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Original Article

Effect of zinc and vitamin A supplementation on immune responses in Indonesian pre-schoolers

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Background and Objectives: Vitamin A and zinc are interrelated, but the effects of zinc on vitamin A supplementation on morbidity are inconsistent and not well understood. We investigated the effects of zinc and vitamin A supplementation on immune responses in Indonesian pre-schoolers. **Methods and Study Design:** In a two-stage study design, 826 children (2-5year old) were randomly assigned to receive daily zinc supplement (10 mg) or placebo for 4 months. At 2 months, both groups received a 200,000 IU vitamin A capsules through national vitamin A program. Data were collected at baseline, two and four months, resulting in 4 groups for comparisons: – no zinc no vitamin A (Placebo), zinc only, vitamin A only, and zinc plus vitamin A. Hair, blood and saliva samples were collected to measure hair zinc and serum retinol (vitamin A) concentration, *ex-vivo* IFN-γ, serum IgG and salivary IgA from 81 children selected randomly from each group. **Results:** At baseline, there were no differences between treatment groups. Zinc supplementation increased ex-vivo IFN-γ production, greatest amongst boys, younger (<3.5 years), normal weight and children with low baseline retinol concentration. Vitamin A supplementation increased IFN-γ only in those with low baseline retinol, with no effect on serum IgG and salivary IgA. After vitamin A supplementation, zinc had an effect on salivary IgA among younger and underweight children. **Conclusions:** Zinc supplementation increased IFN-γ (cellular immune responses) and modified the effect of vitamin A supplementation on salivary IgA (mucosal innate immune response) in younger and underweight children.

Key Words: zinc, vitamin A, supplementation, immune response, preschool children

INTRODUCTION

Vitamin A deficiency is known to have an impact on morbidity in young children,[1] and supplementation is associated with a meaningful decrease in childhood mortality and morbidity associated with a range of common illnesses.[2] However, individual study results are variable,[3-5] potentially affected by pathogen type and other factors that affect immune response including vitamin A and zinc status.

Vitamin A and zinc are metabolically interrelated in the human body, including through zinc's involvement in vitamin A release from the liver, vitamin A absorption and circulation through its role in retinol binding protein (RBP) synthesis.[6] A synergistic effect on micronutrient status from combining zinc with vitamin A supplementation has been shown in several studies. Supplementation of mothers and infants in Indonesia with beta-carotene on its own did not increase serum retinol levels,[7] while adding zinc to beta-carotene improved plasma retinol concentration.[8] A study of vitamin A and zinc supplementation among Bangladeshi children showed the greatest reduction in vitamin A deficiency in the children who received zinc plus vitamin A, increasing both serum retinol and RBP.[9] Similarly, zinc supplementation Mexican children resulted in higher plasma retinol compared to the placebo group, with those more deficient at baseline increasing most.[10]

A similar synergistic effect has been seen some morbidity and growth studies. Zinc modified the effect of mass vitamin A supplementation in Indonesian preschool children with a 34% and 30% decrease in episodes of upper respiratory tract infection and percentage of days ill respectively.[11] Similarly, compared with vitamin A alone, zinc plus vitamin A supplementation is reported to improve outcomes for malaria.[12-13] and diarrhea morbidity,[14]

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reduce infections/inflammation and increase linear growth.[15] But other studies on diarrhea and respiratory morbidity in South Africa,[16] and on acute lower respiratory tract infection in an Australian Indigenous community[17] have not shown this benefit.

These inconsistencies may relate to specific immune responses affected by vitamin A and zinc status, though few studies have examined this, providing an incomplete picture. An Indonesian study showed that children who had vitamin A and zinc deficiency had low levels of ex-vivo interferon gamma (INF-γ) production.[18] A study in Mexico city showed that zinc supplementation increased IFN-γ and Interleukin-2 (IL-2) and reduced clinical symptoms of pneumonia amongst under-five children.[19] Another study in Bangladesh showed that vitamin A supplementation alone increased cellular immunity only on infants who had adequate serum retinol[20] while vitamin A and zinc given together decreased respiratory tract infections.[21]

We report here on a study that looked at the effect of zinc supplementation on immune response in Indonesian children aged 2-5 years before and after they received vitamin A supplementation as part of the routine national program. Following guidance on the suitability of markers for assessing immune response in nutrition interventions,[22] we used *ex-vivo* IFN-γ production as a marker of cell mediated immunity, serum Immunoglobulin G (IgG) as markers of humoral immunity and salivary secretory IgA as a marker of local mucosal immunity. Other factors are expected to influence the immune response i.e. age, baseline vitamin A status, anthropometric status[22,23] and gender.[22,24] Therefore, we also assessed the effects in subgroups based on these factors.

