 15 mg is recommended during the last 2 trimesters of pregnancy.[24,25] Moreover, we assumed that only 20% of the dietary zinc was potentially available for absorption[24] because it came mainly from cereals that are high in phytate and pulses, and because the intake of zinc and protein from animal products was low.

After 3 mo of supplementation, serum zinc concentrations tended to be higher in the zinc-supplemented group than in the placebo group, but the difference was small. Increased blood zinc concentrations or a reduced number of low zinc values were reported after zinc supplementation in some[13,17] but not all[11,15] of the previous trials. It is recognized that the specificity of serum zinc as an indicator of body zinc status has limitations,[24,37] and we believe that supplementation in our study was successful in improving maternal zinc status. Compliance with taking the supplements was good; the supplements were consumed between meals to avoid potential competition for absorption and the zinc tablets substantially increased the daily dietary zinc intake.

Supplementation with 30 mg elemental Zn/d during the last 2 trimesters of pregnancy did not result in improved pregnancy outcomes. These findings were unexpected because our study was performed in a very malnourished population in which the dietary zinc intake was low and poorly bioavailable. Our supplementation provided twice the recommended dietary zinc intake for pregnant women, which is similar to or even higher than the dosages used in most of the other zinc-supplementation trials. Effects on birth weight and IUGR were observed in trials with use of dosages of 22.5 and 25 mg elemental Zn/d.[16,17]

As expected in this highly mobile urban population, the dropout rate was substantial. More nulliparous women were lost to follow-up than were women who had previously given birth, which may have resulted in a lower number of LBW infants. However, because the percentages and reasons for women being lost to follow-up were not different between the zinc-supplemented and placebo groups, it is unlikely that the high dropout rate affected the outcome of our study. In addition, the final sample size of 410 infants was still sufficient to enable a difference in birth weight as small as 110 g to be detected.

Surprisingly, in our population only 4 women had serum zinc concentrations <9.18 μmol/L, which was used as the reference point for zinc deficiency in other studies.[13,38] Food intake may have contributed to the relatively high serum zinc concentrations observed in our study population because blood samples were taken during morning hours under nonfasting conditions. Serum zinc is known to vary by 15-20% within individuals throughout the day, primarily as a result of food intake.[37] The amount of time between collection and separation is also known to influence final serum zinc concentrations of samples because zinc leaks from cells into serum.[39] In our study, this delay of a maximum of 6 h may have caused an increase in serum zinc in some cases. Whether or not the relatively high serum zinc concentrations may reflect an adaptation to habitually low dietary zinc intakes in our study population is unknown. Inverse relations between dietary zinc supply and zinc status were observed in several studies,[25] probably because of lower intestinal excretion of endogenous zinc.[40] Serum zinc is known to be susceptible to contamination during sample collection, storage, and analysis. However, we believe that contamination is not a likely cause of the high serum zinc values observed in our study because special care was given to avoid contamination and because random contamination would not explain the systematically high values we observed.

Even though the poor dietary intake and nutritional status of our study population indicated otherwise, the possibility that this population was not zinc deficient on enrollment and hence did not benefit from additional zinc supplementation must be considered. We therefore also analyzed only women with low serum zinc concentrations to assess whether the zinc status of our population affected the outcome of the study. In a trial among African American women who were already receiving a non-zinc-containing prenatal multivitamin or mineral tablet, Goldenberg et al.[17] observed an increase in birth weight after zinc supplementation in women with low plasma zinc concentrations on enrollment. In our population, only 21 women had serum zinc concentrations comparable with the cutoffs used in Goldenberg's trial (<10.6 μmol/L at 13 wk of gestation), and restricting analysis to those women resulted in no significant differences in mean infant birth weight [2360 ± 195 compared with 2375 ± 602 g in the zinc-supplemented (n = 10) and placebo (n = 11) group, respectively], but the sample was small.

We believe that the absence of any effect of zinc supplementation in our study population was most likely due to the concurrent existence of other nutrient deficiencies that reduced the bioavailability of zinc or limited fetal growth. The median intake of energy was 6.2 MJ/d, which is lower than the recommended maintenance requirements of 7.5 MJ/d for pregnant women with similar body weights.[41] Maternal nutritional status was extremely poor at 4 mo of gestation, and most of the women undoubtedly had entered pregnancy in an already

disadvantaged nutritional state. The average weight gain between 4 and 8 mo of gestation was 4.9 kg, whereas in developing countries, weight gains of 1.5 kg/mo during the last 2 trimesters of pregnancy are recommended.[42,43] A total of 132 women (32.5%) had a decrease in MUAC during gestation, indicating that, instead of laying down fat stores for fetal growth later in pregnancy and for lactation and maternal recuperation, these women were actually depleting already-poor fat and lean tissue stores during gestation. Our data clearly show a population at high risk of having infants with LBW that is apparently not reversible with a single micronutrient supplement.

In summary, zinc supplementation alone during the last 2 trimesters of pregnancy in very poor urban Bangladeshi women did not improve infant birth weight or gestational age despite the fact that supplementation resulted in a marginal improvement in maternal zinc status. It is possible that zinc supplementation earlier in gestation or before conception would have a positive effect. However, in most developing countries it is not feasible to identify pregnant women before 12 wk of gestation. The results of our study therefore indicate that public health interventions with zinc supplementation alone are unlikely to reduce the incidence of LBW in a developing country such as Bangladesh.

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4

Zinc supplementation during pregnancy and effects on growth and morbidity in low birthweight infants: a randomized placebo-controlled trial.

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ABSTRACT

Background: Infant malnutrition and mortality rates are high in less-developed countries especially in low birthweight infants. Zinc deficiency is also widely prevalent in these circumstances.

Objective: The objective of this study was to assess the effect of daily zinc supplements given to pregnant mothers on their infants' growth and morbidity.

Design: We did a double-blind, placebo-controlled, randomized trial in 199 and 221 Bangladeshi infants, whose mothers took 30 mg daily elemental zinc or placebo, respectively, from 12 to 16 weeks' gestation until delivery. Infants were followed up until 6 months of age. We obtained data for morbidity every week by mothers' recall. Infants' anthropometric measurements were done every month, and their serum zinc was assessed at 1 and 6 months of age.

Results: Infants of mothers who received zinc during pregnancy had at age 6 months reduced risks compared with those in the placebo group for acute diarrhoea (risk ratio: 0·84; 95%CI: 0·72,0·98), dysentery (0·36; 95%CI: 0·25,0·84) and impetigo (0·53; 95%CI: 0·34,0·82). These reductions were seen in low birthweight infants but not in those with normal birthweight. There were no differences in infant growth or serum zinc concentrations between treatment groups.

Conclusions: Maternal zinc supplementation during pregnancy resulted in a reduction of the health risks in Bangladeshi low-birthweight infants, although this intervention did not improve birthweight. Whether zinc should be added to usual antenatal supplements in regions with high rates of low birthweight should be reviewed.

INTRODUCTION

Micronutrient deficiency, including zinc, contributes significantly to impaired growth, health, and development of children in less-developed countries.[1] Zinc supplementation during infancy partly reverses the adverse effects of zinc deficiency, and is associated with a small improvement in growth,[2] improved immunity, and reduced morbidity due to infectious diseases.[3] These effects were especially pronounced in low-birthweight (ie, <2500 g) infants, who might be vulnerable to the effects of zinc deficiency because of their reduced liver stores of hepatic zinc metallothionein.[4,5]

Infants with a low birthweight have high rates of morbidity and mortality because of infectious diseases and impaired immunity, and are at increased risk of growth failure and abnormal cognitive development as neonates. They are also estimated to account for about a third of all deaths in the first year of life in less-developed countries.[6] The high prevalence of low birthweight, which is mainly attributable to intrauterine growth retardation in developing countries, is related to intergenerational cycles of malnutrition.[7] Thus, the adverse effects of nutritional deprivation, and perhaps zinc deficiency might begin before birth and the most effective time for an intervention might, therefore, be the antenatal period.

Low maternal blood zinc concentrations during pregnancy are associated with an increased risk of low birthweight and preterm delivery in some[8] but not all studies.[9] Results of studies in developed countries of zinc supplementation and pregnancy outcome are also inconsistent.[10] We have previously reported that zinc supplementation during pregnancy in a very poor, urban population of Bangladesh did not result in improved birth weights.[11] In rhesus monkeys, low maternal plasma zinc concentrations were associated with reduced infant growth.[12] Antenatal zinc deficiency in mice and rhesus monkeys resulted in reduced lymphoid organ sizes, IgG concentrations, and blood lymphocytes in the off-spring.[13] Studies are required in man to investigate the relation between pregnancy and zinc status, prenatal zinc supplementation, and infant growth and health.

We, therefore, did a study in infants from poor urban areas in Bangladesh, whose mothers received either daily zinc supplements or placebo during pregnancy, and assessed the effect of supplementation on the infants' growth and morbidity from infectious diseases during the first 6 months of life. The study was done in a population in whom the proportion of babies with low birthweight was 43%.[11]

SUBJECTS AND METHODS

Study design and population

We did our study in selected poor urban areas of Dhaka, characterized by a very high population density, poor housing, inadequate sewerage, and low socioeconomic status. The population is young and most people are illiterate.[14] Pregnant women were enrolled between 12 and 16 weeks' gestation, stratified by parity and randomly assigned to receive either daily zinc supplements or placebo until delivery. We used a computer-generated random letter to assign the mothers to each group. The codes remained unknown to investigators and participants until the study was completed. Singleton neonates were included in postnatal surveillance.[11] Doctors measured weight and length, and calculated gestational age of the babies within 72 h after delivery.[11]

We calculated gestational ages using date of last menstrual period as recalled by mothers at enrollment and with the Capurro method.[15] We classified the babies as small or appropriate for gestational age using the tenth percentile of a US fetal growth curve as a cutoff,[16] and recorded data on infant morbidity every week. Details of infant feeding were obtained every 2 weeks, and infants were classified as either exclusively (breastmilk only), predominantly (breastmilk and water or sugar water), or partly (breastmilk and other complementary fluids) breastfed, or not breastfed. We also recorded the age that complementary foods were introduced.

A total of 410 singleton infants with known birthweights (low birthweight < 2500 g. normal birthweight ≥ 2500 g) were included in the postnatal surveillance.

We also included another ten infants, whose mothers had taken supplements and were followed up throughout pregnancy, but for whom valid birthweight measurements were not available. Thus, 199 infants of mothers on zinc supplements and 221 of mothers on placebo were enrolled. Sample-size calculations had indicated that a minimum of 360 infants (180 in each group) was needed to detect a 15% difference in mean weight gain during the first 6 months of life, a 15% difference in mean number of episodes of diarrhoea, or a 20% difference in mean serum zinc concentrations between the groups, with 80% power and a type I error of 5%.

Supplements

During pregnancy, mothers on zinc supplements received 30 mg elemental zinc per day. The placebo was a cellulose substance indistinguishable from the zinc supplement in both appearance and taste. All supplements were prepared in bubble packs of ten tablets each (ACME Ltd, Dhaka, Bangladesh) and two laboratories independently confirmed the zinc content of both tablets. Health-care workers gave the women a week's supply of zinc tablets or placebo, every week. The women were instructed to consume 1 tablet daily between meals with no other vitamin or mineral supplements. Consumption of iron and vitamin or mineral supplements was uncommon in this population.[11] At baseline (12-16 weeks' gestation) only 11 (2%) women reported taking iron supplements and 45 (8%) reported taking other vitamin or mineral supplements during the past 14 days. According to general practice in Bangladesh, iron and folate supplements (200 mg ferrous sulphate and 200 μg folate per day) had been provided by the study team to women with a hemoglobin concentration of less than 90 g/L at 4 months' gestation (14 women, 2·5%) and 7 months gestation (40, 8·5%). Compliance with zinc or placebo treatment was 86%, as assessed by counting the remaining tablets at the next weekly visit, and by monthly unannounced checks in a 10% sub-sample.

Blood analyses

We took blood samples from non-fasting infants at 1 and 6 months of age by antecubital venipuncture in the morning using trace-mineral-free plastic syringes, stainless steel needles, and plastic tubes. We separated the serum within 6 h after samples were taken and stored serum at -20 °C. Before analysis we diluted the samples (1/12) with 0·03% polyoxyethylene 23 laryl ether and HNO_3 (10 mmol/L). Zinc concentration was measured by flame atomic absorption spectrophotometry. We established a standard curve with a commercial zinc reference (BDH Laboratory Supplies, London, UK), in concentrations of 0·1, 0·25, 0·5 and 1·0 mg/L, and used commercially obtained serum with known concentrations of zinc as a measure of quality control. The coefficient of variation of the measurements was always less than 5%.

Anthropometry

During monthly home visits, infant weight was measured to the nearest 10 g on beam-balance scales (Seca 725, Seca, Hamburg, Germany) that were daily calibrated against standard weights. Recumbent lengths were measured to the nearest 0·1 cm on a length board, and head, chest, and arm circumferences were measured to the nearest mm with numeral insertion tapes. We recorded for all indices the mean of two readings as the measured value. Anthropometric measurements were done by trained field workers. Intra-observer and inter-observer variations were acceptable, with coefficients of variation less than 2% for all anthropometric indices. We defined underweight (low weight for age), stunting (low length for age) and wasting (low weight for length) as standard deviation scores (Z scores) of less than -2·00 compared with the US National Center for Health Statistics reference charts[17] using EPI Info software (version 6·1, 1994, Centers for Disease Control, Atlanta, GA, USA).

Morbidity assessment and outcome definitions

During home visits trained non-medical interviewers asked detailed questions on the infant's history during the past week of respiratory infections, diarrhoea, fever, skin infections, and other illnesses. To distinguish impetigo from other skin diseases, interviewers asked mothers whether the infants had any signs of this disease, using photographs showing clinical signs of impetigo of various degrees of severity as a guide. Infants were examined for signs of dehydration during monthly visits. The respiration rate was recorded, and a second record was taken for infants with a respiration rate of more than 60 per min.

The same trained fieldworkers obtained the data, which registered nurses checked manually and coded. Doctors examined infants who required medical treatment using standard treatment protocols, and referred them to appropriate health-care facilities if necessary. Information was recorded about standard infant vaccinations received through the expanded programme of immunisation or other sources. We defined an infant as having acute lower respiratory infection if reported symptoms included cough, difficult breathing, or both, with or without fever, lasting more than 1 day, and rapid breathing or chest indrawing. Upper-respiratory-tract infection (URI) was diagnosed if the infant had reported symptoms of cough or difficult breathing, with or without fever for more than 1 day, which was not associated with rapid breathing or chest indrawing, or a reported cough for more than 1 day with nasal discharge. Acute diarrhoea was defined as symptoms of unusually loose or unusually frequent stools, or both, as reported by the mother. If the stool contained blood the episode was classified as dysentery. Diarrhoea lasting for 14 days or longer was defined as persistent diarrhoea.

We calculated the actual number of surveillance days by subtracting the days on which no recall data were available from the total days of follow-up. When a mother had been absent for more than 15 days (two consecutive visits), recall data was only collected for the 14 days before the previous interview. Infants were classified as recovered if they were symptom free for at least 3 consecutive days.

Statistical analysis

We did a multiple stepwise regression to identify variables that contributed significantly to the variation in the different outcome variables and those that required adjustment as confounders in the multivariate analysis on the effect of zinc supplementation. Possible interactions between treatment and infant's birthweight, sex, or serum zinc status were assessed by introduction of separate interaction terms in the linear regression models. On the basis of these outcomes, separate models were used for subgroups of birthweight.

We assessed differences in mean, total weight and length gain between infants of mothers who took zinc supplements or placebo using ANCOVA with the outcome variables (eg, total weight gain) as dependent variables and treatment group (zinc or placebo group) as independent variable. Baseline values were added as covariates (SPSS version 7.5). We tested differences in anthropometric indices and Z scores at 1, 2, 3, 4, 5, and 6 months of age, with ANCOVA for repeated measurements.

To compare differences between treatment groups in frequency of diseases, we used a Poisson regression model with number of episodes as dependent variable, treatment group as independent variable and total days of actual surveillance as off-set term included in the model. We added potential confounders to the regression models as additional independent variables (epidemiological graphics estimation and testing package, EGRET, SERC, Seattle, WA98105, 1991).

Duration of disease was calculated for all infants as percentage of actual surveillance days ill. We used the non-parametric Kruskal-Wallis test to compare differences in duration of illness between infants from zinc and placebo groups. Since the distribution of length of illness was highly skewed, we used log-transformed values in the multivariate regression models to enable control for covariates. Before log-transformation a constant was added to all duration values to eliminate zero values. P values less than 0·05 were regarded as statistically significant.

RESULTS

After randomisation 139 women were lost to follow-up, including 7 twin births and 19 infant deaths. Of the infants that were included in the follow-up after birth 22 died and 13 were lost to follow-up because their families moved away from the study area. The mothers of two infants refused further participation in the study. No differences were recorded between the groups in numbers or reasons for not completing the study (figure 1). The maternal baseline characteristics were similar for infants lost to follow-up and those who completed follow-up (table 1). Mothers of infants who completed follow-up were significantly older (p=0·016) at enrolment in the zinc supplement group than the placebo group (table 1). No other differences in baseline characteristics were seen between the two treatment groups. 184 infants in the zinc and 199 in the placebo group completed 6 months' follow-up (figure 1). Infants lost to follow-up were included in the analysis of morbidity for the days on which observations were available.

All infants were breastfed throughout the study but the rate of exclusive breastfeeding was low because of early introduction of water given with breastmilk. At 24 weeks of age, 55 (13%) of all infants were exclusively or predominantly breastfed, and 353 (84%) were partly breastfed.

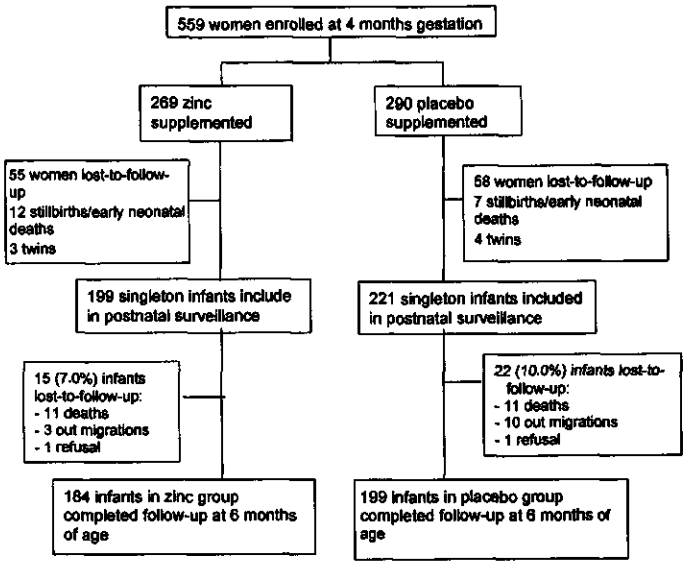

Figure 1. Trial profile.

There was no relation between breastfeeding status at 1 or 6 months of age and main outcome variables in stepwise multiple regression analysis to identify potential confounding variables. There was a relation with age at water introduction for some variables and with that at complementary food introduction for other outcome variables. Therefore, those variables were controlled for in the multivariate analysis. No differences in infant serum zinc concentrations were seen at 1 month (15·1 μmol/L [SD 5·3] *vs.* 15·8 μmol/L [5·5]) or at 6 months of age (11·4 μmol/L [2·5] *vs.* 11·7 μmol/L [2·5]).

Between birth and 6 months of age changes in weight (3·49 kg [0·77] *vs.* 3·56 kg [0·77]), length (15·2 cm [2·0] *vs.* 15·4 cm [2·1]) and head circumferences (8·0 cm [1·2] *vs.* 8·1 cm [1·3]), did not differ between infants of mothers on zinc or placebo. Average weight increase (0·58 kg/month [0·13] *vs.* 0·59 kg/month [0·13]) and linear growth (2·5 cm/month [0·3] vs. 2·6 cm/month [0·4]) did not differ between infants from the zinc and placebo groups, respectively.

Table 1. Selected baseline characteristics of 420 pregnant mothers.

	Zinc-supplement		Placebo	
Maternal Characteristic	Lost to follow-up (n=15)	Follow-up completed (n=184)	Lost to follow-up (n=22)	Follow-up completed (n=199)
Maternal age (years)	22·0 (4·8)	23·8 (5·8)	23·5 (5·5)	22·4 (5·5)
Nulli-parae	31%	19%	27%	24%
Socioeconomic status:				
-Poor	25%	39%	18%	41%
-Very poor	56%	40%	55%	35%
Body Mass Index (kg/m^2)	19·2 (2·8)	18·8 (2·5)	19·5 (3·4)	19·1 (2·3)
MUAC (mm)	224 (27)	227 (24)	231 (33)	226 (23)
Hemoglobin (g/L)	113 (15)	115 (15)	116 (12)	116 (14)
Serum zinc concentration (μmol/L)	14·4 (2·8)	16·1 (5·4)	15·0 (4·9)	16·4 (6·1)

Values are mean (SD) unless otherwise indicated; MUAC = mid upper arm circumference.

At 6 months of age, Z scores for infants in the two groups were closely similar for mean length for age (-1·47 [0·91] *vs.* -1·41 [0·96]), weight for age (-1·36 [0·87] *vs.* -1·28 [0·92]), and weight for length (-0·29 [0·77] *vs.* -0·28 [0·82]). There were no interactions with anthropometric outcomes and infant birthweight, sex, or serum zinc status.

The 420 infants were followed up for a total of 65771 (93%) of a possible 70560 days (31378 days in the zinc supplement and 34393 days in the placebo group). Infants of mothers taking zinc supplements had fewer episodes of acute watery diarrhoea, dysentery, and impetigo than those of mothers in the placebo

Table 2. Frequency of diseases for infants from zinc supplement and placebo groups during first 6 months of life.

Disease	Zinc supplement	Placebo	RR (95% CI)	p
Acute diarrhoea				
– Total episodes	267	346		
– Episode/infant				
– All infants*	1·5 (1·9)	1·9 (2·1)	0·84 (0·72;0·98)	0.037
– LBW infants#	1·4 (1·8)	2·2 (2·3)	0·68 (0·53;0·87)	0.002
– NBW infants¶	1·6 (1·9)	1·6 (1·9)	1·04 (0·84;1·29)	NS
Persistent diarrhoea				
– Total episodes	49	49		
– Episode/infant				
– All infants*	0.3 (0.7)	0.3 (0.6)	1.13 (0.76;1.68)	NS
– LBW infants#	0.2 (0.5)	0.3 (0.6)	0.88 (0.46;1.71)	NS
– NBW infants¶	0.3 (0.8)	0.2 (0.6)	1.47 (0.86;2.50)	NS
Dysentery				
– Total episodes	7	22		
– Episode/infant				
– All infants*	0.04 (0.2)	0.1 (0.4)	0.36 (0.25;0.84)	0.019
– LBW infants#	0.04 (0.2)	0.1 (0.4)	0.26 (0.07;0.99)	0.049
– NBW infants¶	0.04 (0.3)	0.1 (0.4)	0.46 (0.14;1.44)	NS
Cough				
– Total episodes	668	768		
– Episode/infant				
– All infants*	3.9 (1.9)	4.1 (1.8)	0.95 (0.68;1.06)	NS
– LBW infants#	4.2 (2.4)	3.9 (2.1)	0.99 (0.84;1.16)	NS
– NBW infants¶	3.7 (1.7)	4.0 (1.8)	0.94 (0.82;1.08)	NS
Acute lower respiratory infection	106	134		
– Total episodes				
– Episode/infant	0.7 (1.2)	0.8 (1.3)	0.89 (0.69;1.15)	NS
– All infants*	0.6 (1.2)	1.5 (6.6)	0.97 (0.64;1.45)	NS
– LBW infants#	0.7 (1.1)	0.7 (1.0)	0.87 (0.62;1.24)	NS
– NBW infants¶				
Impetigo				
– Total episodes	30	61		
– Episode/infant				
– All infants*	0.2 (0.6)	0.3 (0.8)	0.53 (0.34;0.82)	0.005
– LBW infants#	0.1 (0.5)	0.3 (0.8)	0.39 (0.18;0.85)	0.018
– NBW infants¶	0.2 (0.7)	0.3 (0.8)	0.64 (0.37;1.10)	NS

RR = risk ratio estimated with Poisson regression adjusted for socioeconomic status, parity, baseline serum zinc, age at introduction of water. Values are in mean (SD) unless otherwise indicated. *n=196 (zinc) and 214 (placebo). # LBW= < 2500g birthweight (n=88 and 84). ¶ NBW = ≥ 2500g birthweight (n=106/129). Birtweight was unknown in three infants.

group. For infants who were followed up for 15 days or longer the risk of these diseases was reduced by 16% for acute diarrhoea, 64% for dysentery, and 47% for impetigo (table 2). Infants of mothers on zinc had fewer days ill with dysentery and impetigo than those whose mothers were on placebo (table 3). Maternal zinc

supplementation had no effect on frequency and duration of cough or acute lower respiratory infections in the infants.