METHODS

Children, aged 2-5 years, registered with community health posts (CHP) for one of the primary health centres in Semarang, Indonesia were invited to participate in the study. Moderate and severely malnourished children (weight-for-height Z score (WHZ) <-2 of the WHO/NCHS reference) were excluded. Of 1047 children present at recruitment, 826 children were included in the study. Figure 1 shows the study design and provides detail on included participants.

Children were randomly divided at CHP level into 2 groups by lottery. They received a daily supplement of either zinc sulphate (10 mg elemental zinc) or placebo syrup for 4 months. Both groups also received a single dose of vitamin A (200,000 IU capsule) after 2 months of zinc supplementation as part of the routine national vitamin A supplementation program (For ethical reasons, the study could not prevent the children from receiving vitamin A supplementation). Consequently, comparisons are made based on zinc versus placebo, evaluated at two different times, before and after vitamin A supplementation. Thus four comparison groups are defined as placebo before vitamin A (A), zinc before vitamin A (B), placebo after vitamin A (C) and zinc after vitamin A (D).

At baseline, serum retinol (vitamin A), C-reactive protein (CRP), RBP, albumin, hair zinc level and anthropometric status were measured in all children. Socio-economic data for families were collected by interview using structured questionnaires. Two months after zinc supplementation commenced and just before vitamin A supplementation, a sub-sample of 81 children in each group was randomly selected from children without illness on the day of sample collection and blood samples were collected. At the end of zinc supplementation (2 months after vitamin A supplementation), another sub-sample of 81 children in each group was selected and the same procedures as at 2 months repeated. Dietary intakes of the children were also measured using 30-d quantitative food frequency questionnaires. Subsampling was done in this way to avoid sampling any individual child on three occasions, as this was not acceptable to the community. Three children were excluded from analysis as their ages were not in the range of 2-5 years. Therefore, only 321 children were included in the final analysis.

The sample size was based on 80% power and a 5% significance level to detect a 20% difference in immune response (at least half of a standard deviation for our measures). Allowing a 20% drop out rate, a sample size of at least 76 in each group was required.

There was no difference between the zinc and placebo syrups in taste or appearance. The syrups were given fortnightly to mothers by specially trained health workers, to be taken daily. These health workers visited and supervised syrup consumption every third day.

The ethical clearance for this trial was approved by the Ethical Committee of Medical Research, Medical Faculty, Diponegoro University, Semarang, Indonesia with the clearance number 04/EC/FK/RSDK/2003 and Medical Research Ethics Committee of The University of Queensland, Australia with the clearance number 2003000137. Written informed consent was obtained from the mothers of all participants. This trial has been registered by Australia New Zealand Clinical Trials Registry (ANZCTR) with the number of ACTRN12611000659909.

Sample collection and analysis

At baseline, 3 ml of venous blood was drawn by venepuncture between 9.00-12.00 am to minimize the diurnal variation and immediately transferred to a labelled centrifuge tube, covered with aluminium foil to prevent sunlight exposure, and placed in an ice-cooled box. Blood samples were then centrifuged at 10,000 x g for 10 minutes within 6 hours of blood collection. Serum was separated and aliquots were transferred to Eppendorf tubes for serum retinol, RBP, albumin, CRP and IgG measurements. All samples were then stored at -20℃ and moved to a -70℃ freezer the day after. Samples for serum retinol analysis were transferred to the Institute of Nutrition Mahidol University (Thailand), while the blood samples for all other parameters were measured in laboratories of the Medical Faculty of Diponegoro University (Indonesia).

Serum retinol concentration was determined using High Performance Liquid Chromatography (HPLC). The baseline and final assays were performed in the same batches. The intra assay coefficient variation (CV) was 3.5%, and the inter assay CV was 6.1%.