When data were analysed separately for infants with low and normal birthweight (for whom birthweights were available) larger reductions were seen in low-birthweight but not normal-birthweight infants for the frequency of acute diarrhoea, dysentery, and impetigo in the zinc supplement than the placebo group. In low-birthweight infants of mothers receiving zinc, the risk of disease was reduced by 32% for acute diarrhoea, 74% for dysentery, and 61% for impetigo. By contrast, in normal-birthweight infants no differences in frequency of disease were seen between the zinc and placebo groups.

Table 3. Proportion of days ill during first 6 months of life.

Infant Morbidity	Proportion of days ill (%)				p
	Zinc supplement		Placebo		
	Mean (SD)	Median (range)	Mean (SD)	Median (range)	
Acute diarrhoea					
– All*	4·8 (6.0)	2.5 (0·0-29·7)	5·0 (6.3)	2·9 (0·0 ; 36·1)	NS
– LBW infants#	4·4 (6.1)	1·5 (0·0-29·7)	6·0 (7.1)	3·5 (0·0 ; 36·1)	0.008
– NBW infants¶	5·1 (6.0)	3·1 (0·0-24·8)	4·2 (5.6)	2·3 (0·0 ; 21·1)	NS
Persistent diarrhoea					
– All*	3·2 (7.8)	0·0 (0·0-42·9)	2·9 (7.8)	0·0 (0·0 ; 58·6)	NS
– LBW infants#	2·8 (6.7)	0·0 (0·0-30·3)	2·7 (6.2)	0·0 (0·0 ; 29·5)	NS
– NBW infants¶	3·6 (8.6)	0·0 (0·0-42·9)	2·6 (7.8)	0·0 (0·0 ; 58·6)	NS
Dysentery					
– All*	0·2 (1.2)	0·0 (0·0-10·5)	0·5 (2.0)	0·0 (0·0 ; 14·8)	0.047
– LBW infants#	0·1 (1.0)	0·0 (0·0- 8·8)	1·0 (2.3)	0·0 (0·0 ; 14·8)	NS
– NBW infants¶	0·2 (1.4)	0·0 (0·0-10·5)	0·4 (1.6)	0·0 (0·0 ; 10·6)	NS
Cough					
– All*	37·6 (24.1)	32·5 (0·0-91·8)	37·7 (24.2)	36·3 (0·0 ; 100·0)	NS
– LBW infants#	39·5 (25.1)	36·4 (0·0-91·8)	36·5 (26.1)	36·2 (0·0 ; 96·5)	NS
– NBW infants¶	35·4 (22.8)	29·9 (0·0-91·1)	36·3 (22.9)	36·3 (0·0 ; 100·0)	NS
Acute lower respiratory infection					
– All*	7·7 (16.0)	0·0 (0·0-86·3)	8·8 (15.4)	0·0 (0·0 ; 100·0)	NS
– LBW infants#	7·0 (13.4)	0·0 (0·0-60·5)	10·8 (18.5)	0·0 (0·0 ; 100·0)	NS
– NBW infants¶	8·0 (17.6)	0·0 (0·0-86·3)	7·2 (12.5)	0·0 (0·0 ; 55·3)	NS
Impetigo					
– All*	0·9 (3.2)	0·0 (0·0-24·1)	1·5 (4.4)	0·0 (0·0 ; 39·0)	0.013
– LBW infants#	0·6 (2.5)	0·0 (0·0-18·1)	1·8 (5.4)	0·0 (0·0 ; 39·0)	0.047
– NBW infants¶	1·0 (3.6)	0·0 (0·0-24·1)	1·4 (3.6)	0·0 (0·0 ; 21·2)	NS

p values are from linear regression analysis with log transformed values, controlled for birtweight, gestational age, age at introduction of complementary food, maternal baseline body mass index, household socioeconomic status, maternal baseline serum zinc. *n=199 [zinc] and 221 [placebo]. #LBW = < 2500g (n=90 and 88). ¶ NBW = ≥ 2500g (n=106 and 129). Only infants with known birthweight were included in subgroup analysis.

Likewise, low-birthweight infants in the zinc group had significantly fewer days ill with acute diarrhoea and impetigo than those in the placebo group, whereas no differences between the groups were seen in the normal-birthweight infants (table 3).

In low-birthweight infants born at term but with intrauterine growth retardation, we recorded significant reductions in zinc versus placebo groups for acute diarrhoea (risk ratio 0·59; 95%CI: 0·44-0·79), all diarrhoeal episodes (0·61; 95%CI: 0·46-0·81) and impetigo (0·27; 95% CI: 0·10-0·76) whereas no differences between the groups were seen in preterm low-birthweight infants (< 37 weeks' gestation n=46), for acute diarrhoea (RR: 1·19 {0·68-2·10}, for all diarrhoea (1·10; 0·66-1·83) or for impetigo (0·95; 0·22-4·10).

DISCUSSION

In our study population, zinc supplementation during the last two trimesters of pregnancy reduced the frequency and duration of acute diarrhoea, dysentery and impetigo especially in low-birthweight infants, but had no effect on infant growth during the first 6 months of life. Previously reported results from this study showed that prenatal zinc supplementation did not affect maternal weight gain during pregnancy or infant birthweight in this malnourished population.[11] 181 (43%) of our infants had low birthweight (< 2500g) and were, therefore, at increased risk of malnutrition and premature mortality mainly because of diarrhoea and respiratory tract infections. [6,18] The improvements in morbidity were substantial, (32% and 74% reduced risk of acute diarrhoea and dysentery, respectively), and greater than the 18% reduction in frequency of diarrhoeal disease attributed to zinc supplementation during childhood.[3] The reductions in our study were also larger than the 28% in number of days ill due to diarrhoea reported with infant zinc supplementation in an intervention study in low-birthweight, full-term infants in Brazil.[4]

The improvements in morbidity in low-birthweight infants were due to reductions in intrauterine-growth-retarded and not preterm low-birtweight infants, although sample sizes for premature infants were too small for firm conclusions. Infants who are full-term with intrauterine growth retardation and those who are premature show different patterns of growth, morbidity, and mortality. Premature infants have better growth potential and gradually approach a normal weight.[7] By contrast, intrauterine-growth-retarded infants who receive optimum feeding achieve a limited catch-up growth only in the first few months of life.[19]

We noted effects on both frequency and duration of diarrhoeal diseases and impetigo, perhaps through enhancement of immune function *in-utero*, as suggested by results in animals. Antenatal zinc deprivation in rats resulted in low immunoglobulin serum concentrations, especially IgA, IgG_2 and IgM in offspring at 6

months of age; these abnormalities can persist into adulthood.[13] Maternal zinc status during pregnancy might also affect *in-utero* acquisition of antibodies, because of the importance of zinc in placental transport.[20]

Zinc supplementation in infants and children is known to have beneficial effects on both prevalence and incidence of diarrhoeal diseases and pneumonia. These effects are thought to be attributable to improved immunity and intestinal mucosal regeneration and function, including decreased intestinal permeability.[3] The effects of zinc supplementation on impetigo might also be attributable to improved epidermal barrier function.[21]

Maternal zinc supplementation in our study population had an effect on postnatal morbidity but not on infant birthweight or postnatal growth. Maternal protein and energy supplementation in East Java also benefited infant growth but did not affect birth weight.[22] However, trials in Mexico[23] and Jamaica[24] of infant rather than prenatal zinc supplementation resulted in reduced infant morbidity and hospital admissions, but had no effect on infant growth. Results from a study in Peru also suggest that zinc supplementation during pregnancy has no effect on birthweight, but improves maternal and infant zinc status,[25] and might have enhanced immunity, as shown by increased concentrations of immunoglobulins in cord blood.

The effect of zinc on growth might be attributable to a direct role of zinc in protein synthesis and gene expression,[2] but may also result secondarily from reductions in morbidity and an increase in appetite.[26] In our study the 6-month follow-up might have been too brief to detect this effect.

Findings from studies of zinc supplementation during childhood have shown a small effect on growth, especially in children with low height for age or low plasma zinc concentrations.[2,26] By contrast, in undernourished populations in Mexico,[23] Jamiaca,[24] and Uganda,[25] zinc did not have an effect on infant growth. Zinc might not have been the primary growth-limiting nutrient in these populations, or in our study population.

Serum zinc concentrations at 1 and 6 months of age in our population were similar to those recorded in older infants and children in Ethiopia and Chile;[26,28] however, they were higher than those for children in India[29] and for infants who were small for gestational age in Chile.[5] Mothers in our study had high serum zinc concentrations during pregnancy. Although serum zinc is not a good measure of body zinc status,[30] these concentrations suggest that our infants were not profoundly zinc deficient. This possibility could account for the absence of a growth effect of zinc in our population. Most infants in our study were predominantly or partly breastfed. In more-developed countries, zinc deficiency is rare in full-term breastfed infants during the first few months of life.[31]

Our results suggest that infants of mothers supplemented with zinc during pregnancy are still born small, but might have a less compromised immune system

than those of mothers who are not supplemented. Low birthweight, therefore, might be mainly an indicator of risk rather than direct cause of morbidity and mortality. In this respect, a small neonate would not be as disadvantaged if it were not for the adverse health outcomes later in life that are associated with low birthweight.[7] In intervention studies with the aim of reducing the frequency of low birthweight, investigators have restricted their assessments mainly to an effect on birthweight rather than the consequences of low birthweight.[10] Our findings suggest that future antenatal intervention studies should continue observations beyond the neonatal period.

Our results show a positive and clinically relevant effect of maternal zinc supplementation during pregnancy on infant morbidity, especially in low-birthweight infants, and might potentially contribute to a reduction in the disadvantaged health outcomes of these infants. Our findings could have important implications for child health and survival programs in less-developed countries and, consequently, consideration should be given to the addition of zinc to regular antenatal supplements in regions with a high prevalence of low birthweight.

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5

The effect of zinc supplementation during pregnancy on immune response to childhood vaccines in Bangladesh.

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ABSTRACT

Background: An essential role for zinc in development of the fetal immune system has been documented. However, the effect of antenatal zinc supplementation on infants' postnatal immune response to vaccinations is unknown.

Objective: To evaluate the effect of zinc supplementation during pregnancy on immune response to tuberculin skin test and the combined diphtheria, tetanus toxoid and pertussis (DTP)-*Haemophilus influenzae* type-b (Hib)- conjugate vaccine in poor Bangladeshi infants.

Design: We immunized 405 infants whose mothers were supplemented daily with 30mg elemental zinc or placebo beginning at 12-16 weeks gestation with the standard BCG vaccine at birth. A subcohort of 203 infants was in addition immunized at one month-intervals with three doses of DTP-Hib vaccine starting at 9 weeks of age. The delayed hypersensitivity (PPD) skin test was performed in 345 infants at 24 weeks of age. Hib polysaccharide (PRP) antibodies were assessed for 91 infants at 4 and 24 weeks of age.

Results: In infants born with low birth weight (LBW) a lower proportion of negative responses to PPD skin test were observed in the zinc (66.2%) compared to placebo (78.5%) group (p=0.07). No differences were observed in normal birth weight infants. There were no differences in proportion of infants above the protective thresholds for anti-PRP antibodies between zinc (81%) and placebo (89%) group. Geometric mean PRP antibody titres at 4 and 24 weeks of age were not different between groups.

Conclusions: Zinc supplementation during pregnancy did not enhance immune response to Hib-conjugate vaccine but improved the delayed hypersensitivity immune responses to BCG-vaccine in Bangladeshi LBW infants.

INTRODUCTION

The response to childhood vaccinations varies in different populations. For example, the immune response to the pure polysaccharide *Haemophilus influenzae type b* (Hib) vaccine of Native American populations in the USA is known to be lower than in the general US population.[1] Similarly, the immunogenicity of three doses of live attenuated, trivalent oral polio vaccine (TOPV) is lower in developing countries than in industrialized countries[2] and the effect of prior bacillus Calmette-Guerin (BCG) vaccination on the tuberculin skin test is also known to vary in different populations.[3]

Malnutrition and the occurrence of several micronutrient deficiencies are one important explanation for the depressed immune responses to vaccines in infants of developing countries. Undernourished infants have impaired immunity[4] and several micronutrients, of which zinc is among the most notable, are known to affect the immune system in characteristic ways.[5]

The essential role for zinc in the non-immune and immune host defenses has been demonstrated.[5,6] In zinc deficiency, impaired epithelial barrier functions, atrophy of lymphoid organs, a reduced number of T- and B-lymphocytes, and decreased macrophage functions have been observed,[7] as well as depressed

antibody responses.[8] A depressed delayed hypersensitivity reaction resulting in anergy[6] and decreased $CD4^+$ lymphocytes have been found in zinc deficient children which was reversed after zinc supplementation.[9]

Maternal zinc deficiency during gestation may contribute to an impaired immune function in the offspring. In animal models perinatal zinc deficiency resulted in impaired development of the fetal immune system, including a reduced antibody response to certain bacterial antigens. These abnormalities might even persist into adulthood.[5] Maternal zinc status may also affect *in-utero* acquisition of antibodies due to the role of zinc in placental transport.[10]

Very little information exists on the effect of maternal zinc status or antenatal zinc supplementation in humans on infants' immune response. While there is a report of zinc supplementation during pregnancy in Peru, resulting in a 35% increase of IgG3 levels in cord blood,[11] we are not aware of any reports on the effect of maternal zinc supplementation on infants' postnatal immune response to childhood vaccines.

We therefore evaluated the effect of maternal zinc supplementation in Bangladeshi urban poor women during the last two trimesters of pregnancy on humoral sero-response of their infants, using *Haemophilus influenzae* type b conjugate vaccine as a marker and on the tuberculin skin reaction to BCG as a marker for cell mediated immune response.

SUBJECTS AND METHODS

Study design and study population

This study was a nested study within a community-based, randomized controlled trial, to evaluate the effect of zinc supplementation during pregnancy on birth weight and infant morbidity from infectious diseases.[12, 13] Details on the study population, intervention and data collection procedures have been reported elsewhere.[12] The study was approved by the Ethical Review Committee of ICDDR,B and the Committee on Human Research of the Johns Hopkins School of Hygiene and Public Health.

A total of 559 women from selected areas of Dhaka city slums were enrolled between 12 and 16 weeks gestation after obtaining written informed content. Enrolled mothers were stratified by parity and randomly assigned to two treatment groups, to receive either 30 mg elemental zinc per day (twice the RDA, as zinc acetate) or a cellulose placebo. Individual randomization was achieved by a computer-generated random letter assignment and the codes remained unknown to both investigators and participants until the study was completed. The supplements were prepared in bubble packs of ten tablets each (ACME Ltd, Dhaka)

and zinc content of both zinc and placebo tablets were independently confirmed by two different laboratories. Compliance with tablet consumption was 86%, as assessed by counting the remaining tablets in the bubble packs during unannounced home visits. Supplementation continued until delivery.

A total of 420 singleton infants born to these women were included in the postnatal surveillance and followed to 24 weeks of age. Information on morbidity was collected weekly by mother's recall, infant anthropometrics were measured monthly and serum zinc was assessed at 4 and 24 weeks of age.[13] Prior to the onset of the nested study, we had calculated required sample sizes to detect differences between treatment groups with 80% power and a type 1 error 5%. There was no prior data available on the immune response to *H. influenzae* type b vaccines in developing countries. We however assumed a lower immune response in the Bangladeshi infants and estimated that approximately 25% of Bangladeshi infants (25% less than the general US population) would have an antibody level of ≥ 0.15 μg/ml to PRP if they were given three doses of the vaccine. We also assumed that with zinc supplementation, their response would be the same as the US infants. Anticipating that 15% would not complete the follow-up a required sample size of 75 was calculated to be enrolled in each group.

Immunization

A total of 405 infants (194 in zinc, 211 in placebo groups respectively) were vaccinated with 0.05 ml reconstituted live freeze-dried vaccine of Bacille Calmette Guerin (BCG) by study physicians within 72 hours after birth.

A subcohort of 203 infants (96 in zinc, 107 in placebo groups respectively) who had not yet reached the age of 12 weeks at the time of the onset of the nested study, were in addition vaccinated with the combined diphtheria, tetanus toxoid and pertussis (DTP)- *Haemophilus Influenzae* type b (Hib) vaccine (Diphtheria CRM, 97 protein conjugate), [TETRAMUNE® Wyeth-Lederle, NY] at 9, 13 and 17 weeks of age (± 3 weeks). The second and third round of vaccinations were given at 4 week intervals (± 2 days). Infants were given the trivalent oral polio vaccine (TOPV) simultaneously.

Post-vaccination safety surveillance was carried out from day 1 through day 5 post-vaccination during home visits by trained study nurses. Data was collected and recorded daily on rectal temperature, local swelling and redness, generalized rash, seizures, general health of the infant, feeding history and any other unexpected reactions.

Assessment of cell mediated immune response.

Cell mediated immune response was tested at 24 weeks of age by delayed hypersensitivity skin test (DTH) using the purified protein derivate (PPD) test (Staten Serum Institute, Copenhagen, Denmark). Tuberculin solution (0.1 ml) was

applied by trained nurses to the volar surface of the forearm intradermally using an Omega glass PPD syringe with platinum needles. After 72 hours the size of the induration in millimeters developed was read as the transverse diameter. A cutaneous reaction was considered positive when an induration of > 5mm was observed.[14] Six nurses were extensively trained and standardized in the administration and interpretation of PPD skin testing. Reproducibility of skin test reading was tested prior and during the study in hospitalized patients with tuberculosis attending the Institute of Chest Diseases, Chest Hospital, Dhaka. Variability between reading by the same nurse and between the different nurses was measured. Overall, Pearson correlation coefficients were high, ranging between 0.98 ($p < 0.000$) and 0.73 ($p \approx 0.03$) and consistent with inter-rater agreements observed in similar studies.[15]

Assessment of humoral immune response and serology

Antibody response to *H. influenzae* b polysaccharide (polyribosylribitol phosphate (PRP)) was assessed pre-vaccination at 4 weeks of age and post-vaccination at 24 weeks of age. Non-fasting blood specimens (5 ml) were obtained by antecubital venipuncture in the morning hours. Serum was separated a maximum of 6 hours after collection and stored at -20C until analysis. Antibodies to PRP were measured by enzyme linked immunosorbent assay (ELISA). All assays were performed at the laboratory of Wyeth Lederle Vaccines (NY, USA) using standard procedures.[16] Geometric mean antibody titres were calculated as the antilog of the mean of the logarithms of titres. Mean paired differences between pre- and post-immunization titres and percentage of infants with PRP antibody levels ≥ 0.15 μg/ml, associated with immediate protection,[17] and ≥ 1.0 μg/ml, associated with long-term protection[18] were calculated.

Statistical methods

Differences between groups in proportion of anergic infants from the Tuberculin skin test and proportion of infants with antibody titres above the protective thresholds before and after immunization were assessed with the Chi-square test and Mac Nemar's test respectively. Differences between treatment groups in mean size of induration, geometric mean antibody titres and paired differences between pre and post immunization titres were assessed with the Mann-Whitney U-test.

Analysis of Covariance (ANCOVA) and Logistic Regression Analysis were performed to control for potential confounders (SPSS7.5 FOR WINDOWS; SPSS Inc, Chicago). Separate models were made based on subgroups for birth weight and gender. P-values of <0.05 were considered statistically significant.

RESULTS

Description of the study population.

Of the 405 singleton infants (194 in zinc and 211 in placebo group) who were born to mothers from the trial of zinc supplementation in pregnancy and immunized with the BCG vaccine at birth, 39 (21 or 10.8% in zinc-supplemented and 18 or 8.5% in placebo- supplemented group) were lost-to-follow-up before the end of the study due to the following reasons: infant deaths (n=10), migration from the study area (n=11) or refusal to further participate (n=18). Reasons for lost-to-follow-up were not different for infants from zinc and placebo groups. An additional 7 infants could not receive the PPD skin-test at 24 weeks of age because they were sick or absent at the time of the test. Therefore, 359 infants (167 zinc, 192 placebo) were administered with the PPD skin test at 24 weeks of age. For 14 infants, skin test readings could not be performed within 72 hours, thus, the final sample size for the effects on the tuberculin skin test consisted of 345 infants (163 in zinc , 182 in placebo group). A comparison of baseline characteristics (table 1) revealed that infants included in the tuberculin skin test had significantly greater body weights and larger gestational age at birth compared to infants who were lost-to-follow-up. In the multivariate analysis we therefore controlled for infant's birth weight and gestational age.

A sub-cohort of 203 infants (96 in zinc and 107 in placebo supplemented group) were eligible for inclusion in the Hib-immunization trial since they had not yet reached the age of 12 weeks. A total of 27 infants (16 or 16.7% in zinc supplemented and 11 or 10.3% in placebo group) were lost-to-follow-up before the end of the study for the following reasons: infants deaths (n=5), migration from the area (n=8), refusals (n=10) and infants who had received immunizations through another source (n=4). There were no differences between treatment groups in reasons for lost-to-follow-up. A total of 176 infants (80 in zinc and 96 in placebo group) completed the course of three doses of the combined diphtheria, tetanus toxoid and pertussis (DTP)- *Haemophilus influenzae* type b (Hib) vaccine and provided pre- and post-vaccination serum samples for immune assays. Median ages at pre- and post-immunization blood sampling were 4.0 and 24.1 weeks respectively and median ages at the three rounds of immunization were 9.6, 13.9 and 18,1 weeks. Serum samples of 85 infants were insufficient for analysis resulting in a final sample of 91 infants (38 zinc, 53 placebo group) for analysis of Hib-response.

Compared to infants who were lost-to-follow-up for the Hib assays, the 91 infants who were included in the Hib-trial had significantly higher gestational ages at birth (table 1) and their mothers had significantly lower Body Mass Indexes (BMI) at baseline (i.e., 4 months gestation). We controlled for those variables in the multivariate analysis.

Table 1. Selected baseline and birth characteristics for mothers at 4 months gestation and infants included in analysis on immune response or lost-to-follow-up for analysis on immune response for 405 infants enrolled in BCG trial and 203 infants enrolled in DTP-Hib trial.

	Infants lost for PPD skin test (n=64)	Infants included for PPD skin test (n=345)	Infants lost for Hib-immune assay (n=112)	Infants included for Hib-immune assay (n=91)
Infants:				
Birthweight (g)	2427 (484)[1]	2558 (367)*	2534 (404)	2536 (351)
LBW (%)[2]	54.1	40.6*	43.1	42.7
Gestational age (wk)	38.0 (2.8)	39.0 (2.1)*	38.6 (2.3)	39.7 (1.9)#
IUGR (%)[3]	75.7	73.7	71.9	82.0#
Gender: male (%)	57.3	53.3	54.4	52.7
Mothers:				
Age (yr)	23.1 (5.6)	23.0 (5.5)	23.0 (5.5)	23.3 (5.7)
Nulliparous (%)	24.0	21.7	21.6	24.2
Socioeconomic status Poor/very poor (%)[4]	81.3	76.5	79.0	71.5
BMI[5]	18.9 (2.8)	19.0 (2.4)	19.1 (1.6)	18.4 (1.9)#
MUAC[6]	224 (27)	227 (23)	228 (25)	223 (21)
Hemoglobin (g/L)	112 (13)	115 (13)	114 (13)	116 (12)
Serum zinc (µmol/L)	15.1 (3.8)	15.5 (4.6)	15.6 (4.7)	14.7 (3.1)
Treatment: zinc (%)	48.0	47.2	48.9	41.8

[1] Values in mean (SD)
[2] LBW= low birth weight (<2500 g)
[3] IUGR = Intra Uterine Growth Retarded (<10% of fetal growth chart; 13)
[4] Based on an index of household assets (13)
[5] BMI= Body Mass Index
[6] MUAC= Mid Upper Arm Circumference
* Different from infants dropped for PPD (purified protein derivate) skin test ($p<0.05$)
# Different from infants dropped for Hib (*Haemophilus influenza* type b)-immune assays ($p<0.05$)

Effect of zinc supplementation on cellular immune response.

At 24 weeks of age, 60.9% (210 of 345) of all infants showed a negative tuberculin skin response (induration of ≤ 5 mm) in response to the PPD skin test. There were no differences in proportion of negative responses between treatment groups (zinc: 58.9%; placebo: 62.6%; table 2). A significantly higher percentage of infants born with low birth weights (LBW; <2500 g) showed negative skin responses compared to infants born with normal birth weights (NBW; 71.9% vs. 52.7% for LBW and NBW groups respectively, $p<0.0001$).

Table 2. Cellular immune response to tuberculin (PPD) skin test at 24 weeks of age for infants by birth weight in zinc and placebo groups.

	Zinc group	Placebo group
Anergic (%)[1]		
All infants[2]	58.9	62.6
LBW[3]	66.2	78.5*
NBW[4]	52.3	53.0
Mean size of PPD-induration (mm)[5]		
All infants	11.7 (0.4)	11.8 (0.4)
LBW	11.7 (0.6)	11.2 (0.9)
NBW	11.8 (0.5)	12.0 (0.4)

[1] Anergic = size of PPD induration < 5 mm
[2] All infants (n=163/182)
[3] Low birth weight infants (<2500 g, n=74/65)
[4] Normal birth weight infants (n=87/116)
[5] Mean size of induration of positive responses (SEM)
* Different from zinc supplemented group (p=0.07; Logistic regression controlling for birthweight and gestational age)
PPD=purified protein derivate tuberculosis

When data were analysed separately for 139 LBW-infants, fewer infants in zinc compared to placebo group were not responsive to the tuberculin skin test (66.2% vs. 78.5%) with the difference being of marginal statistical significance (p=0.07). No differences in response to the tuberculin skin test were observed between treatment groups for the 203 normal birth weight infants, or for infants born premature (n=50) or at-term (n=295). The mean size of induration among the positive responses was 11.8 mm (SEM 0.4) and did not differ for LBW and NBW infants of zinc and placebo groups (table 2).

Effect of zinc supplementation on response to Hib conjugate vaccine.

Geometric mean PRP antibody titres before and after immunization and mean paired differences between titres pre- and post-immunization are shown in table 3. There were no statistically significant differences in geometric mean PRP titres at 24 weeks of age (post-immunization) between infants from zinc (6.78 μg/ml; 95% CI: 3.36;12.01) and placebo groups (10.15 μg/ml; 95% CI: 6.29;16.14).

Table 3. Geometric mean PRP antibody titres pre- and post-immunization for infants in zinc and placebo group[1]

	Zinc group (n=38)	Placebo group (n=53)
Pre-immunization (μg/ml)[2]	0.22 (0.15;0.33)	0.21 (0.15;0.29)
Post-immunization (μg/ml)[3]	6.78 (3.36;12.01)	10.15 (6.29;16.14)

[1] Geometric mean titres calculated as the antilog of the mean of the logarithms of values (values in brackets: 95% confidence intervals)
[2] Pre-immunization at 4 weeks of age
[3] Post immunization at 24 weeks of age
PRP=polyribosylribitol phosphate.

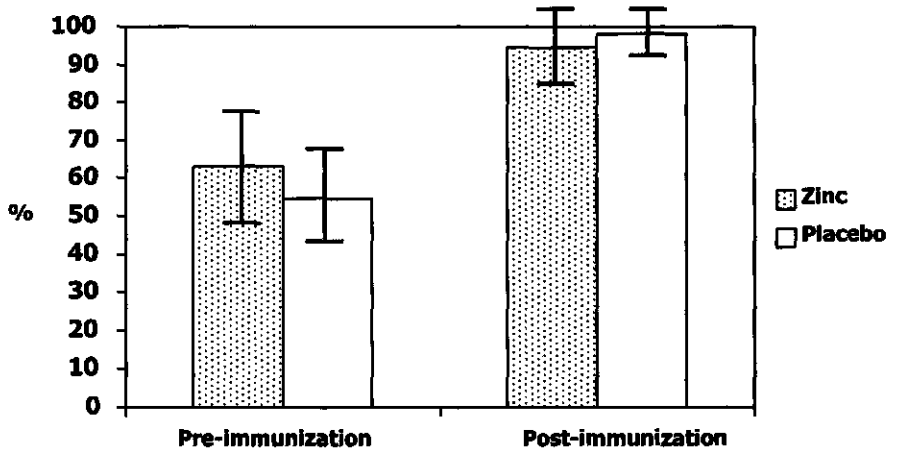

Figure 1. Proportion of infants with PRP antibody titres > 0.15 μg/ml pre- and post-immunization (and 95% confidence intervals) for zinc and placebo groups.

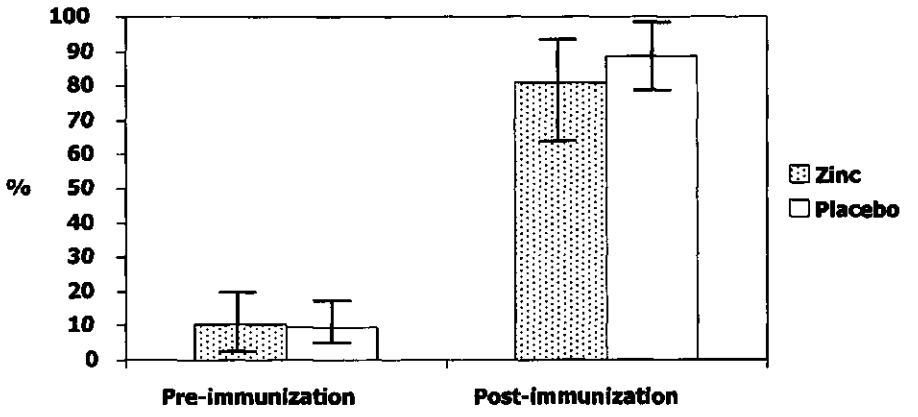

Figure 2. Proportion of infants with PRP antibody titres > 1.0 μg/ml pre- and post-immunization (and 95% confidence intervals) for zinc and placebo groups.

Nearly all infants (95% in zinc and 98% in placebo supplemented group, difference NS) achieved protective antibody titres correlated with immediate protection (≥0.15 μg/ml; figure 1). Furthermore, the large majority (81% in zinc and 89% in placebo group, difference NS) achieved titres ≥ 1 μg/ml associated with long-term protection (figure 2). No differences were observed between treatment groups in geometric mean PRP antibody titres for LBW (n=38) and NBW (n=51) infants but sample sizes were small.

Infants from zinc supplemented mothers had significantly fewer days of fever (>38.0 C) during post-immunization safety surveillance compared to infants from placebo supplemented mothers (means (SEM): 0.98 (0.11) vs. 1.23 (0.12); p<0.05). No other differences in potential adverse effects following DTP-Hib immunization were observed between treatment groups (table 4).

Table 4. Adverse events in the 5 days following immunization for three rounds of DTP-Hib vaccine for infants in zinc and placebo group.

	Zinc group (n=89)	Placebo group (n=94)
Swelling/redness at injections site		
- n (%)	84 (94.6)[1]	90 (96.1)
- days	4.27 (0.27)[2]	3.95 (0.26)
Crying more than usual		
- n (%)	89 (100)	93 (99.0)
- days	4.01 (0.20)	3.97 (0.20)
Less active		
- n (%)	80 (90.2)	85 (90.2)
- days	2.57 (0.29)	2.36 (0.16)
Lethargic		
- n (%)	13 (14.1)	13 (13.7)
- days	0.21 (0.06)	0.21 (0.07)
Fever measured		
- n (%)	56 (62.9)	66 (70.2)
- days[3]	0.98 (0.11)	1.23 (0.12)*

[1] Number (Proportion) of infants showing the event on at least one day [2] Values in means (SD)
[3] Fever: rectal body-temperature > 38°C * p < 0.05; Student's t-test

DISCUSSION

Zinc supplementation with 30 mg elemental zinc/day during the last two trimesters of pregnancy resulted in a positive albeit small effect on the response to the delayed hypersensitivity skin test on BCG vaccine at 24 weeks of age in infants born with low birth weight (LBW). No differences were observed among infants born with normal birth weights (NBW). There were no differences in antibody responses to the *Haemophilus influenzae* type b (Hib) vaccine between the groups. However, fewer days with fever were observed in the 5 days post-Hib-vaccination in infants born to zinc-supplemented compared to placebo-supplemented mothers.

We previously reported results from the same cohort of infants demonstrating that maternal zinc supplementation during pregnancy did not improve birth weight but reduced episodes of acute diarrhea, dysentery and impetigo during the first six months of life among LBW infants.[13] The prevalence of LBW in Bangladesh is among the highest in the world and in our study a total of 43% of all live births were LBW.[12] LBW infants are known to be at-risk for impaired immunity and increased morbidity later in life.[19] The results of this current study suggest that an improved cellular immunity might be a possible mechanism for the observed reductions in morbidity among LBW infants after maternal zinc supplementation.

Beneficial effects of zinc supplementation on indicators of cellular immune response such as the delayed hypersensitivity skin response to PPD or CMI, number or functioning of circulating T-lymphocytes and thymus size have been reported previously in studies supplementing infants and children.[9, 20-23] Animal models of zinc deficiency suggest that improved delayed-type hypersensitivity after zinc repletion may be caused by a restored interaction between effector T-cells and macrophages.[7] Zinc deficiency is also known to be associated with atrophy of lymphoid organs and a reduced number of T-lymphocytes in animal models, all of which respond positively to a repletion of zinc.[5]

Zinc deficiency has in addition been shown to affect elements of the immunologic memory as reduced T-dependent and, to lesser extent, T-independent antibody responses have been observed in zinc deficient mice.[8] Antenatal zinc deprivation in rats resulted in lower immunoglobulin serum concentrations, in particular IgA, IgG2 and IgM in the off-spring at 6 months of age.[5] Evidence for an effect of zinc supplementation on humoral immune response in humans is less and not conclusive. Increased serum IgA and salivary IgA concentrations have been observed after zinc supplementation in two studies among Chilean infants.[20,21] However, serum IgG and IgM concentrations were not affected by zinc supplementation among malnourished infants in Chile.[20] Similarly, no effects of zinc supplementation on salivary IgA were observed in young rural Gambian children.[24]

We observed a lower number of febrile days during the first five days post-immunization after maternal zinc supplementation. Negative correlations between plasma zinc and number of febrile days have been observed previously in malnourished Chilean infants.[20] These findings may be the result of a depressed acute phase response mediated by cytokines during zinc deficiency.[25] Zinc repletion is expected to restore these functions.

In our study we did not observe effects of maternal zinc supplementation on infants' antibody response to the Hib vaccine. The polysaccharide Hib-conjugate vaccine that we used in our study contains a T-dependent antigen (HbOC) that induces an anamnestic response after repeated infections and is predominantly of

the IgG class.[16] We had anticipated a beneficial effect of zinc supplementation for this specific antigen since it is known that T-dependent B-lymphocytes are more affected by zinc deficiency than T-independent ones.[5] Therefore our findings are somewhat unexpected. Our sample sizes were small and only sufficient to detect a difference of 4.6 μg/ml in PRP titres at 24 weeks or a difference of 74% in proportion of post-immunization titres above the protective threshold (≥ 1 μg/ml) between treatment groups assuming 80% power and a type I error of 5%.

The Hib-conjugate vaccine that was used in this study has been shown to be more immunogenic than the unconjugated pure polysaccharide vaccine. In addition, the Hib conjugate vaccines, unlike the pure polysaccharide vaccines are known to reduce Hib carriage in the upper respiratory tract, especially among younger children in the US and The Gambia.[26,27] The highly potent conjugate vaccine may have induced immune response in all our infants and therefore overcome any blunting of immune response resulting from zinc deficiency.

In our population, geometric mean PRP antibody concentrations post-immunization were 7.89 μg/ml which is substantially higher than levels observed in infants in developed countries such as the UK (3.65 μg/ml) after immunization with a similar conjugate vaccine.[28] Although our sample sizes were small, it is possible that frequent exposure to the *Haemophilus influenzae* type b-antigen even at this young age, may have caused an anamnestic response if the child came in contact with the organism after receiving the primary series of the vaccine. Recent studies confirm that the incidence of invasive *Haemophilus influenzae* type b (Hib) diseases are high in South Asia with an estimated 50 cases per 100.000 compared to 22-109 per 100.000 in pre-vaccination USA and Europe.[29] Moreover, a 700% increase in Hib incidence over the period 1987-1994 has been reported from a hospital in Bangladesh.[30] We hypothesize therefore that high infection rates of *Haemophilus influenzae* type b in these settings may have contributed to the humoral immune response to the vaccine in nearly all our infants which may have masked any potential beneficial effects of zinc supplementation.

Prior to immunization at the age of 4 weeks, 58% of our infants had PRP antibody concentrations of ≥ 0.15 μg/ml compared to only 30% observed in UK infants of similar age.[28] At 4 weeks of age, concentrations primarily reflect maternal Hib-specific immunoglobulins and the high maternal antibody concentrations in our population are another indication of a high degree of exposure to Hib organism.

In conclusion, in our cohort of poor Bangladeshi infants we were not able to demonstrate an effect of maternal zinc supplementation on the immune response to *Haemophilus influenzae* type b vaccine. To our knowledge this study is the first to report the effect of maternal zinc supplementation on infant's immune response to vaccines, but our findings seem to be in contrast with the observed improvements in neonatal humoral immunity by increased cord-blood

immunoglobulin concentrations after zinc supplementation during pregnancy in a similar trial in Peru.[11] Additional research is required to define the exact role of zinc in the development of the immune system in infants in developing countries.

Finally, the findings of our current study suggest that an improved cellular immune response may have caused observed reductions in morbidity among infants born with LBW after maternal zinc supplementation during the last two trimesters in pregnancy.[13] LBW infants are known to have impaired immunity and an increased burden of infectious diseases during infancy,[19] and maternal zinc supplementation might be an effective way to reverse some of these disadvantageous outcomes.

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6

The effect of zinc supplementation between 1 and 6 months of life on growth and morbidity of Bangladeshi infants in urban slums.

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ABSTRACT

Background: Evidence for an effect of zinc supplementation on growth and morbidity in very young infants in developing countries is scarce and inconsistent.

Objective: To assess the effect of zinc supplementation from 4 to 24 weeks of age on growth and morbidity in poor Bangladeshi infants.

Design: Infants of Dhaka slums were enrolled at 4 weeks of age and randomly assigned to receive daily 5 mg elemental zinc (n=152) or placebo (n=149) until 24 weeks of age. Infants were followed weekly for information on compliance and morbidity while anthropometric measurements were performed monthly. Serum zinc was assessed at baseline and 24 weeks of age.

Results: At 24 weeks of age serum zinc levels were higher in the zinc compared to placebo supplemented infants (13.3 (SD 3.8) vs. 10.7 (2.9)μmol/L;p<0.001). Significantly greater weight gains were observed in zinc group compared to placebo group for 43 infants who were zinc deficient (<9.18μmol/L) at baseline (3.15 (0.77) vs. 2.66 (0.80) kg, p<0.04). In the other infants no differences were observed in mean weight and length gains between 4-24 weeks. Zinc-deficient infants also showed a reduced risk for incidence of Acute Lower Respiratory Infection (ALRI) after zinc supplementation (RR:0.30;95%CI:0.10,0.92) whereas among the non-zinc deficient infants there were no differences between treatment groups.

Conclusions: Improvements in growth rate and reduced incidence of ALRI were observed in zinc-deficient Bangladeshi infants after zinc supplementation. However, among non-zinc deficient infants there were no improvements in growth or morbidity, although zinc supplementation improved serum zinc status.

INTRODUCTION

Zinc deficiency has been associated with reduced growth, impaired immunity and increased prevalence and incidence of infectious diseases among infants and children in developing countries.[1-3] Evidence for a causal relationship with morbidity has been provided by randomized controlled intervention trials in infants above 6 months of age and children in both industrialized and developing countries showing improved immune functions[4,5] and reduced morbidity[2] after zinc supplementation. These effects are thought to be more attributable to a correction of the zinc deficiency status that causes an impaired immunity and intestinal mucosal damage[6] rather than to a pharmacologic effect of zinc[7]. Evidence for an effect of zinc supplementation on growth of children came from a recent meta-analysis concluding that zinc supplementation during childhood is responsible for a small but statistically significant effect on growth particularly among growth retarded children.[1]

Most of the intervention trials studying the effect of zinc on growth or morbidity were performed in children above 6 months of age when the period of highest growth velocity has already passed. It has been hypothesized that earlier interventions might be more effective in preventing growth faltering and reducing

morbidity patterns among children at-risk.[8] Unfortunately, only limited information is available on the effect of zinc supplementation in younger infants and the results are not conclusive. Beneficial effects on growth and morbidity were observed after zinc supplementation among low birth weight (LBW) and small for gestational age (SGA) infants in Brazil[9] and Chile[10] respectively. However, in India no effect of zinc supplementation on diarrheal morbidity was observed in children among 6-11 months of age in contrast to the strong beneficial effects observed among older children.[11] Different prevalences of zinc deficiency among younger age groups caused by varying rates of prematurity, intrauterine growth retardation (IUGR), exclusivity of breast-feeding and early morbidity may explain the varying responses to zinc supplementation in very young infants.

To investigate the effect of zinc supplementation on growth and morbidity from infectious diseases in infants below 6 months of age, we performed an intervention trial among 4-24 weeks old Bangladeshi infants living in Dhaka urban slums. Because the incidence of low birth weight among this population is as high as 43%, which is mostly (76%) due to IUGR[12], a large proportion of these infants can be considered to have impaired immunity, increased morbidity and be at-risk of growth faltering later in life.[13]

SUBJECTS AND METHODS

Study population

The study was performed in three selected areas of Dhaka city slums, in a representative sample of households from Dhaka's slum population.[14] The slum areas are characterized by high population density, poor housing, multi-family latrines and water sources, poor sewerage and drainage facilities and irregular garbage collection. The population is relatively young and mostly illiterate.[15] Malnutrition is known to be widely prevalent and the prevalence of malnutrition among children of the urban slums is even higher than among children of the rural poor.[16]

Singleton infants were eligible for inclusion in the study if they met entry criteria of age (4 ± 1 week) and residence, were in good general health without known underlying illnesses, were not included in any other intervention trial and/or had not received immunizations through other sources. A total of 301 infants aged 3-5 weeks (152 in zinc and 149 in placebo supplemented groups) were identified through an established household-surveillance system and enrolled in the study between April and June 1997. Prior to enrollment, a written informed consent was obtained from the infant's parents.

The study was approved by the Ethical Review Committee of ICDDR,B and the Committee on Human Research of the Johns Hopkins School of Hygiene and Public Health.

Study design

Infants were randomly allocated to a liquid 5 mL daily dose with or without 5 mg elemental zinc (as zinc acetate). Both liquids contained sucrose, flavors and preservatives, were indistinguishable in both appearance and taste and were prepared and coded by Opsonin Chemical Industries Ltd. Dhaka. The zinc content of both supplement and placebo was independently confirmed by two different laboratories. Health workers delivered a bottle containing a one-week supply of 40 ml of the supplement at the houses of the participants and instructed mothers to give a daily dose to their infants using a marked dropper or feeding spoon. Compliance with supplement consumption was 85% (of total days) and the average daily consumption was 4.2 (SD 1.3) ml as assessed by measuring liquid levels at the weekly visits. Unannounced compliance checks between the regular visits were also performed monthly in a 10% subsample of the population. No differences in compliance were observed between the two treatment groups. The codes of the supplements were unknown to both participants and study staff and broken only after data editing and cleaning was completed.

Prior to randomization, information was collected on household's socio-economic status, demographic characteristics, maternal and infant anthropometry, characteristics of the delivery (place, duration, complications) and infant vaccination history. The infants were provided with the standard immunizations by the study nurses. The Bacille Calmette Guerin (BCG) vaccine was administered at enrollment and infants were immunized with the Trivalent Oral Polio vaccine (TOPV) and the combined diphtheria, tetanus toxoid and pertussis (DTP)-*Haemophilus Influenza type B* (hib) vaccine (TETRAMUNE, Wyeth Lederle Vaccines, NY, USA) at 8, 12 and 16 weeks of age (± 2 weeks). A subsample of 171 infants who had not yet reached the age of 4 months ± 15 days at the time of arrival of the vaccine, received in addition the 7-valent Pneumococcal conjugate vaccine (Wyeth Lederle Vaccines, NY, USA) at 4 weeks (± 2 weeks) intervals.[17]

Infants' serum zinc was determined at 4 and 24 weeks of age. Infants were followed weekly for assessment of compliance and morbidity of diarrheal and respiratory diseases. Information on infant feeding practices was collected every 2 weeks and infants were classified as either exclusively, predominantly (infant receiving breast milk and water/sugar water), or partially breastfed or not breastfed at all. The age of introduction of complementary foods was also determined. Anthropometric measurements (weight, length, arm-, head- and chest-circumference) were performed monthly until the infants were 6 months old.

Data collection procedures

During monthly home visits, infant weight was measured to the nearest 10 g on beam-balance scales (Seca 725) that were regularly calibrated against standard weights. Recumbent lengths were measured to the nearest 0.1 cm on a length board and head-, chest- and arm circumferences were measured to the nearest millimeter using numeral insertion tapes. The mean of two measurements was recorded as the observed value for all indices. Intra- and inter-observer variations were assessed and found to be acceptable with coefficients of variation below 2% for all anthropometric indices. Underweight (low weight-for-age), stunting (low length-for-age) and wasting (low weight-for-length) were defined as standard deviation scores (Z-scores) of < -2.00 compared with the US NCHS reference charts using EPI Info software (version 6.1, 1994, CDC, Atlanta, GA, USA).[18]

A detailed history during the past week of respiratory infections, diarrhea, fever and other illnesses was recorded during weekly home visits. Infants were investigated for signs of dehydration during monthly visits. Infants who required medical treatment were examined by study physicians following standard treatment protocols and referred to appropriate healthcare facilities if necessary. All data were collected by the same trained fieldworkers and manually checked and coded by registered nurses.

Acute lower respiratory infection (ALRI) was defined as a history of cough and/or difficult breathing, with or without fever, lasting more than 1 day and accompanied by at least one of the following symptoms: rapid breathing or chest indrawing. Upper respiratory tract infection (URI) was defined as a) history of cough or difficult breathing, with or without fever, for more than one day not associated with rapid breathing or chest indrawing, or b) cough for more than one day with nasal discharge.

Acute diarrhea was defined as unusually loose and/or unusually frequent stool according to the mother's perception. If the stool contained blood the episode was classified as dysentery. Persistent diarrhea was defined as diarrhea lasting for 14 days or more.

The number of actual surveillance days was counted by subtracting the days on which no recall data were available from the total days of follow-up. When a mother had been absent for more than 15 days (2 consecutive visits), recall data was only collected for the 14 days prior to the first interview after the period of absence. An episode of illness was considered resolved if the child was free of symptoms for at least 3 consecutive days according to definitions used in similar studies.[2,11]

Specimen collection and laboratory procedures

At 4 and 24 weeks of age, non-fasting blood specimens were obtained by antecubital venipuncture in morning hours for serum zinc determination using

trace mineral-free plastic syringes, stainless steel needles and plastic tubes. Serum was separated at a maximum of 6 hours after collection and stored at -20C until analysis. Prior to analysis, the serum samples were diluted (1:12) with 0.03% poly-oxyethylene 4 lauryl ether (Brij 30) and HNO_3 (10 mmol/L). Zinc concentration was measured using flame atomic absorption spectrophotometry[19] (AA-6501S Atomic Absorption Flame Emission Spectrophotometry, Shimadzu, Japan). A standard curve was established using a commercial zinc reference (BDH Laboratory Supplies, UK) in concentrations of 0.1, 0.25, 0.5 and 1.0 mg/l. Commercial serum with known concentrations of zinc were used as a measure of quality control. The coefficient of variation of the measurements was always < 5%.