There were 37 participants with serum CRP values >5 mg/L, indicative of inflammation.[25] As there were no differences in results of key variables between

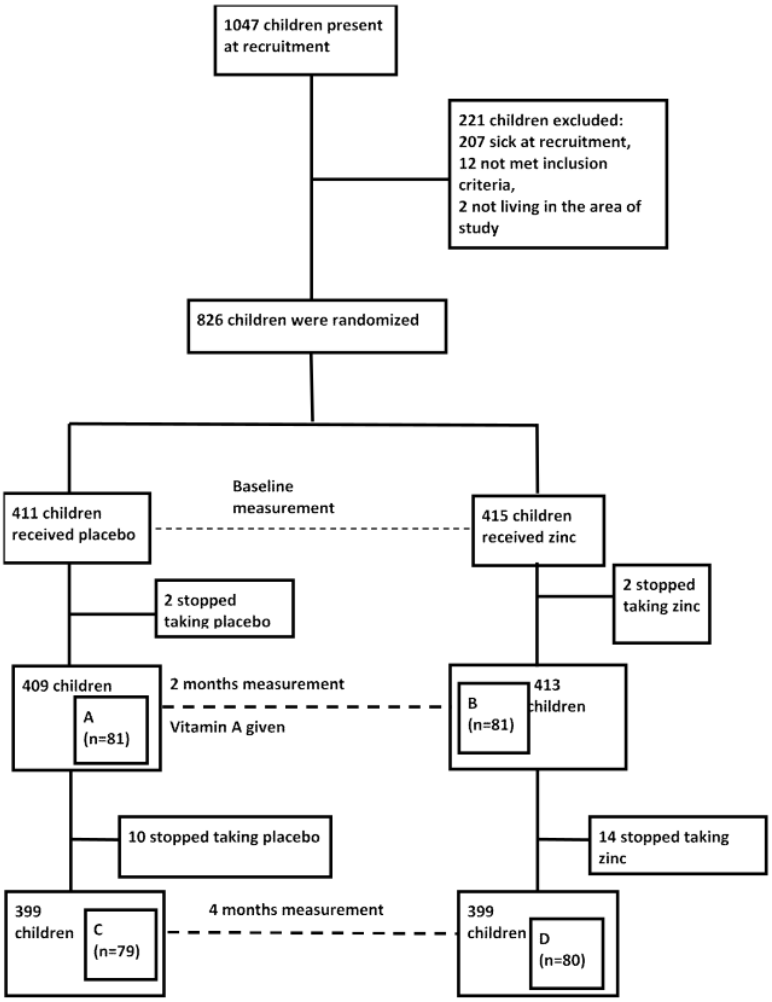

Figure 1. Study design and included participants

participants with and without inflammation, all participants were included in the results presented here. For reasons of haemolysis and an inadequate volume of blood from some participants, the number of samples differs slightly across analyses.

Approximately 50 mg hair samples were taken close to the scalp from the occipital area using stainless steel scissors. The hair samples were stored in labelled polyethylene zip-locked bags at room temperature.[26] Hair zinc analysis was carried out in the Inorganic Chemistry Laboratory, Queensland Health Scientific Services (Australia). After being washed by ethanol, rinsed with de-ionized water, dried, weighted, the samples were digested with HNO_3.[26] The analysis of zinc content was done on the Varian Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES). A hair sample with certified value was included in all runs. The intra and inter assays CV were 4.7% and 8.6% respectively. For summer season, low hair zinc level is defined as of <70 µg/g, while the winter cut-off point is <110 µg/g).[27]

At two and four months, 5 ml of whole blood was drawn and processes repeated for the measures above. For IFN-γ measurement, aliquots were put in an EDTA sterile vacutainer tube. Within 6 hours of blood collection, the EDTA treated whole blood samples were transferred into well plates, 500 µL RPMI and 10 µg/mL PHA were added to each well[28] and the well plates were incubated for 48 hours. After being centrifuged, the supernatant was then transferred into a cryotube, labelled and put into a -70℃ freezer before assessment.[29] Ex-vivo IFN-γ production measurement was conducted using a commercially available human IFN-γ ELISA kit (Pelikine, CLB, The Netherlands). The intra assay CV was 9.5% and the inter assay CV was 19.8%, which are within the acceptable range.

Saliva samples were taken after fasting for half an hour, with the child spitting into a sterile bottle after tooth examination by assistant dentists. Saliva samples were kept in a cooled box and transferred to Eppendorf tubes in the lab before storage at -20℃.

Serum IgG and salivary IgA were assessed using commercial kits within 6 months of collection and followed recommended procedures. Serum IgG measurement used an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Immundiagnostik AG, Bensheim, Germany, catalogue no. K6510). The intra-assay CV was 7.5% and inter-assay CV 16.8%, both within acceptable range. Salivary IgA used an indirect enzyme immunoassay kit (Salimetrics, LLC). The intra-assay coefficient variation CV was 3.4% and inter-assay CV 20.3%. Salivary protein levels of samples were measured using the Bradford method by Bio-Rad Protein Assay reagents (Kit II). Standards were reconstituted and measurements taken following procedures from the manual and absorbance measured in a spectrophotometer at 595 nm.

Statistical analysis

The analysis was conducted using SPSS 13.0 software. Potential confounders considered were socio-demographic status, gender, compliance, initial anthropometric status, zinc and vitamin A status, DEFT (defect, erupted, filled, total) index for teeth[30] and salivary protein level.[31]

Salivary IgA level was adjusted for salivary protein level in all steps of analysis. As the distributions of IFN-γ and salivary IgA variables were not normal, we used log transformations which were normally distributed. The results were converted to the original values using inverse logarithm.

The effects of vitamin A and zinc were assessed in linear regression using General Linear Models (GLM) for three dependent variables: *ex-vivo* IFN-γ, serum IgG and salivary IgA. Treatment (zinc or placebo group) and time (before and after vitamin A supplementation) variables were set as fixed factors and potential confounding variables as covariates. Those confounders which showed conventional statistically significance as judged by *p*-value <0.05 were included. Because two treatments were being investigated, we first assessed whether or not there was an interaction between the treatments; reported as the 'interaction models'. Main effects were then examined; reported as the 'main effects models'. The effects of using a single supplement were also estimated, and reported as 'vitamin A / zinc alone'. The effects were also assessed in terms of impact on the proportion of children with low serum IgG (<5 mg/L) or low salivary IgA (<100 µg/mg protein) using the Chi-square test.

Analyses were also stratified by gender, age, baseline retinol and anthropometric status to identify any differences in effects for sub-groups using the following criteria: younger children (<3.5 years); children with low baseline vitamin A status (serum retinol <30 µg/dL); underweight children (weight for age Z score (WAZ) <-2).

RESULTS

Participants were from a generally healthy population (sick children at baseline were not included in the study) in a relatively poor suburban area (about 70% under the poverty line). Education levels were low, but most parents were literate. Fathers were mostly blue-collar workers, while mothers were housewives (Supplementary table 1).

The characteristics of children at baseline are shown in Table 1. There were no significant differences in characteristics between groups at baseline. Mean serum retinol in both overall sample and sub-samples were in the normal range, and the prevalence of vitamin A deficiency (serum retinol <20 µg/dL) was very low (2.3%). However, a significant proportion of children (32.4%) had inadequate level of vitamin A (serum retinol <30 µg/dL). Only 5.3% had low hair zinc levels using the summer cut off point, but 20.3% of children if the winter cut-off point is used. There were no differences in energy (p=0.79), protein (p=0.96), zinc (p=0.71) and vitamin A (p=0.31) intake between the groups (details not reported here).

Table 2 shows the effect of vitamin A and zinc supple-

Table 1. Child characteristics at baseline of sub-samples selected for nutritional status and immune response sub-studies[†‡]