Statistical methods

Prior to the main analysis, potential confounders that contributed significantly to the variation in outcome variables, were identified by a multiple stepwise regression. Possible interactions between treatment group and infant's sex, baseline nutritional status or serum zinc status were evaluated by introducing separate interaction terms in the linear regression models. Significant interactions between treatment and baseline serum zinc and between treatment and gender were observed for some of the outcome variables. We therefore created separate models based on subgroups for gender and baseline serum zinc < or ≥ 9.18 μmol/L. The cut off for zinc deficiency was selected according to the literature.[2]

Differences in mean and total weight and length gain between zinc- and placebo-supplemented infants were assessed using Analysis of Covariance (ANCOVA) and selected baseline values were added as covariates (SPSS7.5 FOR WINDOWS; SPSS Inc, Chicago). The covariates had been selected prior to the ANCOVA through a multiple stepwise regression identifying variables that contributed significantly to the variation in outcome variable. Differences in anthropometric indices and Z-scores at 1, 2, 3, 4, 5 and 6 months of age were tested using ANCOVA for repeated measurements.

Differences between groups in incidence of diseases were assessed with Poisson regression models (Epidemiological GRaphics Estimation and Testing Package, EGRET, SERC, Seattle, WA98105, 1991) including number of episodes as dependent, treatment group as independent and total days of actual surveillance as off-set term in the model.[20] Potential confounders were added to the regression models as additional independent variables.

Longitudinal prevalence of diseases was calculated for each individual as percentage of actual surveillance days with illness. The non-parametric Mann-Whitney U test was used to compare differences in longitudinal prevalence between infants from zinc and placebo groups. Log transformed values of prevalence were used in the multivariate regression models since the distribution of longitudinal prevalence was highly skewed. Prior to the log-transformation a

constant ('one') was added to all values to enable log-transformation of the zero-values. P values of <0.05 were considered statistically significant. Results are presented as means (standard deviations) unless otherwise indicated.

Table 1. Baseline characteristics of infants in zinc and placebo groups

	Zinc-supplemented[1] (n =152)	Placebo-supplemented[1] (n =149)
Age (months)	0.89 (0.13)	0.90 (0.12)
Gender: male (%)	41%	48%
Socio-economic status[2]:		
-Poor	41%	44%
-Very poor	30%	34%
Weight (kg)	3.47 (0.48)	3.44 (0.50)
Length (cm)	51.3 (2.3)	51.1 (2.1)
MUAC (mm)	104 (9)	104 (9)
Head circumference (cm)	35.6 (1.4)	35.6 (1.3)
Serum zinc (μmol/L)	11.9 (2.9)	11.7 (3.0)

[1]Values in means (SD)
[2] Based on an index of household assets (12)
MUAC, mid upper arm circumference.
None of the differences statistically significant

RESULTS

During the course of the study a total of 31 infants (10%) were lost-to-follow-up (14 (9%) in the zinc supplemented and 17 (11%) in the placebo group; NS). Fifteen out of the 31 infants either permanently or temporarily out-migrated, 8 infants died during the study period, 4 parents refused further participation and 4 infants had received immunizations through other sources and were excluded from further participation. There were no differences in reasons for lost-to-follow-up between the two treatment groups. Infants who were lost-to-follow-up from the study still contributed to the analysis for morbidity for the number of days on which there were observations.

A total of 270 infants (138 in the zinc and 132 in the placebo group) completed the study until 24 weeks of age. This final sample size was sufficient to detect the following differences with 80% power and type I error of 5% between zinc and placebo groups for the main outcome variables: 1.2 μmol/L (10%; SD 3.5) for serum zinc at 24 weeks of age, 0.24 kg (9%; SD 0.7) for increase in body weight

Table 2. *Changes in anthropometric indicators between 4 and 24 weeks of age by baseline serum zinc status for infants in zinc and placebo groups.*

	Zinc-supplemented[1]	Placebo-supplemented[1]
Weight growth (kg)		
-All infants[2]	2.85 (0.73)	2.79 (0.72)
-Serum zinc < 9.18 μmol/L[3]	3.15 (0.77)	2.66 (0.80)*
-Serum zinc ≥ 9.18 μmol/L[4]	2.79 (0.71)#	2.81 (0.72)
Weight growth (g/kg/month)		
-All infants[2]	131 (29)	129 (29)
-Serum zinc < 9.18 μmol/L[3]	144 (29)	130 (33)
-Serum zinc ≥ 9.18 μmol/L[4]	128 (28)#	128 (28)
Linear length growth (cm)		
-All infants[2]	11.5 (1.9)	11.4 (2.1)
-Serum zinc < 9.18 μmol/L[3]	12.5 (2.0)	11.5 (2.2)
-Serum zinc ≥ 9.18 μmol/L[4]	11.3 (1.8)#	11.4 (2.1)
Change in head circumference (cm)		
-All infants[2]	5.1 (1.1)	5.2 (1.1)
-Serum zinc < 9.18 μmol/L[3]	5.9 (1.0)	5.1 (1.2)*
-Serum zinc ≥ 9.18 μmol/L[4]	5.0 (1.1)#	5.2 (1.1)
Change in chest circumference (cm)		
-All infants[2]	6.8 (2.1)	6.6 (2.1)
-Serum zinc < 9.18 μmol/L[3]	7.8 (2.1)	6.7 (2.4)
-Serum zinc ≥ 9.18 μmol/L[4]	6.6 (2.0)#	6.6 (2.1)
Change in MUAC (cm)		
-All infants[2]	2.5 (1.1)	2.6 (1.0)
-Serum zinc < 9.18 μmol/L[3]	2.9 (1.2)	2.5 (1.1)
-Serum zinc ≥ 9.18 μmol/L[4]	2.5 (1.0)	2.6 (1.0)

[1] Values in mean (SD).
[2] All infants, n=138/133 for zinc and placebo groups respectively.
[3] Baseline serum zinc < 9.18 μmol/L (n=21/16).
[4] Baseline serum zinc ≥ 9.18 μmol/L (n=117/115)
* Different from value in zinc supplemented group; $p < 0.05$ (ANCOVA control for gender, baseline length-for-age-Z-score, baseline infant weight, baseline serum zinc, household income).
# Different from value in serum zinc < 9.18 μmol/L group; $p < 0.05$ (ANCOVA control for gender, baseline length-for-age-Z-score, baseline infant weight, baseline serum zinc, household income).
MUAC, mid upper arm circumference

between 4 and 24 weeks of age and 0.7 episodes/child/6 months (12%; SD 0.2) for incidence of acute diarrhea. Baseline characteristics of infants in zinc and placebo group were not different (Table 1). At baseline a total of 43 infants (14%) had serum zinc levels < 9.18 μmol/L (22 (15%) in zinc and 21 (14%) in placebo group; NS).

Serum zinc

At 24 weeks of age, serum zinc concentrations in the zinc supplemented infants were significantly higher compared to infants in the placebo group (13.3

[3.8] vs. 10.7 [2.9] μmol/L; p < 0.001). Serum zinc concentrations significantly decreased between 4 and 24 weeks in the placebo group (-1.1 [4.1] μmol/L) whereas they increased in the zinc supplemented infants (1.5 [4.7] μmol/L, p-value of difference < 0.001). At 24 weeks of age a total of 28 (22%) infants in the placebo group were zinc deficient (serum zinc < 9.18 μmol/L) compared to 12 (9%) infants in the zinc group (p<0.005).

Assessment of growth

No differences were observed for changes in weight, length and head circumferences between 4 and 24 weeks of age for infants from zinc and placebo groups respectively (Table 2). Mean weight and length at 24 weeks of age were 6.33 (0.94) kg and 62.7 (2.6) cm for infants supplemented with zinc and 6.23 (1.01) kg and 62.5 (2.7) cm respectively for placebo supplemented infants (difference NS). Mean length-for-age, weight-for-age and weight-for-length Z-scores between 4 and 24 weeks of age were not different between treatment groups (data not shown).

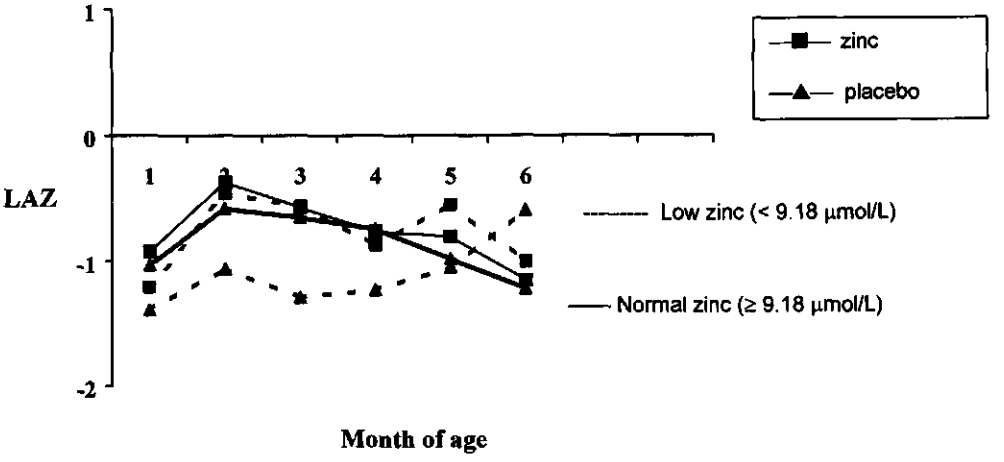

Figure 1. Mean length-for-age Z-scores (LAZ) between 1 and 6 months of age for infants in zinc and placebo supplemented groups.

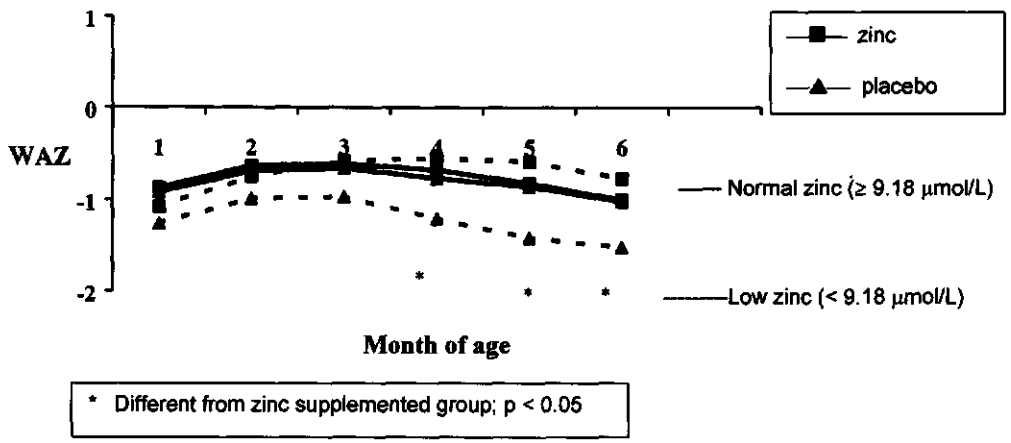

Figure 2. Mean weight-for-age Z-scores (WAZ) between 1 and 6 months of age for infants in zinc and placebo supplemented groups.

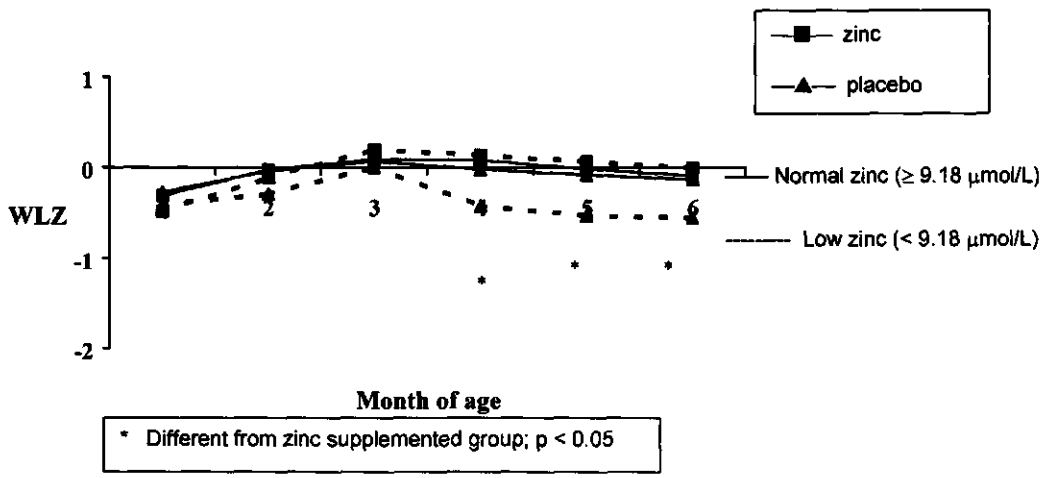

Figure 3. Mean weight-for-length (WLZ) Z-scores between 1 and 6 months of age for infants in zinc and placebo supplemented groups.

When data were analyzed separately for infants with (n=43) or without (n=257) zinc deficiency at baseline (serum zinc values < 9.18 μmol/L), significant differences were observed after zinc supplementation for total weight gain between 4 and 24 weeks of age and weight at 24 weeks of age (6.52 (0.85) vs. 5.82 (0.99) kg in zinc and placebo groups respectively; $p<0.05$) in 43 infants that were zinc deficient at baseline (Table 2). A similar trend was observed for total length gain but this difference did not reach statistical significance. Weight-for-age and weight-for-length Z-scores at 4, 5 and 6 months of age were significantly higher in zinc supplemented infants compared to the placebo supplemented infants (Figure 1-3). After zinc supplementation significantly greater changes in weight, length, head-, and chest-circumferences were observed in infants that were zinc deficient compared to their non-zinc deficient counterparts (Table 2). No differences were observed in placebo infants or between treatment groups in infants with normal baseline serum zinc values (≥ 9.18 μmol/L; Table 2 and Figure 1-3).

Table 3. Longitudinal prevalence of morbidity between 4 and 24 weeks of age for infants in zinc and placebo groups

Morbidity	Percentage of days ill			
	Zinc group[1]		Placebo group[1]	
Acute diarrhea				
– All infants[2]	5.9	4.8 (0.0-37.0)	5.8	3.6 (0.0-33.3)
– Serum zinc < 9.18 μmol/L[3]	5.8	5.2 (0.0-26.4)	6.7	4.1 (0.0-26.5)
– Serum zinc ≥ 9.18 μmol/L[4]	6.0	4.4 (0.0-37.0)	5.6	3.6 (0.0-33.3)
Persistent diarrhea				
– All infants	4.2	0.0 (0.0-48.1)	4.7	0.0 (0.0-86.4)
– Serum zinc < 9.18 μmol/L	4.3	0.0 (0.0-30.8)	3.1	0.0 (0.0-28.0)
– Serum zinc ≥ 9.18 μmol/L	4.2	0.0 (0.0-48.1)	5.0	0.0 (0.0-86.4)
Dysentery				
– All infants	0.7	0.0 (0.0-18.6)	1.1	0.0 (0.0-73.0)
– Serum zinc < 9.18 μmol/L	0.0	0.0 (0.0-0.0)	0.2	0.0 (0.0-4.1)
– Serum zinc ≥ 9.18 μmol/L	0.8	0.0 (0.0-18.6)	1.3	0.0 (0.0-73.0)
Cough (URI)[5]				
– All infants	36.9	35.3 (0.0-100.0)	35.1	32.2 (0.0-94.6)
– Serum zinc < 9.18 μmol/L	38.5	40.6 (0.0-100.0)	38.5	44.6 (2.00-94.6)
– Serum zinc ≥ 9.18 μmol/L	36.6	33.8 (0.0-91.8)	34.1	29.1 (0.0-89.8)
ALRI[6]				
– All infants	7.6	0.0 (0.0-93.9)	7.5	0.0 (0.0-87.2)
– Serum zinc < 9.18 μmol/L	5.8	0.0 (0.0-69.4)	8.0	0.0 (0.0-45.5)
– Serum zinc ≥ 9.18 μmol/L	7.9	0.0 (0.0-93.9)	7.4	0.0 (0.0-87.2)

[1]First column: mean % of days ill; second column: median (range)
[2] All infants (n=152/149) only children included with >= 15 days follow-up
[3] Baseline serum zinc < 9.18 μmol/L (n=22/21). [4] Baseline serum zinc ≥ 9.18 μmol/L (n=130/127)
[5]URI, upper respiratory infection [6]ALRI= acute lower respiratory infection.
None of the differences statistically significant (Linear regression with log transformed values).

In male infants, total weight gain between 4 and 24 weeks of age (3.21 (0.68) vs. 2.99 (0.62) kg in zinc and placebo groups) was higher in the zinc supplemented compared to placebo supplemented infants. However, this difference was not statistical significant after controlling for confounding variables in the multivariate analyses. No differences in growth were observed between treatment groups among female infants or among different subgroups based on infant's nutritional status.

Morbidity

No differences were observed between the two treatment groups for percentage of days ill and number of episodes of diarrheal and respiratory diseases (Table 3 and 4). However, when data were analyzed separately for zinc-deficient children (baseline serum zinc < 9.18μmol./L), fewer number of episodes with acute lower respiratory infections (ALRI) were observed in the zinc supplemented (0.3 [0.7] episodes/child/6months) compared to the placebo supplemented infants (0.9 [1.3] episodes/child/6months; RR: 0.30; 95%CI:0.10;0.92). No significant differences were observed between zinc and placebo groups for infants with normal baseline serum zinc values (≥ 9.18μmol./L; Table 4).

There were no differences in the effect of zinc on morbidity for infant's gender.

DISCUSSION

Zinc supplementation with 5 mg elemental zinc/day between 4 and 24 weeks of age did not improve infant growth nor reduced morbidity from diarrheal and respiratory diseases in Bangladeshi infants with normal serum zinc values. However, in infants with low serum zinc concentrations (<9.18 μmol/L) at baseline, beneficial effects on growth in terms of a significant greater weight gain and improved though not significant linear growth and on morbidity in terms of significant fewer number of episodes of Acute Lower Respiratory Infections (ALRI) were observed after zinc supplementation. These findings were somewhat unexpected since we had anticipated a beneficial effect of zinc supplementation on growth and morbidity among all infants in this deprived population. The study was performed among young infants below 6 months of age who are at high risk for growth faltering and/or contracting diseases, in a population where zinc deficiency is thought to be common.

Table 4. Incidence of diseases between 4 and 24 weeks of age for infants in zinc and placebo supplemented groups.

	Zinc group	Placebo group	RR (95% C.I.)[1]
Total surveillance days	20547	20238	
Acute diarrhea			
- Total # episodes	244	216	
- Episode/child/6mth[2]			
-All infants[3]	2.1 (2.0)	2.0 (2.1)	1.13 (0.94;1.36)
-Serum zinc < 9.18 µmol/L[4]	2.1 (2.0)	2.6 (2.1)	0.87 (0.60;1.72)
-Serum zinc ≥ 9.18 µmol/L[5]	2.1 (2.1)	1.9 (2.1)	1.14 (0.93;1.39)
Persistent diarrhea			
- Total # episodes	40	38	
- Episode/child/6mth[2]			
-All infants[3]	0.4 (0.8)	0.4 (1.5)	1.17 (0.74;1.83)
-Serum zinc < 9.18 µmol/L[4]	0.4 (0.7)	0.3 (0.6)	1.58 (0.31;7.96)
-Serum zinc ≥ 9.18 µmol/L[5]	0.4 (0.9)	0.5 (1.6)	1.04 (0.64;1.69)
Dysentery			
- Total # episodes	10	13	
- Episode/child/6mth[2]			
-All infants[3]	0.09 (0.3)	0.12 (0.4)	0.72 (0.31;1.66)
-Serum zinc < 9.18 µmol/L[4]	0.0 (0.0)	0.1 (0.3)	N.A.[6]
-Serum zinc ≥ 9.18 µmol/L[5]	0.1 (0.3)	0.1 (0.5)	0.77 (0.33;1.78)
Cough (URI)[7]			
- Total # episodes	511	500	
- Episode/child/6mth[2]			
-All infants[3]	3.4 (1.7)	3.4 (1.5)	1.00 (0.89;1.14)
-Serum zinc < 9.18 µmol/L[4]	4.5 (2.1)	4.5 (1.9)	0.95 (0.65;1.39)
-Serum zinc ≥ 9.18 µmol/L[5]	4.6 (2.0)	4.6 (2.2)	1.01 (0.88;1.15)
ALRI[8]			
- Total # episodes	75	74	
- Episode/child/6mth[2]			
-All infants[3]	0.7 (1.2)	0.7 (1.2)	0.99 (0.71;1.37)
-Serum zinc < 9.18 µmol/L[4]	0.3 (0.7)	0.9 (1.3)	0.30 (0.10;0.92)*
-Serum zinc ≥ 9.18 µmol/L[5]	0.7 (1.3)	0.7 (1.2)	1.10 (0.77;1.56)

[1] RR= rate ratio estimated with Poisson regression with 95% CI in parentheses, adjusted for baseline infant weight, length, socio-economic status, problems during delivery reported, delivery duration, age introduction of water.
[2] Values in mean (SD)
[3] All infants (n=150/149) only children included with >= 15 days follow-up
[4] Baseline serum zinc < 9.18 µmol/L (n=22/21).
[5] Baseline serum zinc ≥ 9.18 µmol/L (n=128/127)
[6] N.A.= Not applicable, could not calculate RR due to empty cells
[7] URI, upper respiratory infection.
[8] ALRI, Acute Lower Respiratory Infection.
* $p < 0.05$

We believe that a lack of effect in the apparently non-zinc deficient infants (based on serum zinc concentrations ≥ 9.18 µmol/L) may be due to one or more of the following reasons: (a) zinc might not have been the primary growth-limiting nutrient for these predominantly breastfed infants, (b) the age of these infants did

not allow us to demonstrate an effect of zinc supplementation on morbidity due to the low incidence of diseases among this young age group and the fact that at this age predominantly breastfed infants have probably not yet developed a zinc deficiency, and (c) the dosage we provided may not have been sufficient.

We provided 5 mg elemental zinc per day, the recommended daily allowance for this age group.[21] Since the infants in our study were pre-dominantly breast-fed during most of the study period, most of the dietary zinc intake was from breastmilk and differences between zinc and placebo supplemented infants in dietary zinc intake, other than the supplement, were therefore thought to be random and due to natural variability in breastmilk zinc.[22] Compliance with supplement-consumption was good and we observed significant improvements in serum zinc values in the zinc supplemented but not in the placebo supplemented group, indicating that the zinc supplementation was successful in improving the zinc status of these infants.

We do not know whether a higher dosage of zinc would have given different results in our population. Positive effects of zinc supplementation on growth or morbidity of infants or children have been observed in studies using higher[23-26] but also similar[27,28] or even lower dosages[10] than used in our study. A significantly higher mortality was observed among severely malnourished children in Bangladesh receiving a high-dose (6 mg/kg bodyweight/day) zinc treatment, suggesting that some caution is warranted in supplementing malnourished children with high dosages of zinc.[29]

The lack of effect on growth in the general, i.e., non-zinc deficient population as observed in our study after zinc supplementation is in contrast with findings of other studies among the same age group showing improvements in growth after supplementation with 3 and 5 mg elemental zinc/day in Chile and France respectively.[10,28] Beneficial effects of zinc supplementation on linear and ponderal growth have also been observed among older infants, both in well and malnourished populations.[1,23] However, some studies among older children in malnourished populations[24,27,30] were not able to show an effect of zinc supplementation on growth. It has been hypothesized that zinc was probably not the primary limiting nutrient to growth in these populations[31] and single nutrient interventions might therefore not improve growth in populations with multiple nutrient deficiencies.[32]

We believe that the very young age of our population is the most likely explanation for a lack of effect of zinc supplementation as observed among the general population of infants in our study. Firstly, we supplemented infants between 4 and 24 weeks of age and the overall burden of morbidity in this age group was low. A stronger reduction in morbidity after zinc supplementation has been observed in older compared to younger age groups[11,25] possibly due to the higher incidence of diseases in older age groups where exposure to antigens is

usually higher and the protective effect of maternal antibodies is waning. However, reductions in morbidity after zinc supplementation have been observed even during the first six months of life in a study among low birth weight (LBW) infants in Brazil.[9] The dosage used in this study was similar to our dosage but the supplementation in the Brazilian study started at birth and continued for 12 weeks only.

Secondly, in our study we observed statistically significant differences in growth and morbidity after zinc supplementation among infants that were zinc deficient at baseline. These findings and the fact that at 4 weeks of age only 14% of our population had serum zinc concentrations < 9.18 μmol/L, suggest that most infants of this age had not yet developed a zinc deficiency. At 24 weeks of age, almost all infants in our study (96%) were still pre-dominantly or partially breast-fed. In developed countries zinc deficiency is known to be relatively rare among term breast-fed infants during the first months of life[33] due to large concentrations of highly bio-available zinc in early breastmilk and utilization of hepatic metallothionein as a source of zinc during the first months of life.[34] The results of our study indicate that similar mechanisms may also occur in less affluent societies in breast-fed children below 6 months of age although more research is required to confirm this.