Child' characteristics	Placebo (A group) Mean±SE (n)	Zinc (B group) Mean±SE (n)	Vitamin A (C group) Mean±SE (n)	Zinc plus vitamin A (D group) Mean±SE (n)
Male/Female ratio	1.13	1.19	0.80	1.16
Age (years)	3.68±0.80 (81)	3.57±0.84 (81)	3.40±0.87 (79)	3.66±0.77 (80)
Anthropometric status				
WAZ scores	-1.64±0.08 (81)	-1.68±0.08 (81)	-1.41±0.11 (79)	-1.52 ± 0.11 (80)
HAZ scores	-1.81±0.09 (81)	-1.88±0.10 (81)	-1.68±0.11 (79)	-1.65 ± 0.13 (79)
WHZ scores	-0.61±0.10 (81)	-0.65±0.08 (81)	-0.45±0.12 (79)	-0.60 ± 0.09 (79)
Vitamin A and zinc status				
Serum retinol (µg/dL)	34.0±0.8 (81)	34.3±0.8 (80)	33.1±0.8 (76)	33.6 ± 0.8 (79)
Low serum retinol (<1.05 µmol/L) proportion (%)	28.4 (81)	30.0 (80)	34.2 (76)	32.9 (79)
RBP (mg/L)	22.3±0.5 (73)	22.4±0.6 (71)	21.9±0.6 (76)	22.3±0.6 (72)
Serum albumin (g/dL)	4.4±0.04 (72)	4.4±0.04 (76)	4.4±0.05 (70)	4.4±0.04 (70)
Hair zinc level (µg/g)	159±10.8 (75)	174±11.4 (74)	184±11.3 (75)	155±8.6 (76)
Percent days of syrup consumption (%)				
<75	3.7	1.2	1.3	2.6
75-100	96.3	98.8	98.7	97.5

[†]Total N=321.
[‡]Analysis was done by one way ANOVA and post hoc comparisons for continuous variables and chi square for categorical variables.

mentation on *ex-vivo* IFN-γ production. The interaction model showed no significant interaction effect between zinc and vitamin A, while there was a significant zinc main effect with an increase of 2.4 ng/L in ex-vivo IFN-γ in zinc supplemented group combined across the before and after vitamin A supplementation phases. When the interaction term was dropped from the model this zinc main effect on ex-vivo IFN-γ strengthened to 2.6 ng/L. The effect of zinc supplementation alone was not significant, and vitamin A supplementation did not have a significant effect on *ex-vivo* IFN-γ. There were no significant covariates in these models.

In stratified analysis, zinc supplementation had a significant effect on ex-vivo IFN-γ production levels in boys, in younger children (<3.5 years), in those normal-weight, and in children with low baseline retinol. As the main effect, vitamin A supplementation showed a significant effect on *ex-vivo* IFN-γ production among children with low baseline serum retinol. There were no significant interactions between treatments in stratified analyses.

Overall, the interaction effect between vitamin A and zinc supplementation on serum IgG was not significant, and neither vitamin A nor zinc supplementation had significant main effects on serum IgG (Table 3). Comparisons of the prevalence of low serum IgG at the end of treatment (<5 mg/L) also showed no significant difference between groups. Stratified analysis showed an interaction effect between zinc and vitamin A supplementation in younger children (<3.5 years). Thus, for younger children, the interaction model was used as a final model. This showed that in younger children zinc supplementation alone decreased serum IgG (comparison of zinc and placebo groups), while with vitamin A supplementation, zinc supplementation increased serum IgG levels (comparison of zinc plus vitamin A and vitamin A groups). Among older children (≥3.5 years), there was no such effect.

There was no significant interaction effect between vitamin A and zinc supplementation on salivary IgA levels. Similarly, neither vitamin A nor zinc supplementation showed a main effect on salivary IgA levels (Table 4). Comparisons of prevalence of low salivary IgA among groups at the end of treatment also showed no significant differences. In stratified analysis, there was no significant interaction effect between vitamin A and zinc supplementation on salivary IgA levels, though there was an increasing trend for salivary IgA levels in younger (<3.5 years) and underweight children.

DISCUSSION

The study was conducted among relatively low-income families in a suburban population in Indonesia. At recruitment, these children were generally underweight (WAZ <-1) and short (HAZ <-1) but not thin (WHZ<-2 children were excluded), had low serum retinol and mild zinc deficiency.