In conclusion, zinc supplementation with daily 5 mg elemental zinc between 4 and 24 weeks of age improved zinc status, but did not improve growth or reduce morbidity in poor Bangladeshi infants, who were not zinc deficient at 4 weeks of age. However, improvements in growth and morbidity were observed in infants with low serum zinc values at 4 weeks of age suggesting that zinc supplementation might be beneficial in some infants who are zinc deficient at this very early age.

We previously reported results from another study in the same population showing that zinc supplementation during pregnancy with 30 mg elemental zinc/day during the last two trimesters reduced morbidity from diarrheal diseases, particularly among low birth weight infants during the same age period.[12,35] The differences in results between our two studies indicate that compared to infant supplementation, zinc supplementation during pregnancy might perhaps be a more effective way to reduce some of the increased health risks associated with low birth weight or zinc deficiency in infants below 6 months of age.

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7

Immunization with the heptavalent pneumococcal conjugate vaccine and other childhood vaccines in Bangladeshi infants and effects of zinc supplementation.

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ABSTRACT

Background: Zinc deficiency is known to impair immunologic functions. However, the effect of zinc supplementation on the immune response to polysaccharide vaccines is not known.

Objective: To determine the immunogenicity of the heptavalent PNC vaccine in poor Bangladeshi infants and to assess the effect of zinc supplementation on immune response to PNC and other childhood vaccines.

Design: We immunized 301 infants with the standard BCG vaccine at 4 weeks of age and the combined diphtheria, tetanus toxoid and pertussis (DTP)- *Haemophilus Influenzae* type b (Hib) vaccine from 9 weeks of age. The infants were supplemented with daily 5mg zinc or placebo from 4-33 weeks of age. A subcohort of 241 infants was in addition immunized with the heptavalent PNC vaccine at 18 ± 1 weeks of age. Response to delayed hypersensitivity (PPD) skin test and antibody response to Hib and each of the seven PNC serotypes were assessed at 4, 24 and 33 weeks of age.

Results: After three doses of PNC, geometric mean titres for the pneumococcal serotypes ranged from 3.68 to 13.34 μg/ml. Titres at 24 weeks of age were significantly higher for infants who had received PNC compared to infants who had only received DTP-Hib. There were no severe adverse events related to vaccination. Zinc supplementation resulted in higher titres for serotypes 9V and 23F but had no effect on other serotypes nor on response to DTP-Hib. Zinc had a marginal negative effect on response to PPD skin test.

Conclusions: A heptavalent PNC vaccine proved to be safe and immunogenic in Bangladeshi infants. Zinc supplementation enhanced the immune response of two Pneumococcal serotypes (9V and 23F).

INTRODUCTION

Acute lower respiratory infections (ALRIs) are estimated to cause the death of 4 million under-5 year-olds annually in the world and most of these deaths are due to pneumonia, accounting for up to 30% of all deaths in children <5 years of age from developing countries.[1,2] Streptococcus pneumoniae (the pneumococcus) is the most important cause of bacterial pneumonia in young children.[3]

The pure pneumococcal polysaccharide vaccines currently licensed in the US and many other countries worldwide, are immunogenic in children above 2 years of age and in adults, but not in infants below 2 years of age in whom the incidence of pneumonia-related death is highest. To overcome this problem new vaccines have been developed in which the T-independent capsular polysaccharide is covalently conjugated to a T-dependent protein carrier.[4] A recently developed heptavalent pneumococcal conjugate vaccine (Prevanar; Wyeth-Lederle-PNCRM7) was found to be safe, immunogenic and highly efficacious in preventing invasive disease in US infants beginning at 2 months of age.[5,6] This vaccine contained saccharides of pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F and 23F.

There is only limited data from developing countries regarding the immune responses of infants to this pneumococcal conjugate vaccine. In a pilot trial in the Gambia a pentavalent pneumococcal conjugate vaccine was found to be safe and immunogenic but sample sizes were small (n= 30) and the vaccine contained only five pneumococcal serotypes.[7] If the 7-valent pneumococcal conjugate vaccine is to be used routinely in developing countries it is important to know if the immune responses are similar to infants in developed countries. If the antibody responses are found to be lower, it is important to know if the impaired immune responses can be overcome through supplementation with micronutrients that are known to affect the immune system, such as zinc. Zinc plays an essential role in the non-immune and immune host defenses[8] and beneficial effects on immunity and morbidity have been demonstrated in infants and children after zinc supplementation.[9-11] However, the effect of zinc supplementation in infants on the immune response to vaccines is not known.

We assessed the immunogenicity of a heptavalent pneumococcal conjugate vaccine in infants from a very poor, urban area in Bangladesh where ALRI is estimated to account for 23% of all childhood mortality[12] and evaluated the effect of zinc supplementation during 4-33 weeks of age on the immune response to this vaccine.

SUBJECTS AND METHODS

Study design and study population

This study was a nested study within a community-based, randomized controlled trial, to evaluate the effect of zinc supplementation on growth and morbidity from infectious diseases among infants aged 4 to 24 weeks. Details on the study population, intervention and data collection procedures have been reported elsewhere.[13] The study was approved by the Ethical Review Committee of ICDDR,B and the Committee on Human Research of the Johns Hopkins School of Hygiene and Public Health.

Singleton infants living in three selected areas of Dhaka city slums were eligible for inclusion in the study if they met the entry criteria of age (4 ± 1 week), were in good general health without known underlying illnesses, were not included in any other intervention trial and/or had not received immunizations through other sources. A total of 301 infants aged 4±1 week (152 in zinc and 149 in placebo supplemented groups) were identified through an established household-surveillance system and enrolled in the study between April and June 1997 after obtaining written informed consent from the parents. Infants were randomly allocated to a liquid 5 mL daily dose sucrose liquid with or without 5 mg elemental zinc (as zinc acetate). Both liquids contained sucrose, flavors and preservatives,

were indistinguishable in both appearance and taste and were prepared and coded by Opsonin Chemical Industries Ltd. Dhaka. The zinc content of both supplement and placebo was independently confirmed by two different laboratories. Compliance with supplement consumption was 85% and the average daily consumption was 4.2 ± 1.3 ml as assessed by measuring liquid levels at weekly visits combined with unannounced compliance checks between the regular visits. No differences in compliance were observed between the two treatment groups. The codes of the supplements were unknown to both participants and study staff and broken only after data editing and cleaning was completed. Supplementation started at 4 ± 1 weeks of age and continued until the end of data collection at 24 ± 1 or 33 ± 2 weeks of age respectively. Due to a delayed arrival of the 7-valent pneumococcal conjugate vaccine, only 241 infants who at that time had not yet reached the age of 4 months ± 15 days were eligible for pneumococcal immunization. For these infants supplementation and compliance monitoring continued until 33 weeks of age to allow for a final blood sampling at least one month after the third dose of the vaccine.

Infants were followed weekly for assessment of compliance and morbidity of diarrheal and respiratory diseases. Anthropometric measurements (weight, length, arm-, head- and chest-circumference) were performed monthly until the infants were 6 months old. Infants' serum zinc was determined at 4 and 24 weeks of age.[13]

Immunization

At enrollment 0.05 ml reconstituted live freeze-dried vaccine of Bacille Calmette Guerin (BCG) was administered and infants were immunized with the Trivalent Oral Polio vaccine (TOPV) and combined diphtheria, tetanus toxoid and pertussis (DTP)- *Haemophilus Influenzae* type b (Hib) vaccine (Diphtheria CRM, 97 protein conjugate), [TETRAMUNE® Wyeth-Lederle, NY] at 9, 13 and 18 weeks of age (± 3 weeks). A total of 241 infants who had not yet reached the age of 4 months ± 15 days at the time of the arrival of the vaccine, received in addition the 7-valent pneumococcal conjugate vaccine (Prevanar; PNC, Wyeth Lederle Vaccines, NY, USA) at 18, 24 and 28 weeks (± 4 weeks). The pneumococcal conjugate vaccine contained 2 μg each of saccharides of the following serotypes: 4, 9V, 14, 18C, 19F and 23F and 4 μg of 6B coupled to a protein carrier (CRM197). The second and third round of vaccinations were given at 4 week intervals (± 2 days). When possible the pneumococcal conjugate vaccine was administered conjointly with the TETRAMUNE vaccine intramuscularly into the opposite leg. Vaccines not given concomitantly were given at least 2 weeks apart. All immunizations were given by study nurses.

Post-vaccination safety surveillance was carried out during home visits from day 1 through day 5 post-vaccination by trained study nurses. Data was collected and recorded daily on rectal temperature, local swelling and redness, generalized

rash, seizures, general health of the infant, feeding history and any other unexpected reactions.

Assessment of immune response to H. Influenza type b and Pneumococcal conjugate vaccine.

Antibody response to *H. Influenzae* b polysaccharide (polyribosylribitol phosphate (PRP)) was assessed pre-vaccination at 4 weeks of age and post-vaccination at 24 weeks of age. Immunogenicity of the 7-valent pneumococcal conjugate vaccine was assessed pre-vaccination at 4 weeks of age, after the second dose of vaccination at 24 weeks of age and one month after the third dose at 29 weeks of age. Non-fasting blood specimens (5 ml) were obtained by antecubital venipuncture in the morning hours. Serum was separated a maximum of 6 hours after collection and stored at -20C until analysis. Antibodies (IgG) to PRP and to the seven pneumococcal vaccine serotypes were measured by enzyme linked immunosorbent assay (ELISA). All assays were performed at the laboratory of Wyeth Lederle Vaccines (NY, USA) following standard procedures.[14] The study codes were not known to the laboratory personnel until all assays were completed. Geometric mean antibody titres were calculated as the antilog of the mean of the logarithms of titres. Mean paired differences between pre- and post immunization titres and percentage of infants with antibody levels ≥ 0.15 μg/ml and ≥ 1.0 μg/ml were determined.

Assessment of immune response to tuberculin skin test.

Immune response to the BGC vaccine was tested at 24 weeks of age by delayed hypersensitivity skin test (DTH) using the purified protein derivate (PPD) test (Staten Serum Institute, Copenhagen, Denmark). Tuberculin solution (0.1 ml) was applied by trained nurses to the volar surface of the forearm intradermally using an Omega glass PPD syringe with platinum needles. After 72 hours the transverse diameter of the induration developed was read in millimeters. A cutaneous reaction was considered positive when an induration > 5mm was observed.[15] Six nurses were extensively trained and standardized in the administration and interpretation of PPD skin testing. Inter-rater agreements as assessed prior and during the study in hospital patients were found to be satisfactory with Pearson correlation coefficients ranging between 0.98 ($p < 0.000$) and 0.73 ($p=0.03$) and consistent with inter-rater agreements observed in similar studies.[16]

Statistical methods

Results are presented as means and standard deviation except when otherwise stated. Differences between groups in proportion of infants with an induration of ≤ 5 mm from the tuberculin skin test and proportion of infants with

antibody titres above the protective thresholds before and after immunization were assessed with the Mac Nemar's test. Differences between groups in mean size of induration, geometric mean antibody titres and paired differences between pre- and post- immunization titres were assessed with the Mann-Whitney U-test and the Wilcoxon paired signed rank sum-test respectively. Separate models were made based on subgroups for baseline serum zinc < or ≥ 9.18 μmol/L. The cut-off for zinc deficiency was selected according to the literature.[11]

Analysis of Covariance (ANCOVA) and Logistic Regression Analysis were performed to control for potential confounders (SPSS7.5 FOR WINDOWS; SPSS Inc, Chicago). P-values of <0.05 were considered statistically significant and P-values of <0.09 were considered marginally significant.

RESULTS

Description of the study population.

Of the 301 infants enrolled and immunized with BCG vaccine at enrollment, a total of 33 (11%) infants (15 or 10% in zinc group and 18 or 12% in placebo group, difference NS) were lost-to-follow-up before 24 weeks of age. Eight infants had died, 15 infants had migrated from the study area, the parents of six infants refused to further participate and four infants had received immunizations through other sources and were excluded from further participation. Reasons for lost-to-follow-up were not different for infants from zinc and placebo group. The PPD skin test could not be administered in an additional 10 infants (4 in zinc and 6 in placebo group) at 24 weeks of age because they refused, were sick or not available at the time of the test. For 13 infants readings (10 in zinc and 3 in placebo group) could not be performed within the required 72 hours and the final sample size for the effects on the tuberculin skin test therefore consisted of 245 infants (123 in zinc, 122 in placebo group).

For the assessment of immune response to the combined diphteria, tetanus toxoid and pertussis (DTP)- *Haemophilus influenza* type b (Hib) vaccine, an additional ten infants were lost-to-follow-up because they had not received three doses of vaccinations (n=3) or because their caretakers refused the final blood sampling (n=7). A total of 258 infants (133 in zinc, 125 in placebo group) completed the course of three doses of the DTP-Hib vaccine and provided pre- and post-vaccination serum samples for immune assays. Thirty of these infants (15 in zinc and 15 in placebo group) had only received the DTP-Hib vaccine, whereas the remaining 228 infants had received one or two doses of the 7-valent pneumococcal PNC) vaccine in addition. Median ages at pre- and post-immunization blood sampling were 4.0 and 24.0 weeks respectively and median ages at the three rounds of immunization were 8.7, 13.7 and 18.3 weeks. Serum samples of 76

Table 1. Selected baseline characteristics for infants included in analysis on immune response and infants lost-to-follow-up for analysis on immune response for 301 infants enrolled in Hib trial and 241 infants enrolled in Pneumococco trial.

	Infants lost for Hib trial (n=119)	Infants included in Hib trial (n=182)	Infants lost for PnC trial (n=135)	Infants included in PnC trial (n=106)
Age infant (months)	0.88 (0.12)	0.91 (0.12)#	0.89 (0.12)	0.88 (0.13)
Gender: male (%)	49.6	41.2	48.1	39.6
Weight (kg)	3.39 (0.48)	3.49 (0.49)	3.46 (0.50)	3.43 (0.47)
Length (cm)	51.1 (2.2)	51.3 (2.1)	51.0 (2.2)	51.1 (2.1)
Z-scores:				
Length-for-age	-1.07 (0.95)	-0.99 (0.86)	-1.10 (0.91)	-1.05 (0.86)
Weight-for-age	-0.87 (0.76)	-1.04 (0.75)#	-0.93 (0.76)	-0.93 (0.76)
Weight-for-length	-0.25 (0.58)	-0.41 (0.78)#	-0.23 (0.64)	-0.31 (0.57)
Serum zinc (µmol/L)	12.0 (2.9)	11.7 (3.0)	12.1 (3.0)	11.3 (2.6)*
Age mother (yrs)	25.6 (6.2)	23.9 (5.5)∀	24.8 (6.1)	24.6 (5.8)
Weight mother (kg)	44.0 (6.4)	44.4 (6.4)	43.8 (6.3)	44.5 (6.5)
Parity: primiparae (%)	26.1	25.3	23.0	27.4
Socio-economicstatus: poor/very poor (%)	81.5	69.8	79.2	71.7

[1] Values in mean (SD)
[2] LBW= low birth weight (<2500 g)
[3] IUGR = Intra Uterine Growth Retarded (<10% of fetal growth chart; 13)
[4] Based on an index of household assets (13)
[5] BMI= Body Mass Index
[6] MUAC= Mid Upper Arm Circumference
* Different from infants lost-to-follow-up for Pneumococco assays test ($p<0.05$)
∀ Different from infants lost-to-follow-up for Hib (*Haemophilus influenza* type b)-immune assays ($p<0.05$)
Different from infants dropped for Hib (*Haemophilus influenza* type b)-immune assays ($p<0.06$)

infants were insufficient for analysis resulting in a final sample of 182 infants (95 zinc group, 87 placebo group) for analysis of Hib-response. Compared to infants who were lost-to-follow-up for the Hib assays, the 182 infants who were included in the Hib-trial had statistical significant higher weight for age Z-scores at enrollment and their mothers were significantly younger (table 1). We controlled for those variables in the multivariate analysis.

A total of 241 infants (121 in zinc supplemented group and 120 in placebo supplemented group) received the first dose of the PNC vaccine in addition to the combined diphteria, tetanus toxoid and pertussis (DTP)-*Haemophilus influenzae* type b (Hib) vaccine. Of these 241 infants, 8 (7%) in zinc and 15 (13%) in placebo group, difference NS, were lost-to-follow-up before the final blood drawing at 33 weeks of age for the following reasons: 3 infants died, 6 infants migrated out of the study area, 10 refused to further participate in the study and 4 infants did not complete the three-dose course of the PNC vaccine. There were no differences for reasons of lost-to-follow-up between zinc and placebo group. Median ages at the final post-three dose blood sampling was 32.7 weeks and median ages at the three

rounds of immunization were 18.1, 23.9 and 28.9 weeks. Of the 218 infants who provided a final blood sample after the third vaccine dose, a total of 112 serum samples were insufficient for analysis and pneumococcal antibody titres at 33 weeks of age are therefore known for 106 infants (53 in zinc and 53 in placebo group). Antibody titres of these 106 infants were compared with titres of 30 infants (15 in zinc and 15 in placebo group) who had not received the PNC vaccine The 106 infants with known PNC antibody titres had statistical significant lower serum zinc concentrations at enrollment compared to the infants who were lost-to-follow-up (table 1) and we therefore controlled for baseline serum zinc in the multivariate analysis.

Immunogenicity of pneumococcal conjugate vaccine

Geometric mean antibody titres for the seven pneumococcal serotypes for infants who received only the DTP-Hib vaccine compared to infants who received both the DTP-Hib and the PNC vaccines are shown in table 2. Prior to immunization at 4 weeks of age, there were no differences in titres between the groups. Geometric mean antibody titres declined significantly ($p<0.001$ Wilcoxon paired-rank test) at 24 weeks of age for all seven serotypes in infants who had only received the DTP-Hib vaccine. At 24 weeks of age, 71 (42%) infants had received the first PNC dose only while 100 (58%) infants had received two doses of PNC.

Geometric mean antibody titres for all seven serotypes were significantly higher at 24 weeks of age ($P < 0.001$; Mann-Whitney U test) for infants that had received two PNC doses compared to the titres of infants that had just received one PNC dose at that time (table 2). For infants that had received one PNC dose at 24 weeks of age, geometric mean titres had increased between 4 and 24 weeks of age for serotypes 4, 9V and 18C whereas only the increase in serotype 4 was statistically significant. A similar but more pronounced pattern was observed for infants who had received two PNC doses at 24 weeks of age (table 2). In these infants, geometric mean titres increased significantly for serotypes 4, 9V, 18C and 23F between 4 and 24 weeks. After three doses of the PNC vaccine geometric mean titres for all seven serotypes had increased further. The geometric mean titres ranged from 3.68 to 13.34 μg/ml with the highest titres for serotypes 6B and 14 and the lowest for serotype 9V.

Comparisons between groups were only possible at 24 weeks of age, after all three doses of DTP-Hib vaccine and one or two doses of the PNC vaccine. Geometric mean titres for all seven PNC serotypes were significantly higher in infants that had received two or even only one dose of PNC at 24 weeks of age compared to infants who had only received the DTP-Hib vaccine.

Table 2. Geometric mean titres to each pneumococcal serotype pre- and post-immunization for infants receiving the Pneumococcal conjugate vaccine combined with Tetramune and infants receiving only the Tetramune vaccine[1].

Pneumococco sero-type	Tetramune only (n= 30)	PnC conjugate and Tetramune (n= 106)
4		
Pre-immunization[2]	0.67 (0.42;1.09)	0.60 (0.49;0.74)
Post-1 dose immunization[3]	0.07 (0.04;0.13)	4.92 (3.41; 7.10) **#
Post- 2 dose immunization[4]	-do-	8.19 (6.38;10.52) **#
Post-3 dose immunization[5]	--	8.61 (7.51;9.86)
6B		
Pre-immunization	2.73 (1.76;4.24)	2.37 (1.97;2.86)
Post-1 dose immunization	0.26 (0.17;0.40)	0.56 (0.40;0.78) *¶
Post- 2 dose immunization	-do-	1.25 (0.83;1.89) **
Post-3 dose immunization	--	13.34 (11.14;15.98)
9V		
Pre-immunization	1.37 (0.93;2.00)	1.34 (1.15;1.57)
Post-1 dose immunization	0.19 (0.11;0.32)	0.94 (0.67;1.33) **
Post- 2 dose immunization	-do-	2.30 (1.73;3.05) **#
Post-3 dose immunization	--	3.68 (3.20;4.24)
14		
Pre-immunization	3.23 (2.17;4.80)	3.32 (2.73;4.04)
Post-1 dose immunization	0.26 (0.15;0.44)	1.02 (0.70;1.39) *¶
Post- 2 dose immunization	-do-	2.13 (1.44;3.16) **
Post-3 dose immunization	--	10.23 (8.57;12.21)
18C		
Pre-immunization	0.79 (0.52;1.18)	0.93 (0.77;1.12)
Post-1 dose immunization	0.09 (0.05;0.15)	0.73 (0.50;1.07) **
Post- 2 dose immunization	-do-	1.73 (1.17;2.56) **#
Post-3 dose immunization	--	5.14 (4.39;6.02)
19F		
Pre-immunization	3.16 (2.11;4.74)	2.89 (2.41;3.47)
Post-1 dose immunization	0.49 (0.30;0.82)	1.00 (0.71;1.40) *¶
Post- 2 dose immunization	-do-	3.33 (2.34;4.74) **
Post-3 dose immunization	--	5.75 (4.77;6.92)
23F		
Pre-immunization	1.06 (0.73;1.54)	1.06 (0.89;1.25)
Post-1 dose immunization	0.14 (0.08;0.24)	0.68 (0.48;0.98) **
Post- 2 dose immunization	-do-	1.65 (1.08;2.51) **#
Post-3 dose immunization	--	6.65 (5.52;8.01)

[1] Geometric mean titres calculated as the antilog of the mean of the logarithms of values (values in brackets: 95% confidence intervals)
[2] Pre-immunization at 4 weeks of age
[3] Post- 1 dose immunization at 24 weeks of age (n=71), after 1 doses of PnC vaccine and 3 doses of Tetramune vaccine
[4] Post-2 dose immunization at 24 weeks of age (n=100), after 2 doses of PnC vaccine and 3 doses of Tetramune vaccine.
[5] Post- 3 dose immunization at 33 weeks of age (n=106), after 3 doses of PnC vaccine
* Titre differs from Tetramune only group (p<0.01; ANCOVA, control for age infant at enrollment, age mother, prevalence of upper respiratory infection between 4-24 weeks of age)
** Titre differs from Tetramune only group (p<0.005; ANCOVA, control for age infant at enrollment, age mother, prevalence of upper respiratory infection between 4-24 weeks of age)
# Titre increased compared to titre pre-immunization (p< 0.001; Wilcoxon signed-rank test)
¶ Titre decreased compared to titre pre-immunization (p < 0.001; Wilcoxon signed-rank test).

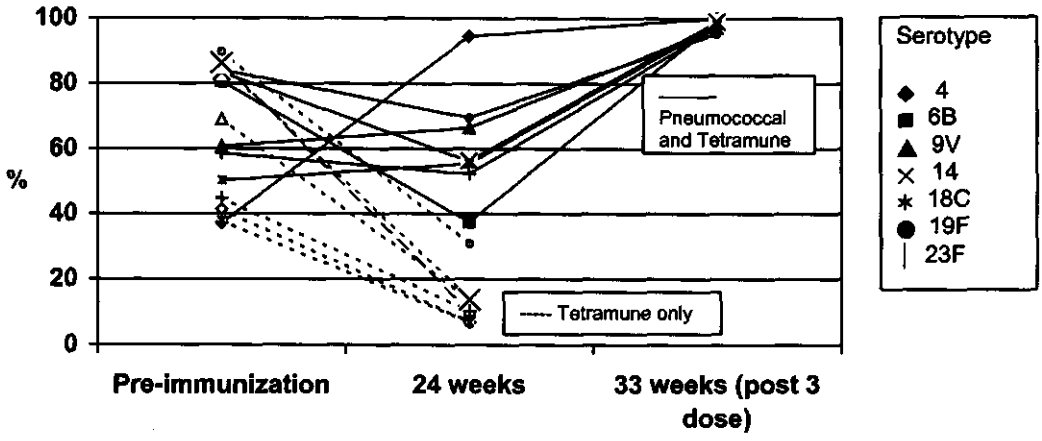

Figure 1. Proportion of infants with Pneumococcal antibody titres > 1.00 μg/ml

After the third dose of PNC vaccine, all infants had received protective antibody levels (> 1 μg/ml) for serotype 4 whereas nearly all had received the protective threshold for the other serotypes: 6B (99%), 9V (97.1%), 14 (99%), 18C (98.1%), 19F (95.2%) and 23F (97.1%) (figure 1). At 24 weeks of age, for all seven serotypes significantly higher proportions of infants who had received two or even one dose of the PNC vaccine had reached the protective thresholds compared to infants who had only received the DTP-Hib vaccine ($p < 0.001$; Logistic Regression). Prior to immunization at 4 weeks of age, no differences in proportions above protective thresholds were observed between groups (figure 1).