Our study showed that zinc supplementation has a significant main effect (effect of zinc combined across the before and after vitamin A supplementation phases) on ex-vivo IFN-γ production, one of the indicators of cell mediated immune response, with the larger effect in boys than girls. This result is in line with other studies showing that zinc supplementation increases cell mediated immunity,[19,32,33] and that zinc supplementation benefits boys more.[34,35]

Helper T cell sub-types (Th1 and Th2) play an important role in regulating the immune reaction, such as the production of cytokine IFN-γ.[36] Studies have shown that zinc deficiency is related to the decrease of Th1 cell, which in turn reduces the production of IFN-γ, and zinc supplementation can reverse this condition.[37] Further, an earlier study reported that males usually have a better Th1 response to zinc supplementation that can change the balance of Th1 and Th2 responses and so may be more beneficial for boys.[38]

Younger (<3.5 years) children also benefited more. Two out of four studies that have shown a significant effect of zinc supplementation on cell mediated immunity were conducted on infants,[39-40] while the other studies showed effects on children 12-59 months[32] and 84-132 months.[33] Of note, zinc is very important in maintaining immune response as zinc strengthens the immune system via its role in the maintenance of epithelial and tissue structure by promoting cell growth and reducing apoptosis.[37] Younger children have immature immune systems, including cell mediated immunity, which develops throughout the childhood.[41] This possibly explains why the younger children benefited more from zinc supplementation.

In the present study, there were also subgroup differences for the effect of zinc supplementation depending on anthropometric (normal weight children benefited most) and vitamin A status. Among the children with low baseline retinol, vitamin A as well as zinc supplementation showed significant effects on ex-vivo IFN-γ, indicating that these effects are dependent on vitamin A status. This is supported by Wieringa et al who showed that vitamin A deficiency resulted in lower *ex vivo* IFN-γ production in vitamin A deficient infants.[18] Kinoshita et al[42] also showed a similar effect in rats, and other studies have shown that vitamin A deficiency is related to cell mediated immunity.[20,43]

These results are generally consistent with morbidity studies where the effect of zinc supplementation is clearest for infections that involve IFN-γ responses, such as antiviral therapy in rhinovirus, and common cold viruses.[43]

There were no main effects for vitamin A or zinc supplementation on total serum IgG levels in our study. IgG is produced by B cells, which are part of the Th2 response.[45-46] The Th2 cell can produce cytokine IL-4 and the delivery of IL-4 to B cells by Th2 cells can activate B cells to produce most of the antibodies against antigens including IgE and some classes of IgG, such as IgG1 antibody.[47] In general, vitamin A deficiency has been shown to result in a reduction of Th2 response and normal or slightly higher Th1 response.[1] Animal studies show that among vitamin A deficient animals antigen-specific IgG is decreased but not total IgG.[48] This is reflected in a preschool children study in Indonesia where vitamin A supplementation resulted in increased serum IgG levels for Tetanus antigen.[49] Thus, these results are consistent with the (limited) literature, whereby zinc supplementation has more effect on Th1 cells, while serum IgG is the product

Table 2. *Ex-vivo* IFN-γ production (pg/mL) in each treatment group from several data analysis models, with estimated effect sizes[†‡§]

Type of model	Placebo (A group) Mean±SE (n)	Zinc (B group) Mean±SE (n)	Vitamin A (C group) Mean±SE (n)	Zinc plus vitamin A (D group) Mean±SE (n)	Covariates	Effect size (*p*-values) Interaction effect (D+A)-(B+C)	Effect size (*p*-values) Zinc main effect (D+B) -(A+C)
Interaction model	5.2±0.4 (75)	6.6±0.5 (77)	5.8±0.5 (79)	6.8±0.6 (80)	-	-0.4 (0.65)	2.4** (0.02)
Main effect model	5.3±0.4 (75)	6.5±0.5 (77)	5.6±0.4 (79)	7.0±0.5 (80)	-		2.6** (0.02)
4 group ANOVA	5.2±0.5 (75)	6.6±0.5 (77)	5.8±0.5 (79)	6.8±0.6 (80)	-		
Sub group analysis main effect models							
Boys	5.4±0.5 (39)	6.8±0.6 (42)	5.4±0.5 (35)	6.8±0.6 (43)	CHP		2.8* (0.05)
Younger (<3.5 yrs)	5.2 ±0.5 (32)	6.8±0.6 (37)	5.1±0.5 (40)	6.7±0.6 (36)	CHP		3.2** (0.02)
Low baseline retinol	4.2±0.6 (22)	6.3±0.8 (24)	5.8±0.7 (26)	8.5±1.1 (26)	-		4.8** (0.03)
Normal weight	5.1±0.4 (55)	7.1±0.6 (51)	5.5±0.4 (62)	7.6±0.7 (52)	-		4.1** (0.002)
Sub group analysis 4 group ANOVA							
Boys	5.0±0.5 (39)	7.4±0.8 (42)	5.9±0.7 (35)	6.4±0.7 (43)	CHP		
Younger (<3.5 yrs)	5.6±0.6 (32)	6.3±0.7 (37)	4.8±0.5 (40)	7.1±0.8 (36)	CHP		
Low baseline retinol	4.1±0.6 (22)	6.5±1.0 (24)	6.0±0.9 (26)	8.2±1.2 (26)			
Normal weight	4.8±0.5 (55)	7.6±0.8 (51)	5.8±0.5 (62)	7.1±0.7 (52)			

Type of model	Effect size (*p*-values) Vitamin A main effect (C+D) - (A+B)	Zinc only effect (B-A)	Zinc effect in the presence of vitamin A (D-C)	Vitamin A only effect (C-A)	Vitamin A effect in the presence of zinc (D-B)	Zinc plus vitamin A effect (D-A)
Interaction model	0.8 (0.42)					
Main effect model	0.8 (0.43)					
4 group ANOVA		1.4** (0.049) (0.201)§	1.0 (0.17)	0.6 (0.38)	0.2 (0.80)	1.6** (0.03) (0.115)§
Sub group analysis main effect models						
Boys	0 (1.00)					
Younger (<3.5 yrs)	-0.2 (0.90)					
Low baseline retinol	3.8* (0.08)					
Normal weight	0.9 (0.55)					
Sub group analysis 4 group ANOVA						
Boys		2.4** (0.02)	0.5 (0.66)	0.9 (0.34)	-0.1 (0.38)	1.4 (0.45)
Younger (<3.5 yrs)		0.7 (0.48)	2.3** (0.01)	-0.8 (0.35)	0.8 (0.45)	1.5 (0.16)
Low baseline retinol		2.4* (0.07)	2.2 (0.17)	1.9 (0.13)	1.7 (0.33)	4.1** (0.005) (0.026)§
Normal weight		2.8**(0.003) (0.018)§	1.3 (0.15)	1.0 (0.23)	-0.5 (0.68)	2.3* (0.011) (0.054)§

†Analysis was conducted on GLM procedures for continuous variables and Chi Square method for the comparison of proportion.
‡Stratified analysis then was conducted on boys & girls, younger & older children, low baseline & normal baseline retinol, underweight & normal weight children.
§Tukey HSD multiple comparison adjustment.
*p-value <0.1, **p-value<0.05, ***p-value<0.01.

Table 3. Serum IgG (g/L) in each treatment group from several data analysis models, with estimated effect sizes[†‡]

Type of model	Placebo (A group) Mean±SE (n)	Zinc (B group) Mean±SE (n)	Vitamin A (C group) Mean±SE (n)	Zinc plus vitamin A (D group) Mean±SE (n)	Covariates	Effect size (*p*-values) Interaction effect (D+A)-(B+C)	Zinc main effect (D+B) -(A+C)
Crude analysis	7.8±0.3 (81)	7.5±0.3 (80)	7.4±0.3 (79)	7.9±0.3 (80)	-		
Interaction model	7.8±0.3 (81)	7.5±0.3 (80)	7.4±0.3 (76)	7.9±0.3 (79)	retinol1[§]	0.8 (0.20)	0.2 (0.75)
Main effect model	7.6±0.2 (81)	7.7±0.2 (80)	7.6±0.2 (76)	7.7±0.2 (79)	retinol1		0.2 (0.77)
4 group ANOVA	7.8±0.3 (81)	7.5±0.3 (80)	7.4±0.3 (76)	7.9±0.3 (79)	retinol1		
Comparison of low serum IgG (<5 mg/L) prevalence at end of treatment	16.0%	12.3%	13.9%	10.0%			
Sub group analysis interaction model							
Younger children	8.2±0.4 (36)	6.8±0.4 (39)	6.9±0.4 (40)	7.7±0.4 (36)	-	2.2*** (0.006)	-0.6 (0.45)