Effect of zinc supplementation on response to pneumococcal conjugate vaccine.

At 4 weeks of age, prior to immunization and prior to the onset of supplementation there were no differences in geometric mean titres for the seven PNC serotypes between infants from zinc and placebo group (table 3). After one or two doses of the PNC vaccine at 24 weeks of age there seem to be higher titres in the zinc compared to the placebo group for serotype 23F (1.88 μg/ml; [95% CI: 1.09;3.2] vs. 1.02 μg/ml; [95% CI: 0.65;1.62] for zinc and placebo group respectively; $p < 0.08$) but the difference was small and only of marginal statistical significance. After three doses of the PNC vaccine at 33 weeks of age significantly higher titres were observed in zinc compared to placebo group for serotype 9V (4.09 μg/ml; [95% CI: 3.27;5.10] and 3.33 μg/ml; [95% CI: 2.79;3.96] for zinc and placebo group respectively; $p < 0.05$). There were no differences between zinc and placebo groups for the other pneumococcal serotypes.

Table 3. Geometric mean titres and % above 'protective' thresholds to each pneumococcal serotype pre- and post-immunization for infants from zinc- and placebo-supplemented groups.

Pneumococcal sero-type	Zinc group (n=53)			Placebo group (n=53)		
	GMT[1]	% > 0.15 mcg	% > 1.0 mcg	GMT	% > 0.15 mcg	% > 1.0 mcg
4						
Pre-immunization[2]	0.74 (0.55;1.00)	85.9	35.9	0.69 (0.50;0.94)	88.4	38.4
Post-2 dose immunization[3]	7.64 (5.31;10.99)			8.18 (6.32;10.59)		
Post-3 dose immunization[4]	8.78 (6.99;11.02)	100	100	8.44 (7.20;9.90)	100	100
6B						
Pre-immunization	2.45 (1.81;3.31)	98.9	81.5	2.90 (2.14;3.93)	98.8	82.6
Post-2 dose immunization	0.98 (0.59;1.64)			1.02 (0.65;1.61)		
Post-3 dose immunization	13.94 (10.83;17.93)	100	100	12.79 (9.80;16.69)	100	98.1
9V						
Pre-immunization	1.47 (1.13;1.92)	98.9	63.0	1.46 (1.12;1.91)	98.8	61.6
Post-2 dose immunization	2.13 (1.40;3.23)			1.41 (0.99;2.00)		
Post-3 dose immunization	4.09 (3.27;5.10)	100	100	3.33 (2.79;3.96)#	100	98.1
14						
Pre-immunization	2.94 (2.18;3.98)	100	84.8	3.45 (2.48;4.78)	97.7	82.6
Post-2 dose immunization	1.59 (0.94;2.70)			1.39 (0.90;2.15)		
Post-3 dose immunization	11.44 (9.25;14.14)	100	100	9.17 (6.87;12.21)	100	98.1
18C						
Pre-immunization	0.98 (0.75;1.27)	94.6	46.7	1.18 (0.87;1.59)	94.2	51.2
Post- 2 dose immunization	1.57 (0.91;2.72)			1.00 (0.64;1.56)		
Post-3 dose immunization	5.47 (4.21;7.09)	100	96.2	4.84 (4.00;5.86)	100	100
19F						
Pre-immunization	3.35 (2.52;4.46)	98.9	83.7	3.63 (2.69;4.89)	100	86
Post-2 dose immunization	2.45 (1.53;3.93)			2.06 (1.40;3.05)		
Post- 3 dose immunization	5.79 (4.32;7.75)	100	94.2	5.70 (4.47;7.28)	100	96.2
23F						
Pre-immunization	1.26 (0.94;1.70)	94.6	56.5	1.30 (1.00;1.68)	97.7	54.7
Post-2 dose immunization	1.88 (1.09;3.24)			1.02 (0.65;1.62)*		
Post-3 dose immunization	6.64 (4.79;9.21)	98.1	94.2	6.66 (5.48;8.10)	100	100

[1] GMT= Geometric mean titres calculated as the antilog of the mean of the logarithms of values (values in brackets: 95% confidence intervals)
[2] Pre-immunization at 4 weeks of age; [3] Post-2 dose immunization at 24 weeks of age; [4] Post-3 dose immunization at 33 weeks of age
* Titre differs from zinc group ($p<0.05$; ANCOVA); # Titre differs from zinc group ($p<0.08$; ANCOVA)

No differences were observed between zinc and placebo supplemented infants post-immunization at 33 weeks of age for all seven pneumococcal serotypes for proportion of infants who had reached the "protective" thresholds of > 0.15 μg/ml or > 1 μg/ml (table 3).

Safety

Adverse events observed after the PNC and DTP-Hib immunization during the five days post-immunization safety follow-up were usually mild and occurred less often than after only the DTP-Hib immunization (table 4). Following the PNC immunization, infants had significantly fewer days with local reactions at the injection site (3.71 ± 2.08 days) during the five days post-immunization for all

three rounds of immunization combined, compared to the DTP-Hib immunization (4.87 ± 2.81 days, p < 0.01; table 4). During the five days following the PNC vaccination fewer days of systemic reactions were reported: crying more than usual (2.64 ± 2.19 vs. 3.89 ± 2.37 days for PNC and DTP-Hib only groups respectively, p<0.005) or less active (1.30 ± 1.38 vs. 2.41 ± 1.89 days for PNC and DTP-Hib only groups respectively, p < 0.005). There was a small and marginal statistically significant (p < 0.06) difference in number of days with fever (>38.0 °C) after receiving the PNC vaccination (0.88 ± 1.05 days) compared to after receiving only the DTP-Hib vaccination (1.26 ± 1.09 days).

Overall, ten infants were hospitalized, one infant was admitted in the out-patient ward and one infant visited the emergency room within one month of receipt of a dose of the PNC vaccine. The reasons for hospitalization were: acute diarrhea (n=6), dysentery (n=2), persistent diarrhea and broncho pneumonia (n=1), septicemia and diarrhea (n=1) and Salmonella type B enteritis (n=1). All were

Table 4. Adverse events in the 5 days following immunization combined for three rounds of DTP-Hib and Pneumococco vaccine for infants receiving Tetramune only or both the vaccines in zinc and placebo group.

	Tetramune only		Pneumococcal and Tetramune	
	Zinc group (n=15)	Placebo group (n=15)	Zinc group (n=113)	Placebo group (n=105)
Swelling/redness at injections site	15 (100)	13 (86.7)	112 (99.1)	96 (91.4)#
- %[1]	5.40 (2.24)	5.11 (3.03)	3.84 (2.07)	3.57 (2.10)#
- days[2]				
Crying more than usual				
- %	15 (100)	14 (95.8)	102 (90.3)	91 (86.7)#
- days	3.63 (1.77)	4.51 (2.98)	2.73 (2.01)	2.55 (2.35)*
Less active				
- %	13 (86.7)	12 (80.0)	81 (71.7)	70 (66.7)*
- days	2.67 (1.88)	2.92 (2.40)	1.33 (1.23)	1.26 (1.53)*
Lethargic				
- %	1 (6.7)	1 (6.7)	14 (12.4)	12 (11.4)
- days	0.17 (0.53)	0.54 (1.15)	0.20 (0.59)	0.15 (0.46)
Fever measured[3]				
- %	11 (73.3)	9 (60.0)	69 (61.1)	52 (49.5)°
- days[2]	1.23 (1.14)	1.10 (1.23)	0.96 (1.05)	0.80 (1.06)^

[1] Values in means (SD)
[2] Fever: rectal body-temperature > 38°C
* Values in zinc and placebo groups combined differ from zinc and placebo group combined titres in Tetramune only group (p < 0.005, Chi-square test or Student's t-test)
# Values in zinc and placebo groups combined differ from zinc and placebo group combined titres in Tetramune only group (p < 0.05, Chi-square test or Student's t-test)
^ Values in zinc and placebo groups combined differ from zinc and placebo group combined titres in Tetramune only group (p < 0.06)
o Values in placebo group of PNC group differ from zinc group (p < 0.07; Student's t-test)

considered by the investigator to be not related or remotely related to the study vaccine with the exception of one case who was judged to be possibly related to the study vaccine. This infant had developed diarrhea one day after the PNC vaccination and was hospitalized five days later with acute diarrhea. The infant recovered completely and was discharged from the hospital after five days. A total of three infants died at home within one month upon receipt of the PNC vaccine due to severe malnutrition, diarrhea associated with fontanel swelling and respiratory tract infection. All deaths occurred more than one week after the vaccination and were judged to be not related or remotely related to the study vaccine.

There were no major differences between infants from zinc and placebo groups in adverse effects following DTP-Hib immunization or PNC immunization. The proportion of infants with fever on any day during the post-immunization

Table 5. Cellular immune response to tuberculin (PPD) skin test at 24 weeks of age for infants by birth weight in zinc and placebo groups.

	Zinc group	Placebo group
Size of induration < 5 mm (%)		
All infants[2]	65.0	55.7*
Serum zinc < 9.18 umol/L[3]	81.8	72.2
Serum zinc ≥ 9.18 umol/L[4]	61.4	53.4
Mean size of PPD-induration (mm)[5]		
All infants	10.21 (0.53)	10.35 (0.39)
Serum zinc < 9.18 umol/L	11.00 (2.08)	9.33 (0.88)
Serum zinc ≥ 9.18 umol/L	10.77 (0.50)	10.56 (0.41)

[1] Anergic = size of PPD induration < 5 mm
[2] All infants (n=123/122)
[3] Baseline serum zinc < 9.18 umol/L (n=22/18)
[4] Baseline serum zinc ≥ 9.18 umol/L (n=101/103)
[5] Mean size of induration of positive responses (SEM)
* Different from zinc supplemented group (p=0.09; Logistic regression controlling for birthweight and gestational age); PPD=purified protein derivate tuberculosis

safety surveillance following the PNC immunization was higher in the zinc (61.1%) compared to the placebo group (50.0%) but the difference was small and only of marginal statistical significance ($p < 0.07$).

Effect of zinc supplementation on immune response to DTP-Hib vaccine and Tuberculin skin test.

At 24 weeks of age, 60.4% (148 of 245) of all infants showed an induration of ≤ 5 mm after the PPD skin test which was considered a negative response. The proportion of infants with a negative response to the tuberculin skin test was higher in the zinc (65.0%) compared to the placebo group (55.7%; table 5) but the difference was small and only marginally significant ($p < 0.09$). A significantly higher percentage of infants with baseline serum zinc values below 9.18 μmol/L

had a negative response on the skin test compared to infants with higher serum zinc values at baseline (79.4% vs. 57.6% for < 9.18 vs. ≥ 9.18 μmol/L respectively, p<0.01). No differences in response to the tuberculin skin test were observed between treatment groups for 40 infants with low (<9.18 μmol/L) and for 204 infants with higher baseline serum zinc values (table 5). The mean size of induration among the positive responses was 10.3 mm (SEM 0.5) and not different for infants of zinc and placebo groups (table 5).
Geometric mean PRP antibody titres before and after immunization are shown in table 6. There were no significant differences in geometric mean PRP titres at 24 weeks of age (post-immunization) between infants from zinc (6.96 μg/ml; 95% CI:

Table 6. Geometric mean PRP antibody titres pre- and post-immunization for infants in zinc and placebo group[1]

	Zinc group (n= 95)	Placebo group (n= 87)
Pre-immunization (μg/ml)[2]	0.28 (0.21;0.36)	0.29 (0.22;0.37)
Post-immunization (μg/ml)[3]	6.96 (4.34;11.15)	9.72 (6.43;14.70)

[1] Geometric mean titres calculated as the antilog of the mean of the logarithms of values (values in brackets: 95% confidence intervals)
[2] Pre-immunization at 4 weeks of age
[3] Post immunization at 24 weeks of age
PRP=polyribosylribitol phosphate.

4.34;11.15) and placebo groups (9.72 μg/ml; 95% CI: 6.43;14.70). Nearly all infants (94% in zinc and 95% in placebo supplemented group, difference NS) achieved protective antibody titres correlated with immediate protection (≥0.15 μg/ml; figure 2) and a large majority (81% in zinc and 89% in placebo group, difference NS) in addition achieved titres ≥ 1 μg/ml associated with long-term protection (figure 2).

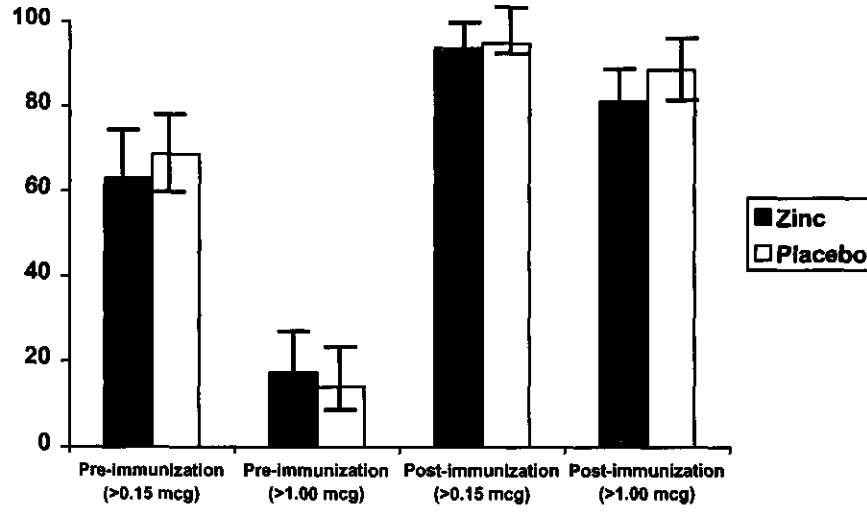

Figure 2. Proportion above protective thresholds for PRP antibody titres

DISCUSSION

Immunization with the 7-valent pneumococcal conjugate (PNC) vaccine was acceptably safe and resulted in significant rises in antibody titres of all 7 serotypes when administered to 4 to 7 month old Bangladeshi infants. Local and systemic reactions during the five days post-immunization were usually mild and occurred less often than after vaccination with the DTP-Hib vaccine. There were no severe adverse events resulting in hospitalization or death that were thought to be related to the vaccination. The mortality rate in this cohort of infants (3/240 or 1.3% in three months of follow-up) was similar to infant mortality rates observed in other studies in Bangladeshi infants.[13,17] The national infant mortality rate for Bangladesh is 79/1000 or 7.9%.[18]

As far as we are aware this is the first trial reporting on the immunogenicity of the heptavalent PNC vaccine in a developing country. A pentavalent Pneumococcal conjugate vaccine, containing serotypes 6B, 14, 18, 19F and 23F proved to be safe and immunogenic in a pilot study in the Gambia.[7] In the US the same heptavalent PNC vaccine that was used in our study was found to be safe, immunogenic and highly efficacious in preventing invasive disease and otitis media in US infants and young children.[5,6] In this study the first dose of PNC vaccine was given at 2 months of age compared to approximately 4 months of age in our study.

After three doses of vaccination, geometric mean titres (GMT) in our study were high and ranged from 3.68 μg/ml for serotype 9V to 13.34 μg/ml for serotype 6B. The concentrations observed in these Bangladeshi infants were substantially

higher than the titres observed in US infants (ranging from 0.98 μg/ml for serotype 9V to 3.48 μg/ml for serotype 14)[6] and higher than titres observed in Gambian infants after three doses of a pentavalent PNC vaccine (ranging from 2.49 μg/ml for serotype 19 to 7.59 μg/ml for serotype 14).[7] It is possible that frequent exposure to the S. Pneumoniae in our population may have caused a booster response if the child has come into contact with the organism after receiving the primary series of the vaccine. A booster dose of the PNC vaccine at 12 to 15 months of age resulted in increases in titres that were greater than the increases observed after the primary three-dose series in US children.[6] Serotype 6B in particular showed a remarkable booster response whereas in previous studies it was one of the least immunogenic serotypes after the primary series of vaccination. In our study, serotype 6B yielded the highest antibody titres after three doses of the vaccine. It has been hypothesized that since the PNC vaccine employs a T-dependent immune response, it effectively induces T cell memory and primes for an anamnestic response to all 7 vaccine serotypes.[6] Our findings suggest that the anamnestic responses may vary for the different serotypes with serotype 6B being particularly responsive. Antibody responses to the different pneumococcal polysaccharides are known to be serotype specific with serotype 14 being the most immunogenic and 9V being the least immunogenic in most studies after the primary series of vaccination.[5,6,7,19,20] The results of our study are consistent with these findings with the remarkable exception of the response to serotype 6B. An interesting finding especially since serotype 6B is the most common pneumococcal serotype in developing countries and thought to be responsible for 14% of all invasive diseases.[21] The frequent exposure to this serotype probably primed the immune system and may have enhanced a booster response. It is also possible that the better immune response to 6B is a result of the twofold higher concentration of this antigen in the vaccine compared to the other serotypes.

Antibody levels associated with protection against pneumococcal serotypes are not known but it has been hypothesized that a minimum antibody titre after the primary series associated with long term protection against invasive disease is in the range of 0.15 to 0.5 μg/ml.[5] In our study all infants had reached antibody concentrations of > 0.15 μg/ml and more than 95% of the infants in addition had reached antibody concentrations of > 1.0 μg/ml for all seven serotypes after three doses of the PNC vaccine. Thus, the vaccine is likely to provide a long-term protection against invasive pneumococcal diseases associated with one of the seven vaccine serotypes. Unfortunately the current heptavalent vaccine is estimated to cover only 52% and 25.5% of the serogroups responsible for invasive pneumococcal diseases in developing countries[21] and Bangladesh[22] respectively. It has been suggested that an effective vaccine for global use should preferably

contain the following nine serotypes: 1, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. Such a formula would cover 50.5% of the serotypes occurring in Bangladesh.[22]

Supplementation with 5 mg elemental zinc per day from 4 weeks to 33 weeks of age resulted in significantly higher titres for serotype 9V after three doses of the PNC vaccine compared to titres in infants who had received a placebo. After one or two doses of the PNC vaccine there were higher albeit only marginal statistically significant titres in the zinc compared to placebo group for serotype 23 F. There were no differences between zinc and placebo groups for the occurrence of adverse events during five days post-immunization nor for the antibody response to the DTP-Hib vaccine. Contrary to expectations, zinc supplementation resulted in a more negative response to the delayed hypersensitivity tuberculin skin test but the effect was small and only of marginally statistical significance.

Zinc deficiency has been associated with reduced numbers of T-and B-lymphocytes, impaired epithelial barrier functions, atrophy of lymphoid organs and decreased macrophage functions resulting in a depressed delayed hypersensitivity reaction.[8] In infants and children most of these abnormalities have been responsive to zinc supplementation.[9,10,23,24] We therefore had anticipated a beneficial effect of zinc supplementation on the antibody response to childhood vaccines. In our study the high immunogenicity of the PNC vaccine in all infants may have masked any potential beneficial effects of zinc supplementation. Indeed, the two serotypes that were responsive to zinc supplementation in our study (9V and 23F) were among the least immunogenic of all seven serotypes.

Previously reported findings from our study demonstrated a beneficial effect of zinc supplementation on the incidence of reported Acute Lower Respiratory Tract Infections (ALRI) but only in infants that had been defined zinc deficient (serum zinc < 9.18 μmol/L) upon enrollment.[13] The biological pathways for this observed effect are as yet unclear. The PNC vaccine may have been protective against at least some of the ALRI infections that were caused by the S. Pneumonia of one of the seven vaccine serotypes. A higher immunogenicity to some of the serotypes after zinc supplementation may have resulted in a more effective protection against the disease in some cases but this hypothesis is obviously preliminary and requires confirmation through controlled trials investigating the efficacy of the PNC vaccine against invasive pneumococcal diseases in developing countries and defining the exact role of zinc in this process.

In conclusion, a heptavalent pneumococcal conjugate vaccine proved to be safe and immunogenic when administered to infants from a poor urban community in Bangladesh. The efficacy of the vaccine in preventing invasive diseases and the effect of micronutrient supplementation on this efficacy are yet to be determined.

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8

General discussion

In this thesis two intervention studies are described to evaluate the impact of zinc supplementation on infant's intra-uterine and postnatal growth, gestational age duration, morbidity and immune response. In the first study, 559 pregnant women from Dhaka city slums were either supplemented with daily 30 mg elemental zinc or received a placebo starting from 12-16 weeks gestation until delivery. In the second study, a separate cohort of 301 four-week old infants from the same study-area were supplemented daily with 5 mg elemental zinc or placebo until 24 weeks of age.

In this chapter the findings of both studies will be evaluated and discussed, the effects of the two interventions will be compared and possible policy implications and recommendations for future research will be discussed. Prior to evaluating the effects of the interventions, this chapter will first reflect on the general zinc status of our study population as estimated from dietary intake data and blood zinc analysis.

ZINC STATUS AND ZINC DEFICIENCY DURING PREGNANCY IN URBAN BANGLADESH.

Dietary intake

The pregnant women enrolled in the intervention study were at-high risk for inadequate energy and zinc intakes (*Chapter 2*). Median intakes of energy (6065 kJ/d) and zinc (6.1 mg/d) were low at 4 months gestation although intakes increased between 4 and 8 months of pregnancy by 11% and 23% for energy and zinc, respectively. Median dietary zinc intakes of 8 to 14 mg/day have been reported from pregnant women in the US and UK[1] while intakes of 6 to 7 mg/day were observed in studies in Brazil,[2] Malawi,[3] and Peru.[4] The intakes observed in Bangladesh were therefore comparable with intakes observed in other less industrialized countries and lower than intakes reported from developed countries.

Zinc requirements during pregnancy to meet increased physiological demands are influenced by usual zinc intakes, possible changes in fractional zinc absorption and/or endogenous zinc excretion during pregnancy and the bio-availability of zinc from the diet. In animals and humans, regulation of zinc absorption and endogenous intestinal excretion are the primary means to maintain zinc homeostasis at varying levels of zinc intakes.[5] In populations with habitually low zinc intakes, conservation of endogenous zinc is thought to be more critical in maintenance of zinc homeostasis than changes in absorption but there are important limitations of adaptation mechanisms in situations of extremely low or chronically low intakes.[6]

The increased physiological zinc requirements during pregnancy may be partly met by an increased fractional absorption.[7] However, the changes in

fractional absorption during human gestation are modest and less than the significant increases that occur during early human lactation.[6,8] Estimates of zinc requirements during pregnancy do therefore not yet take into account these possible metabolic adaptations.[7,9]

Bioavailability of zinc in the diet is influenced by the food source as well as other components of the diet that inhibit or promote absorption of zinc. Phytate:zinc ratios above 15 are associated with suboptimal zinc status.[10] Unfortunately, in our study we were not able to estimate phytate:zinc molar ratio's, because the phytate concentrations of many of the foods consumed in this population were not known. However, most (74%) of the daily zinc intake came from cereals (rice) and pulses and total protein accounted for 12.1% of the daily energy intakes (*Chapter 2*) while the intake of animal protein was considered negligible. Based on these dietary characteristics, we classified the zinc in this diet to be of low bioavailability according to WHO guidelines,[30] and assumed that only 15-20% of the dietary zinc in our population was available for absorption.

Thus assuming a poor bio-availability of dietary zinc, we used the probability approach,[11] to estimate the proportion of women at risk for inadequate dietary zinc intakes (*Chapter 2*). As expected, the percentage at risk for inadequate intakes was high: 98% and 89% for normative and basal requirements[7] respectively.

Serum zinc concentrations

In contrast to these low dietary zinc intakes, mean serum zinc concentrations at 4 and 7 months gestation were higher than expected in our population (15.3 [SD 4.3] and 15.2 [SD 4.3] μmol/L respectively). At 4 weeks gestation only four women had blood zinc concentrations < 9.18 μmol/L (*Chapter 3*), the cut-off used as the reference for zinc deficiency in other studies.[12,13] In adults, a normal range for serum zinc is considered to be between 10.7-15.3 μ mol/L,[14] similar to values observed in children, and these values usually tend to decline during pregnancy. Lower plasma zinc concentrations in pregnant women compared to non-pregnant controls of comparable age have been observed as early as 2 months gestation.[15] At 19 weeks gestation, serum zinc concentrations of 9.7 (SD 1.5) μmol/L were observed in low-income women in the US[16] whereas average plasma concentrations at 10-24 weeks gestation were 10.6 (SD 2.1) and 8.1 (SD 2.3) μmol/L in Peru[17] and Malawi[18] respectively. Several possible explanations for the higher than expected serum zinc concentrations in our study were discussed (*Chapter 3*). These included an increase due to food intake,[14] the delay of up to 6 h between collection and separation of samples,[19] and possible contamination during sample collection or analysis. The relatively high serum zinc concentrations in our population may also reflect an adaptation to habitually low dietary zinc intakes[6] since inverse relationships between dietary zinc supply and zinc status

were observed in several studies.[7] However, this would not explain the discrepancies between serum zinc concentrations observed in our study population and the concentrations reported from populations with similar dietary zinc intakes.[17,18]

The limitations of plasma/serum zinc as an indicator for body zinc status are well known,[7,20] although it is still considered the best available indicator for assessment of zinc status on a population level.[20] Because low dietary intakes of poorly available zinc were observed in our population despite the relatively high serum zinc concentrations, while acknowledging the difficulties with the interpretation of serum zinc values, it was assumed that the study described in this thesis was performed in a population of pregnant women with suboptimal zinc status.

ZINC STATUS AND ZINC DEFICIENCY DURING INFANCY IN URBAN BANGLADESH.

Dietary intake

All infants enrolled in this intervention trial were breast-fed for the entire duration of the study but the rate of exclusive breastfeeding was low due to early introduction of water provided along with breast milk. This practice is very common in Bangladesh and has been reported before.[21] Infants receiving breastmilk and water only were classified as "predominantly" breast-fed and infants receiving breast milk and other complementary fluids and foods were classified as "partially" breast-fed. Using these standard definitions, 16% of all infants were exclusively or predominantly breast-fed (breast milk and water) and 80% were partially breast-fed at 24 weeks of age (*Chapter 6*). Breastmilk was therefore the primary source of dietary zinc in these infants. Unfortunately, we did not assess breastmilk zinc concentrations in our population. In developed countries, the average zinc intake during lactation is thought to be sufficient to ensure adequate breastmilk zinc concentrations and milk zinc output during the first months of lactation.[22] However, compared to the concentrations observed in western women, lower breastmilk zinc concentrations at comparable time points during lactation have been observed in some[23] but not all[24] studies on milk zinc concentrations of women in developing countries.

Serum zinc concentrations.

Average serum zinc concentrations at 1 and 6 months of age in the non-supplemented infants enrolled in our intervention study were 11.7 (SD 3.0) and 10.7 (SD 2.9) µmol/L respectively (*Chapter 6*). These concentrations are comparable to concentrations observed in older infants and children in Ethiopia[25] and Chile,[26] but higher than concentrations found in older infants and children in

India[27] and small-for-gestational-age infants in Chile.[28] In addition to the possibilities already discussed for the high maternal serum zinc concentrations, breastfeeding practices may have contributed to the relatively higher serum zinc concentrations among the infants in this study. In developed countries, zinc deficiency is relatively rare among term breast-fed infants during the first months of life.[29] A high bioavailability of zinc from human milk together with efficient conservation of endogenous zinc by the gastrointestinal tract ensures that dietary zinc intakes from human milk are adequate during the first 6 months of life despite declining breastmilk zinc concentrations.[30]

Because at 4 weeks of age only 4% of the infants in this study had serum zinc concentrations below the cut-off value for zinc deficiency (9.18 μmol/L), similar mechanisms may have protected infants in this study from developing zinc deficiencies (*Chapter 6*), at least for the duration of this study. However, the situation may be different for the period after 6 months of age. Available data of zinc intakes of infants older than 6 months of age in Peru and Mexico suggest that many infants and young children in developing countries are unlikely to meet their zinc requirements and are at-risk of suboptimal zinc status.[31]

THE EFFECTS OF ZINC SUPPLEMENTATION DURING PREGNANCY ON PREGNANCY OUTCOME, GROWTH, MORBIDITY AND IMMUNE RESPONSE.

Supplementation with 30 mg elemental zinc/day during the last two trimesters of pregnancy did not improve birth weight or gestational age (*Chapter 3*) nor did it have an impact on postnatal growth during the first six months of life. However, it resulted in reduced morbidity from diarrheal diseases and impetigo in infants born with low birth weight (LBW; *Chapter 4*). This observed reduction was probably due to an improved cellular immunity or a less compromised immune function. A positive albeit small effect on response to the delayed hypersensitivity skin test on BCG vaccine at 24 weeks of age was observed in LBW infants whose mothers had received zinc during pregnancy (*Chapter 5*).

We are not aware of previous studies that report effects of zinc supplementation during pregnancy beyond the neonatal period. Most trials of maternal zinc supplementation have been performed in developed countries and have limited their assessments mainly to an effect on birth weight. The results of these trials have been inconsistent.[32-34]

However, a similar double-blind, placebo-controlled randomized trial has recently been performed in Peru,[4,17] and the findings from that trial are remarkably comparable to ours. In Peru, 1295 women were enrolled between 10 and 24 weeks of gestation and randomly assigned to prenatal supplements of 60 mg

iron and 250 μg folate with or without 15 mg zinc. The infants were followed until one year of age. In the Peruvian study, zinc supplementation during pregnancy did not improve birth weight or gestational age even though it increased maternal serum zinc and infant cord blood zinc. However, zinc supplementation did seem to affect fetal neurobehavioral activity as measured by fetal movements and heart rate[35] and resulted in increased concentrations of immunoglobulins in cord-blood and a reduced number of febrile episodes in the infants during the first six months of life.[36] Recently reported findings of the Peruvian study suggest that infants whose mothers were supplemented with zinc during pregnancy had better growth in the second half year of life, despite the absence of an effect on intra-uterine or early postnatal growth (Zavaleta N, personal communication). Because infants were not followed beyond six months of age, those findings could not be confirmed in the current study in Bangladesh.

The findings of the study described in this thesis are consistent with other studies showing effects on both prevalence and incidence of diseases after zinc supplementation in infants and children, and are thought to reflect the prominent role of zinc in both immune and non-immune host defense mechanisms.[37] Work with animal models indicate a role of perinatal zinc status in optimal development of the fetal immune system. Reduced lymphoid organ size, gamma globulin concentrations and blood lymphocytes numbers have been observed in off-spring born to zinc deficient mice and rhesus monkeys. Antenatal zinc deprivation in rats resulted in lower serum immunoglobulin concentrations, in particular IgA, IgG2 and IgM in the off-spring at 6 months of age. These abnormalities may persist into adulthood.[38] Maternal zinc status during pregnancy may also affect *in-utero* acquisition of antibodies due to the role of zinc in placental transport of immunoglobulins.[1] It is therefore conceivable that the reduction in morbidity in the LBW infants (*Chapter 4*) was the result of an improved immune system (*Chapter 5*) or perhaps a less compromised immune system in LBW infants during gestation.

The observation of a lack of effect on intra-uterine or postnatal growth despite reductions in morbidity, has been discussed extensively (*Chapter 4*). Although it is appreciated that growth and morbidity are related clinical features, it is believed that in relation to the effect of zinc the two should be considered as separate outcomes. Different biological pathways may explain the role and effect of zinc in the two outcomes. The effect of zinc on growth may be due to a direct effect of zinc on protein synthesis and gene expression,[39] but there can also be an indirect impact of zinc on growth through reductions in morbidity and increased appetite.[25] In our population a growth effect of zinc might not have been observed

because of the time-lag between the observed reductions in morbidity and a possible beneficial effect on growth.

Predictors for an effect of zinc on growth in children are low height-for-age and zinc deficiency defined as low plasma zinc concentrations.[39] Zinc supplementation among 6-21 month old infants in Ethiopia resulted in more pronounced effects on weight and length velocities in stunted compared to non-stunted supplemented infants.[25] Similar predictors might be relevant in the context of intrauterine growth,[33] such that, zinc supplementation during pregnancy might exert a beneficial effect on intrauterine growth primarily in populations that are zinc deficient and at high risk for poor fetal growth. A clinically and statistically significant effect on birth weight and head circumference was observed in a maternal zinc supplementation trial in Alabama in which only women with low plasma zinc concentrations on enrollment were selected.[40] The effects were more pronounced in women with a relatively low Body Mass Index (BMI < 26 kg/m^2). In children however, studies in malnourished populations in Mexico,[41] Jamaica,[42] and Uganda[43] all failed to show an effect of zinc supplementation on growth. It has been suggested that zinc might not be the primary growth limiting nutrient in otherwise nutritionally deprived children.[44] Also in our population of pregnant women zinc might not have been the primary intrauterine growth limiting nutrient. This is suggested by the lack of a growth effect in our study and by the low total energy intakes during pregnancy. In addition, higher energy, but not zinc intakes during pregnancy were related to larger maternal weight gains and a reduced risk of premature deliveries (*Chapter 2*).

THE EFFECTS OF ZINC SUPPLEMENTATION DURING INFANCY ON INFANT GROWTH, MORBIDITY AND IMMUNE RESPONSE.

Supplementation with 5 mg elemental zinc/day between 4 and 24 weeks of age improved growth and reduced the incidence of ALRI in infants who were zinc-deficient (< 9.18 μmol/L) at 4 weeks of age. However, among zinc sufficient infants there were no effects on growth or morbidity, even though zinc supplementation improved zinc status (*Chapter 6*). The biological pathways for this effect in zinc-deficient infants are still to be clarified. Zinc supplementation resulted in a higher anti-body response to some serotypes (9V and 23F) of the 7-valent pneumococcal conjugate vaccine (PNC) but it had no effect on other serotypes nor on the response to the combined diphtheria, tetanus toxoid and pertussis (DTP)-*Haemophilus influenzae* type-b (Hib)- conjugate vaccine while a small, marginally significant ($p < 0.09$) negative effect on the response to PPD skin test was observed (*Chapter 7*).

This study was one of the first to supplement very young infants (< 6 months of age). Most intervention trials assessing the effect of zinc on growth or morbidity have been conducted in children older than 6 months of age,[37,39] i.e., beyond the period of maximal growth velocity. Immunologically, the period between birth and six months of age is characterized by waning passive immunity from maternal antibodies and continued development of the infants immune system and immune response. It has been hypothesized that interventions with zinc earlier in infancy might therefore be more effective in preventing growth faltering and reducing morbidity patterns among children at risk.[45] Infants supplemented before 6 months of age in Chile and France,[28,46] had improved growth while a reduced morbidity but no effect on growth was observed after zinc supplementation during the first six months of life among LBW infants in Brazil.[47] In contrast, greater reductions in morbidity after zinc supplementation have been observed in older compared to younger age groups.[27] In our study, zinc supplementation between 4 and 24 weeks of age did not affect growth or morbidity in those infants who were not zinc deficient at 4 weeks of age while the effects on the immune response were not clear. Despite the rationale for a stronger effect in this young age group, an alternative hypothesis (*Chapter 6*) is that younger infants in fact, will have a reduced response to zinc compared to older infants. It is known that compared to older infants, the overall burden of morbidity in the 0-6 months age group is low. Moreover, most of these infants presumably do not yet develop a zinc deficiency as suggested in our study by a prevalence of only 14% with serum zinc concentrations < 9.18 μmol/L at 4 weeks of age and by the lack of a physiologic response in growth and morbidity to zinc supplementation among the non-zinc deficient infants. The observation that serum zinc concentrations in the placebo group were declining between 4 and 24 weeks of age, while the proportion of zinc-deficient infants increased from 14 to 22% (*Chapter 6*) indicate that the zinc status of these infants was deteriorating over time. After 6 months of age, these infants might therefore be at-risk for sub-optimal zinc status and interventions with zinc might have a greater impact beyond this age.

ZINC NUTRITION THROUGHOUT THE LIFE CYCLE.

In this thesis interventions with zinc are described during two critical phases of the life-cycle, i.e., pregnancy and early infancy. Comparisons of the results between the two studies might provide useful information for future research and policy implications, although it is recognized that comparisons between different studies are difficult to interpret. The two cohorts of pregnant women ("the pregnancy cohort") and infants ("the infant cohort") came from the same study area but might nevertheless have been different in essential baseline

characteristics. Although socio-economic characteristics were not different between the two cohorts, mothers of infants in the infant cohort were found to be older and had better nutritional status (weight, length) at one and six months post-partum compared to mothers in the pregnancy cohort. Likewise, infants in the infant cohort had a better nutritional status at one and six months of age than infants in the pregnancy cohort. The two cohorts therefore were not completely comparable. Based on these differences, one probably would have predicted a greater effect of zinc on growth in the more malnourished pregnancy cohort, since low nutritional status is considered a predictor for a growth-effect of zinc. However, this was not observed in our studies.

Effects on growth

Supplementation with zinc during pregnancy did not improve intra-uterine or postnatal growth or pregnancy duration whereas supplementation with zinc in the infants improved linear and ponderal growth, but only in those infants (14% of the population) who were zinc-deficient at baseline. To better understand the effect of maternal zinc supplementation in the zinc-deficient population data of pregnant women and their infants with low serum zinc concentrations were segregated and analyzed. As reported previously (*Chapter 3*), only 21 (4%) of the pregnant women had low baseline serum zinc concentrations (< 10.6 μmol/L) and no effect on birth weight was observed in these women. However, surprisingly similar findings were observed between the two infant cohorts when subsequently data were segregated and analyzed of the 20 (6%) infants born to study women, who were zinc deficient (<9.18 μmol/L) at one month of age. The fourteen zinc-deficient infants whose mothers received zinc during pregnancy had significantly greater weight gains during the first six months of life compared to the six zinc-deficient infants in the placebo group (3.61 [SD 0.80] kg *vs.* 1.91 [SD 2.28] kg in zinc and placebo groups respectively, $p < 0.05$) even after controlling for nutritional status at birth, gestational age and gender. The small sample sizes are acknowledged, but nevertheless the consistency in the observations between the two studies provides valuable information. Both antenatal and postnatal zinc supplementation may benefit growth but the effects are restricted to infants who for some reason are zinc-deficient in early infancy. For most infants however, zinc supplementation did not benefit growth, and possible explanations for this finding which will have potential implications for future policy and research were already discussed.

First, zinc might not have been the primary growth-limiting nutrient for these women and infants. In that respect, supplementation with multiple micronutrients in addition to zinc, might be more effective. One concern related to the use of multiple micronutrient supplementation has been the potential negative effects of interactions between different nutrients when provided

simultaneously in one supplement.[48] Although there is evidence of interactions among several micronutrients at the metabolic level[49] very little is known about the significance of these interactions for pregnancy outcomes. Prenatal iron supplements for instance impaired zinc absorption in pregnant women in Peru, but the inclusion of zinc in the supplements reduced the potential inhibitory effects of iron.[50] Adding zinc to the iron/folate supplement did not effect hemoglobin levels[51] and led to higher zinc concentrations in pregnant women.[17] Use of a multivitamin/mineral prenatal supplement beginning in the first or second trimester of pregnancy was associated with twofold reductions in risk of LBW and preterm delivery in low-income US women.[52] The supplement used in this study contained, among others, 25 mg zinc, 1 mg folic acid and 65 mg iron. Unfortunately, to date, no controlled intervention trials have examined the effects of multi-micronutrient supplements during pregnancy in less-developed countries where multiple micronutrient deficiencies are common.[49] Several trials are currently underway, including a multi-country trial co-ordinated by UNICEF,[53] which will provide important information on the effect of multiple micronutrient supplementation on pregnancy outcomes.

Secondly, the relatively limited duration of the studies in Bangladesh might have prevented the observation of an effect on growth. As previously discussed, the observed reductions in morbidity might yield a potential indirect effect of zinc on growth beyond 6 months of age. In rural Bangladesh, episodes of dysentery in particular have been associated with lower annual weight and height gains among children under five years of age.[54] In addition, the timing of the antenatal interventions might not have been optimal for a growth-effect. Several studies have indicated that maternal pre-pregnancy weight is the most important predictor for infant birth weight,[55,56] and interventions aimed at improving maternal nutritional status prior to conception might therefore be more effective. Data from studies in East Java, Pakistan and Guatemala further suggest that interventions with nutritional supplements should evaluate overall change in maternal nutritional status across a full reproductive cycle, in conjunction with changes in birth weight and nutritional status between two siblings born during that cycle,[57,58] while taking into account that differential effects might occur, depending on initial maternal nutritional status.[59]

Effects on morbidity

Maternal supplementation with 30 mg elemental zinc/day during the last two trimesters of pregnancy reduced morbidity from diarrheal diseases and impetigo, particularly among LBW infants (n= 176, 43%; *Chapter 3*). By contrast, infant zinc supplementation did not have an effect on morbidity in most infants, except for a lower incidence of Acute Lower Respiratory Infections (ALRI) after

zinc supplementation in infants that were zinc-deficient at 4 weeks of age. Unfortunately, birth weight information was not available for these infants and the interaction of infant zinc supplementation and low or normal birth weight could not be assessed. However, the effect of infant zinc supplementation for various levels of infant's nutritional status at 4 weeks of age could be assessed, but no differences in effects on morbidity were observed (*Chapter 6*).

Zinc plays a distinct and essential role in the development of the immune system[38] and it is believed that supplementation with zinc during pregnancy most likely benefited the immune system of the infants born with LBW, whose immune systems would otherwise have been compromised. Compared to infant supplementation, supplementation with zinc during pregnancy might therefore be a more effective way to reduce certain critical health risks associated with LBW in this population.

The prevalence of LBW in Bangladesh is the highest in the world,[60] and in our study 43% of all live births were LBW (*Chapter 3*), mostly (76%) due to intrauterine growth retardation (IUGR). These figures are comparable to prevalences of LBW and IUGR observed in other studies in Bangladesh.[61,62] Infants born with IUGR are known to be at-risk for impaired immunity, increased morbidity and mortality and abnormal cognitive development later in childhood and adulthood.[63,64] The findings of this study indicate that birthweight and LBW may be considered primarily an indicator of risk rather than a direct cause of morbidity and mortality (*Chapter 4*). In this regard, being born too small would not be so disadvantageous if it were not for the adverse health outcomes later in life that are associated with LBW. Intervention studies aiming to reduce incidences of LBW should therefore continue to observe infant outcomes beyond the neonatal period.

The findings described in this thesis indicate that maternal zinc supplementation during pregnancy may contribute to a reduction in the disadvantaged health outcomes of LBW infants. This could potentially lead to important changes in health and nutritional status throughout the life-cycle of these infants. These findings suggest that zinc supplementation during pregnancy might enhance the development of the fetal immune system in infants born with LBW which subsequently leads to a reduced morbidity from infectious diseases in these infants. Potentially, this could result into an improved health and growth beyond six months of age and, eventually, in a healthier and better nourished child, adolescent, and woman of child-bearing age. Evidence from other studies suggest that zinc supplementation during childhood might further improve the child's growth and health. It was hypothesised in this discussion that supplementation with zinc during other critical phases of the life cycle, i.e., adolescence might perhaps result in an improved pre-pregnancy nutritional status and eventually in improved pregnancy weight gains and birth weights.

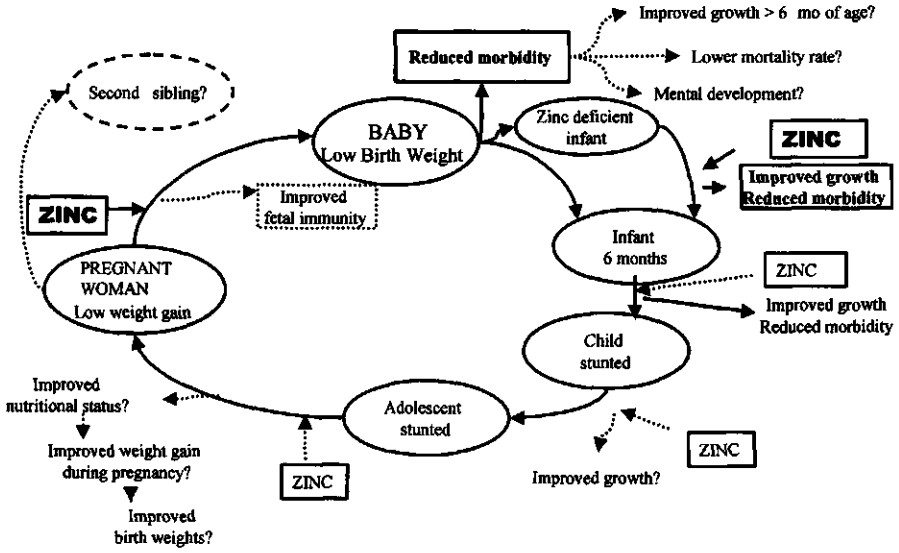

Figure 1. Zinc nutrition throughout the life cycle.

In an attempt to describe the potential role of zinc throughout the life cycle, the ACC/SCN life-cycle conceptual framework as described in *Chapter 1*, was modified (figure 1), adding the findings of this study, the evidence provided by some other studies and the hypotheses described in this discussion which should be investigated in future research.

SUMMARY OF CONCLUSIONS.

The aim of the first study described in this thesis was to evaluate the effect of supplementation with 30 mg elemental zinc/day during the last two trimesters of pregnancy in poor urban women from Bangladesh (*Chapter 1*). Based on the findings of this study, it was concluded that antenatal zinc supplementation did not improve intra-uterine or postnatal growth but resulted in a reduced morbidity from diarrhoeal diseases and impetigo in the infants during the first six months of life, particularly in infants born with LBW. These effects were most likely due to improved cellular immunity in these infants as indicated by a higher proportion of positive responses to the PPD skin test at 6 months of age.

The second study aimed to evaluate the effects of supplementation with 5 mg elemental zinc/day in Bangladeshi infants from 4 to 24 weeks of age (*Chapter 1*). The conclusions of this study were that zinc supplementation improved linear and ponderal growth and reduced morbidity from Acute Lower Respiratory Infections (ALRI) but only in infants that were zinc deficient (serum zinc < 9.18 μmol/L) at 4 weeks of age. In most infants however, supplementation with zinc did not have any effect on growth or morbidity. There was no clear effect of zinc supplementation on infant's immune response. Zinc improved the antibody response to certain Pneumococcal serotypes whereas a small but negative effect on the response to the PPD skin test was observed.

POLICY IMPLICATIONS

Even though issues related to a potential interaction between micronutrients and programmatic issues regarding the distribution of and compliance to antenatal supplements still need to be resolved, child health and survival programs in developing countries should consider the inclusion of zinc in antenatal multiple micronutrient supplements especially in regions with a high prevalence of LBW (*Chapter 4*). In the study described in this thesis pregnant women were supplemented with daily 30 mg elemental zinc and no apparent adverse effects related to this dosage were observed. In Peru, pregnant women were supplemented with 15 mg zinc/day added to an iron/folate supplement and similar beneficial effects on infant morbidity were observed[36] whereas no evidence for potential adverse effects on iron status were reported.[51] A dosage of 15 mg zinc/day, the current recommended daily allowance,[65] therefore seems to be a reasonable safe and effective dose for most women in developing countries.

More research is required to enable formulation of policy directions regarding the use of zinc supplements in infants during the first months of life. Zinc might be beneficial in infants that are zinc-deficient at a very young age and supplementation of these infants could be considered. Because appropriate or feasible screening techniques to identify zinc deficiency are lacking in most developing country settings, universal zinc supplementation remains the only option. However, there are several concerns related to the universal supplementation of very young, exclusively breast-fed infants in countries like Bangladesh. First, supplementing breast-fed infants under less hygienic conditions should preferably be avoided. A better strategy to prevent zinc deficiency might perhaps be to enhance the promotion of exclusive breast-feeding until the age of six months. Information from our study suggest that breast-feeding might protect most of these infants from developing a zinc deficiency before the age of six months (*Chapters 4 and 6*). Secondly, there is uncertainty regarding the most appropriate dosage of zinc for supplementing malnourished infants below six

months of age. In the study described in this thesis infants were supplemented with daily 5 mg elemental zinc which is the recommended daily allowance for this age group.[65] No evidence for any adverse side effects related to the zinc supplement was found in our study but the higher proportion of negative responses to the PPD skin test in zinc compared to placebo supplemented infants (*Chapter 7*), suggesting a potential negative effect on cellular immunity warrants further research. In addition, a statistically significant lower score on the Mental Development Index (MDI) of the Bailey scale was observed in the zinc-supplemented infants at 13 months of age in an addendum to our study[66] and more research is required to explain these findings. Finally, a significantly higher mortality was observed among severely malnourished children in Bangladesh receiving a high-dose (6 mg/kg bodyweight/day) zinc treatment,[67] probably due to an inhibitory effect of zinc on copper absorption, suggesting that caution is warranted in supplementing malnourished children with high dosages of zinc.[68]

FUTURE RESEARCH

Future research on the role of zinc during pregnancy should include:

- Studies on the efficacy and efficiency of antenatal multiple-micronutrient supplements, including zinc in a dosage of at least 15 mg/day while taking into account possible interactions with protein/energy intakes. Potential interactions between nutrients when provided in one tablet should also be evaluated in these studies.
- Intervention studies with zinc and/or multiple micronutrients, with the aim to improve maternal nutritional status prior to conception, preferably in regions where maternal undernutrition is common. In particular, interventions during phases of the lifecycle when growth is accelerated (i.e., in adolescence) should be explored.
- Studies on the exact role of zinc in the development of the immune system
- More research on the effects of maternal zinc supplementation on cognitive and motor development in infants born with LBW versus NBW and in zinc-deficient versus non-zinc deficient infants.
- All proposed studies on the effect of maternal zinc and/or multiple micronutrient supplements should include assessments of breast milk zinc and continue observations beyond the neonatal period, and preferably throughout a full reproductive cycle.

Future research on the role of zinc during infancy should include:

- Studies on the interaction between breastfeeding practices, breastmilk zinc concentration, maternal dietary zinc intake and infant zinc status during the first

six months of life and beyond in a developing country setting. In particular, it should be explored why certain infants become zinc-deficient at a very early age whereas others do not.

- Studies on the effect of zinc supplementation in infants should preferably be able to distinguish between effects in infants born with LBW and infants born with NBW.
- Research on guidelines for effective and safe dosages of zinc for infants below six months of age in undernourished populations.
- More research on the effects of infant zinc supplementation on cognitive and motor development in infants born with LBW versus NBW and in zinc-deficient versus non-zinc deficient infants.

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Summary

Zinc deficiency may be widely prevalent in many developing countries, but was not recognized as a public health problem until recently. Zinc deficiency has been associated with reduced growth and development, impaired immunity and increased morbidity from infectious diseases. As a constituent of over 200 metallo-enzymes in the human body, zinc is known to play an important molecular role in processes of gene replication, activation and repression, as well as DNA transcription and translation and protein synthesis. The physiological role of zinc during periods of rapid growth and development emphasises its importance during the periods of gestation, fetal life, and early infancy.

The first study described in this thesis aimed to evaluate the effect of zinc supplementation during the last six months of pregnancy on pregnancy outcome, infant growth, morbidity from infectious diseases and immune response to childhood vaccines during the first six months of life. The aim of the second study was to evaluate the effect of zinc supplementation between 1 and 6 month of age in infants on infant growth, morbidity from infectious diseases and immune response to childhood vaccines during the study period. The studies were performed in a population at high risk of growth faltering and increased morbidity in the urban slum areas of Dhaka, Bangladesh; an area where the prevalence of low birth weight (LBW) is high and zinc deficiency is assumed to be common.

In the first study, a total of 559 women were enrolled between 12 and 16 wk of gestation, stratified by parity and randomly assigned to receive 30 mg elemental zinc/d (n=269) or placebo (n=290). Supplementation continued until delivery. Serum zinc was estimated at baseline and 7 mo of gestation. Dietary intake by means of a 24 h recall and anthropometric measurements were assessed monthly from 4 to 8 months of gestation. Weight, length, and gestational ages of 410 singleton infants were measured within 72 h of birth. Infants were followed until 6 mo of age. Information on infant's morbidity from infectious diseases was collected weekly by mother's recall. Infant anthropometrics were measured monthly and serum zinc was assessed at one and six months of age. A total of 383 infants (184 from zinc group and 199 from placebo group) completed the entire 6 months of follow-up. The infants had been immunized with the standard Bacille Calmette Guerin (BCG) at birth. A subcohort of 203 infants were in addition immunized at one month-intervals with three doses of the combined diphtheria, tetanus toxoid and pertussis (DTP)-*Haemophilus influenzae* type-b (Hib)- conjugate vaccine starting at 9 weeks of age. The delayed hypersensitivity (PPD) skin test was performed in 345 infants at 24 weeks of age. Hib polysaccharide (PRP) antibodies were assessed for 91 infants at 4 and 24 weeks of age.

The pregnant women enrolled in the intervention study were at-high risk for inadequate caloric and zinc intakes. Median intakes of energy (6065 kJ/d) and zinc (6.1 mg/d) were low and more than 85% of the women were estimated to be at-risk for inadequate dietary zinc intakes (*Chapter 2*). No significant effect of zinc treatment was observed on infant birth weight (2513 {SD: 390} compared with 2554 {SD: 393} g in zinc and placebo groups respectively), or on gestational age, infant birth length, head-, chest- or arm circumference (*Chapter 3*). However, compared to infants of the placebo group, infants of mothers receiving zinc during pregnancy showed significantly reduced risks for incidence of acute diarrhea (RR: 0·84; 95%CI: 0·72,0·98), dysentery (RR: 0·36; 95%CI: 0·25,0·84) and impetigo (RR: 0·53; 95%CI: 0·34,0·82) between 0 and 6 months of age. The reductions were observed in LBW infants (n=176, 43%) whereas no significant differences were found in normal birth weight infants. There were no differences in infant growth or serum zinc concentrations between treatment groups (*Chapter 4*). In infants born with LBW a lower proportion of negative responses to PPD skin test were observed in the zinc (66.2%) compared to placebo (78.5%) group (p=0.07). No differences were observed in normal birth weight infants. There were no differences in proportion of infants above the protective thresholds for anti-PRP antibodies between zinc (81%) and placebo (89%) group. Geometric mean PRP antibody titres at 4 and 24 weeks of age were not different between groups (*Chapter 5*). We conclude that supplementation with 30 mg elemental zinc/day during the last two trimesters of pregnancy in poor urban women from Bangladesh, did not improve intra-uterine or postnatal growth but resulted in a reduced morbidity from diarrheal diseases and impetigo, only in infants born with LBW during the first six months of life. These effects were most likely due to improved cellular immunity in these infants as indicated by a higher proportion of positive responses to the PPD skin test at 6 months of age.

In the second study, a separate cohort of 301 infants was enrolled at 4 weeks of age and randomly assigned to receive daily 5 mg elemental zinc (n=152) or placebo (n=149) until 24 weeks of age. Infants were followed weekly for information on compliance and morbidity while anthropometric measurements were performed monthly. Serum zinc was assessed at baseline and 24 weeks of age. Infants were immunized with the standard BCG vaccine at 4 weeks of age and with the combined DTP-Hib vaccine from 9 weeks of age. A subcohort of 241 infants was additionally immunized with the heptavalent pneumococcal conjugate (PNC) vaccine at 18 ± 1 weeks of age. Response to delayed hypersensitivity (PPD) skin test and antibody response to Hib and to each of the seven PNC serotypes were assessed at 4, 24 and 33 weeks of age.

At 24 weeks of age serum zinc levels were higher in the zinc compared to placebo supplemented infants (13.3 {SD 3.8} *vs.* 10.7 {SD 2.9} μmol/L; p<0.001). Significantly greater weight gains were observed in zinc group compared to placebo group for 43 infants that were zinc-deficient (<9.18μmol/L) at baseline

(3.15 {SD 0.77} *vs.* 2.66 {SD 0.80} kg, p<0.04). In the other infants no differences were observed in mean weight and length gains between 4-24 weeks. Zinc-deficient infants also showed a reduced risk for incidence of Acute Lower Respiratory Infection (ALRI) after zinc supplementation (RR:0.30; 95%CI:0.10-0.92) whereas among the non-zinc deficient infants there were no differences between treatment groups (*Chapter 6*). After three doses of PNC, geometric mean titres for the pneumococcal serotypes ranged from 3.68 to 13.34 μg/ml. Titres at 24 weeks of age were significantly higher for infants who had received PNC compared to infants who had only received DTP-Hib. There were no severe adverse events related to vaccination. Zinc supplementation resulted in higher titres for serotypes 9V and 23F but had no effect on other serotypes nor on response to DTP-Hib. Zinc had a marginal negative effect on response to PPD skin test (*Chapter 7*). We conclude that supplementation with 5 mg elemental zinc/day from 4 to 24 weeks of age improved linear and ponderal growth and reduced the incidence of ALRI, but only in infants that were zinc-deficient at 4 weeks of age. In most infants however, zinc did not have any effect on growth or morbidity. There was no clear effect of zinc supplementation on infant's immune response.

In *Chapter 8* we evaluated and compared the findings of the two interventions. We concluded that zinc supplementation during early infancy might benefit post-natal growth but the effects are restricted to infants who for some reason are zinc deficient at a very early age. For most infants of this young age however, zinc supplementation did not benefit growth and several explanations for this finding were discussed. First, zinc might not have been the primary growth-limiting nutrient for these women and their infants. Secondly, the limited duration of the studies might have prevented us from observing a growth-effect of zinc, in particularly any potential secondary effects on growth through the observed reductions in morbidity. Supplementation with zinc during pregnancy reduced morbidity from diarrheal diseases and impetigo among LBW infants. By contrast, infant zinc supplementation did not have an effect on morbidity in most infants, except for a lower incidence of ALRI in infants that were zinc-deficient at 4 weeks of age. The differences in results between our two studies indicate that, compared to infant supplementation, zinc supplementation during pregnancy might be a more effective way to reduce some of the increased health risks associated with LBW or zinc deficiency in infants below 6 months of age.

The prevalence of LBW in Bangladesh is the highest in the world and infants born with LBW are known to be at-risk for impaired immunity, increased morbidity and mortality and abnormal cognitive development. Our findings indicate that maternal zinc supplementation during pregnancy may contribute to a reduction in some of these disadvantaged health outcomes. Therefore child health and survival programs should consider the inclusion of zinc in antenatal multiple micronutrient supplements especially in regions with a high prevalence of LBW.

Samenvatting

Alhoewel lage zinkinnemingen veel voorkomen in ontwikkelingslanden, worden de consequenties voor de gezondheid pas sinds kort onderkend. Eerder werd een verband gevonden tussen zinkdeficiëntie en een verminderde groei en ontwikkeling, aantastingen van het immuunsysteem en een verhoogde kans op infectieziekten. Zink is een bestanddeel van meer dan 200 metaalenzymen in het lichaam en speelt als zodanig een belangrijke moleculaire rol in diverse cellulaire processen zoals genreplicatie, -activering en -onderdrukking, DNA transcriptie en eiwitsynthese. De fysiologische rol van zink tijdens groei en ontwikkeling benadrukken het belang van zink tijdens de zwangerschap, foetale groei en de eerste levensmaanden.

De eerste interventiestudie die in dit proefschrift wordt beschreven had tot doel om het effect te evalueren van zinksuppletie tijdens de laatste zes maanden van de zwangerschap op het geboortegewicht, de zwangerschapsduur, de groei van het kind, de ziektedruk en de reactie van het immuunsysteem op vaccinaties tijdens de eerste zes maanden van het leven. De tweede interventiestudie evalueerde het effect van zinksuppletie tussen de eerste en zesde levensmaand op de groei van het kind, ziektedruk en de reactie van het immuunsysteem op vaccinaties tussen de eerste en zesde levensmaand. De studies zijn uitgevoerd in een bevolkingsgroep met een hoog risico op verminderde groei en infectieziekten, in een sloppenwijk van Dhaka, Bangladesh; een gebied waar veel kinderen een te laag geboortegewicht (low birthweight; LBW, < 2500 g) hebben en waarvan verondersteld wordt dat zinkdeficiëntie er veel voorkomt.

In de eerste studie werden 559 vrouwen geworven die minimaal twaalf en maximaal zestien weken zwanger waren. De vrouwen werden ingedeeld naar pariteit, en vervolgens ad-random toegewezen aan de interventie of controlegroep. Vanaf de dag van werving tot aan de bevalling kreeg de interventiegroep 30 mg elementair zink per dag (n=269) en de placebogroep (n=290) een cellulose-placebo. Aan het begin van het onderzoek en bij zeven maanden zwangerschap werd het serumzink bepaald. Iedere maand werd de voedingsinneming van de vrouwen bepaald middels een 24h recall en werden hun gewicht, de lengte en armomtrek gemeten. Binnen 72 uur na de geboorte werden het gewicht, lengte en arm-, borst-, en hoofdomtrek van 410 zuigelingen gemeten. Tevens werd de zwangerschapsduur vastgesteld. De zuigelingen werden hierna gevolgd tot de leeftijd van zes maanden. Iedere week werd de moeder gevraagd naar symptomen van infectieziekten bij de zuigeling gedurende de week voorafgaand aan het interview. De zuigelingen werden maandelijks gewogen en gemeten, en bij één en zes maanden werd het serumzink bepaald. Na zes maanden waren er nog 383 zuigelingen in het onderzoek (184 in de zink- en 199 in de placebogroep). Alle zuigelingen waren bij de geboorte gevaccineerd met het

standaard Bacille Calmette Guerin (BCG) vaccin. Vanaf de leeftijd van 9 weken waren 203 van deze kinderen met maandelijkse intervals tevens gevaccineerd met 3 doseringen van het gecombineerde diphterie, tetanus, en kinkhoest (DTP)-*Haemophilus influenza* type-b (Hib)-conjugaat vaccin. Bij 24 weken werd de tuberculine (purified protein derivate; PPD) huidtest uitgevoerd in 345 zuigelingen. Hib-polysacchariden-(PRP)-antilichamen werden gemeten bij 91 zuigelingen op de leeftijd van 4 en 24 weken.

De zwangere vrouwen die aan dit onderzoek meededen, hadden een hoog risico op inadequate inneming van energie en zink. De mediaan innemingen van energie (6065 kJ/dag) en zink (6,1 mg/dag) waren laag en meer dan 85% van de vrouwen had een verhoogd risico op inadequate zinkinneming (*hoofdstuk 2*). Er werd geen behandelingseffect gevonden van zink op het geboortegewicht (2513 ± 390 en 2554 ± 393 gram in zink en placebogroepen respectievelijk), noch op de zwangerschapsduur, geboortelengte, of op hoofd-, borst- of armomtrek (*hoofdstuk 3*). Echter, zuigelingen van moeders die zink hadden gekregen tijdens de zwangerschap hadden een significant verlaagd risico vergeleken met zuigelingen in de placebogroep op het vóórkomen tijdens de eerste zes levensmaanden van acute diarree (RR: 0,84; 95% betrouwbaarheidsinterval (BTH): 0,72-0,98), dysenterie (RR: 0,36; 95% BTH: 0,25-0,84) en impetigo (RR: 0,53; 95% BTH: 0,34-0,82). De gereduceerde risico's werden alleen geobserveerd in de LBW-zuigelingen en niet in zuigelingen met een normaal (≥ 2500 g) geboortegewicht. Er werden geen verschillen gevonden tussen de twee interventiegroepen in groei van de zuigeling noch in serumzink concentraties (*hoofdstuk 4*). In de LBW zuigelingen werd een lager percentage negatieve reacties op de PPD-huidtest geobserveerd in zuigelingen uit de zinkgroep (66,2%) vergeleken met de placebogroep (78,5%; p=0,07). Bij zuigelingen met een normaal geboortegewicht werden geen verschillen tussen de twee interventiegroepen gevonden. Tevens werden er geen verschillen gevonden in het percentage zuigelingen met PRP-antilichaam-concentraties boven het beschermende niveau tussen zink (81%) en placebogroep (89%). De gemiddelde PRP-antilichaam-concentraties bij 4 en 24 weken waren niet verschillend tussen de twee groepen (*hoofdstuk 5*).

Uit deze bevindingen concluderen wij dat suppletie met 30 mg elementair zink per dag tijdens de laatste twee trimesters van de zwangerschap bij arme vrouwen uit de sloppenwijken van Bangladesh geen verbetering liet zien van de foetale of de postnatale groei. De suppletie resulteerde wel in een lager risico op diarree en impetigo tijdens de eerste zes levensmaanden, met name in LBW zuigelingen. Het hogere percentage positieve reacties op de PPD-huidtest op de leeftijd van zes maanden in deze groep kinderen geeft aan dat deze effecten waarschijnlijk werden veroorzaakt door een verbeterde cellulaire immuniteit.

In de tweede studie werd een nieuw cohort van 301 zuigelingen geworven op de leeftijd van 4 weken en ad-random toegewezen aan dagelijks 5 mg

elementair zink (n=152) of placebo (n=149) tot de leeftijd van 24 weken. De zuigelingen werden iedere week bezocht om informatie te verzamelen over het volgen van het onderzoeksprotocol en de ziektedruk terwijl iedere maand het gewicht, en de lengte, hoofd-, borst-, en armomtrek werden gemeten. Aan het begin van het onderzoek en met 24 weken werd het serumzink bepaald. De zuigelingen werden gevaccineerd met het standaard BCG-vaccin op de leeftijd van 4 weken en met het gecombineerde DTP-Hib-vaccin vanaf de leeftijd van 9 weken. 241 van deze kinderen kregen bovendien nog het heptavalent Pneumococcal conjugaat (PNC) vaccin vanaf de leeftijd van 18 ± 1 week. De reactie op de PPD-huidtest en de hoeveelheid antilichamen voor Hib en elk van de zeven Pneumococcal serotypen werden bepaald op de leeftijd van 4, 24 en 33 weken.

Op de leeftijd van 24 weken waren de serumzink concentraties hoger in de zuigelingen die zinksuppletie hadden ontvangen vergeleken met de zuigelingen die de placebo hadden gekregen (13,3 ± 3,8 vs. 10,7 ± 2,9 μmol/L; p<0,001). Een significant grotere gewichtstoename werd geobserveerd in de zinkgroep vergeleken met de placebogroep bij 42 zuigelingen die aan het begin van het onderzoek "zinkdeficiënt" waren, dat wil zeggen een serum zink concentratie hadden van < 9,18 μmol/L (3,15 ± 0,77 vs. 2,66 ± 0,80 kg, p < 0,04). Voor de overige kinderen waren er geen verschillen tussen de interventie en controlegroep in gemiddelde gewicht- en lengtetoename tussen 4 en 24 weken. Na zinksuppletie hadden zuigelingen die zinkdeficiënt waren tevens een verminderd risico op het vóórkomen van acute ontstekingen aan de lagere luchtwegen ofwel acute lower respiratory infections (ALRI) (RR:0,30; 95% BTH: 0,10-0,92) terwijl er geen verschillen werden aangetroffen tussen de twee zink en placebogroep bij zuigelingen die niet zinkdeficiënt waren (*hoofdstuk 6*). Na drie doseringen met het PNC-vaccin, lagen de gemiddelde Pneumococcal-serotypen-antilichaam concentraties in de orde van 3,6 tot 13,3 μg/ml. Op de leeftijd van 24 weken waren de concentraties significant hoger in de zuigelingen die het PNC-vaccin hadden ontvangen vergeleken met de zuigelingen die alleen het DTP-Hib-vaccin hadden gekregen. Er werden geen ernstige bijwerkingen geconstateerd na de PNC vaccinatie. Zinksuppletie resulteerde in hogere concentraties antilichamen voor de serotypen 9V en 23F maar er was geen effect voor de overige serotypen noch voor de reactie op de DTP-Hib vaccinatie. Zinksuppletie had een marginaal negatief effect op de reactie op de PPD-huidtest (*hoofdstuk 7*).

Uit de bevindingen van deze studie concluderen wij dat suppletie met 5 mg elementair zink per dag op de leeftijd van 4 tot 24 weken, de lengte- en gewichtsgroei verbeterde en het vóórkomen van ALRI verminderde, maar alleen in zuigelingen die zinkdeficiënt waren op de leeftijd van 4 weken. Echter, de meeste zuigelingen lijken geen baat te hebben bij zinksuppletie voor wat betreft de groei en ziektedruk. Er was geen duidelijk effect van zinksuppletie op de immuunreactie van de zuigelingen.

In hoofdstuk 8 evalueren en vergelijken we de bevindingen van de twee studies. We concluderen dat zinksuppletie tijdens de vroege zuigelingentijd voordelen kan hebben op de postnatale groei van de zuigelingen maar alleen in zuigelingen die om de een of andere reden al zinkdeficiënt zijn op een heel jonge leeftijd. Echter, voor de meeste zuigelingen op deze jonge leeftijd gaf zinksuppletie geen effect op de groei. Het is mogelijk dat zink niet het belangrijkste limiterende nutriënt was voor de groei in deze vrouwen en kinderen. Bovendien kan de relatief korte duur van onze studies ervoor gezorgd hebben dat wij een groeieffect van zink niet aan konden tonen en dit geldt met name voor een mogelijk secundair effect op de groei via de geobserveerde reducties in de ziektedruk. Zinksuppletie tijdens de zwangerschap verminderde de ziektedruk van diarree en impetigo, met name in kinderen met een te laag geboortegewicht. Deze bevindingen staan in contrast tot de effecten van zinksuppletie tijdens de vroege kindertijd. Hierbij werden bij de meeste kinderen geen effecten waargenomen op de ziektedruk, behalve bij kinderen die op de leeftijd van 4 weken zinkdeficiënt waren. In deze zuigelingen werd een lagere incidentie van ALRI waargenomen. De contrasterende resultaten van deze twee studies suggereren dat zinksuppletie tijdens de zwangerschap wellicht een effectieve manier is om bepaalde gezondheidsrisico's te verminderen die geassocieerd zijn met LBW of met zinkdeficiëntie.

Het percentage LBW kinderen in Bangladesh is het hoogste ter wereld. Deze kinderen hebben een verhoogd risico op aantastingen van het immuunsysteem, een verhoogde ziektedruk en verhoogde sterfte en een verminderde cognitieve ontwikkeling. Onze bevindingen suggereren dat preventieprogramma's in ontwikkelingslanden op het gebied van kindergezondheid en -sterfte dienen te overwegen om zink toe te voegen aan multi-micronutrienten supplementen voor zwangere vrouwen, met name in gebieden waar LBW veel voorkomt.

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Saskia

About the author

Saskia Osendarp was born on January 10, 1969, in Delft, The Netherlands. In 1987, she completed secondary school at the 'Lodewijk Makeblijde College' in Rijswijk. In the same year she started with her study for a MSc degree in 'Human Nutrition' at the Wageningen Agricultural University. As part of this study she conducted research projects at the Royal Netherlands Cancer Institute, Amsterdam, the regional health services (GGD) for Zeeland, Goes and the department of Human Nutrition in Wageningen. During an internship of 6 months in the Tai region of Ivory Coast she studied the relation between malnutrition and malaria in a Liberian refugee population. In 1993 she received the MSc degree. From November 1993 until June 1994 she worked at the Ragusa Cancer Registry, Sicily, Italy as nutrition researcher for the European Prospective Investigation into Cancer and Nutrition (EPIC). In August 1994 she moved to Dhaka, Bangladesh and worked for five months as data-assistant for Hellen Keller International, Bangladesh. In March 1995 she started as a PhD-fellow at the ICDDR,B: Centre for Health and Population Research, Dhaka, Bangladesh and the Division of Human Nutrition and Epidemiology of Wageningen Agricultural University. She joined the education programme of the Graduate school VLAG (advanced courses in Food Technology, Agrobiotechnology, Nutrition and Health Sciences) and completed the international course on Nutritional and Lifestyle Epidemiology organized by VLAG in 1995. In June 1997 she attended the Summer Graduate Epidemiology Program at the Johns Hopkins University, Baltimore, USA for which she was awarded a Johns Hopkins University scholarship. In October 1998 she attended the Fourth International Graduate Course on Production and Use of Food Composition Data in Nutrition in Wageningen. She was the overall winner of the 1998 Young Investigator Award of the American Society for Clinical Nutrition, at the Experimental Biology '98 meeting in San Francisco, USA.

From November 1997 she worked at the ICDDR,B as a coordinator for the Operations Research Project of the Bangladesh Integrated Nutrition Program (BINP) and continued part-time on the completion of her PhD dissertation. In March 2001 she completed the assignment in Bangladesh and returned to the Netherlands.

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