Type of model	Effect size (*p*-values) Vitamin A main effect (C+D) - (A+B)	Zinc only effect (B-A)	Zinc effect in the presence of vitamin A (D-C)	Vitamin A only effect (C-A)	Vitamin A effect in the presence of zinc (D-B)	Zinc plus vitamin A effect (D-A)
Crude analysis		-0.3 (0.43)	0.5 (0.25)	-0.4 (0.40)	0.4 (0.27)	0.1 (0.75)
Interaction model	0 (0.99)					
Main effect model	0 (0.99)					
4 group ANOVA	-0.3 (0.49)	0.5 (0.26)	-0.4 (0.37)	0.4 (0.36)	0.1 (0.82)	
Comparison of low serum IgG (<5 mg/L) prevalence at end of treatment		-3.7% (0.65)	-3.9% (0.47)	-2.1% (0.83)	-2.3% (0.80)	
Sub group analysis interaction model						
Younger children	-0.4 (0.55)	-1.4	0.8			

[†]Analysis was conducted using GLM procedures for continuous variables and Chi Square method for the comparison of proportions.
[‡]Stratified analysis then was conducted on boys and girls, younger and older children, low baseline and normal baseline retinol, underweight and normal weight children.
[§]Retinol1: serum retinol at baseline (µg/dL).
***$p<0.01$.

Table 4. Salivary IgA (µg/mL protein saliva) in each treatment group from several data analysis models, with estimated effect sizes[†‡]

Type of model	Placebo (A group) Mean±SE (n)	Zinc (B group) Mean±SE (n)	Vitamin A (C group) Mean±SE (n)	Zinc plus vitamin A (D group) Mean±SE (n)	Covariates	Effect size (*p*-values) Interaction effect (D+A)-(B+C)	Zinc main effect (D+B) -(A+C)
Crude analysis	191±10.4 (81)	194±10.5 (81)	192±10.4 (79)	208±11.3 (80)	-		
Interaction model	192±10.3 (81)	195±10.4 (81)	191±10.4 (79)	206±11.2 (80)	HAZ1[§]	11.6 (0.60)	18.6 (0.41)
Main effect model	189±8.9 (81)	198±9.3 (81)	194±9.1 (79)	203±9.5 (80)	HAZ1		18.2 (0.41)
ANOVA 4 groups	192±10.3 (81)	195±10.4 (81)	191±10.4 (79)	206±11.2 (80)	HAZ1		
Comparison of low salivary IgA (<100 µg/mg protein) prevalence at end of treatment	6.2%	9.9%	11.4%	5.0%			
Sub group analysis 4 group ANOVA							
Younger (<3.5 yrs)	203±16.6 (36)	205±16.6 (39)	172±13.6 (40)	216±17.7 (36)	HAZ1		
Under-weight	186±18.8 (24)	181±17.1 (27)	155±18.1 (17)	218±21.6 (28)	HAZ1		

Type of model	Effect size (*p*-values) Zinc main effect (D+B) -(A+C)	Vitamin A main effect (C+D) - (A+B)	Zinc only effect (B-A)	Zinc effect in the presence of vitamin A (D-C)	Vitamin A only effect (C-A)	Vitamin A effect in the presence of zinc (D-B)	Zinc plus vitamin A effect (D-A)
Crude analysis			2.2 (0.89)	15.6 (0.33)	0.5 (0.97)	13.9 (0.38)	16.1 (0.31)
Interaction model	18.6 (0.41)	9.8 (0.68)					
Main effect model	18.2 (0.41)	9 (0.68)					
ANOVA 4 groups			3.5 (0.83)	15.1 (0.34)	-0.9 (0.94)	10.7 (0.51)	14.2 (0.38)
Comparison of low salivary IgA (<100 µg/mg protein) prevalence at end of treatment			3.7% (0.57)	-6.4% (0.16)	5.2% (0.24)	-4.9% (0.37)	
Sub group analysis 4 group ANOVA			1.4 (0.97)	43.6* (0.07)	-3.1 (0.18)	11.2 (0.66)	12.6 (0.63)
Younger (<3.5 yrs)							
Under-weight			-5.2 (0.86)	62.9* (0.052)	-31.4 (0.29)	36.7 (0.25)	31.5 (0.35)

[†]Analysis was conducted using GLM procedures for continuous variables and Chi Square method for the comparison of proportions.
[‡]Stratified analysis then was conducted on boys and girls, younger and older children, low baseline and normal baseline retinol, underweight and normal weight children.
[§]HAZ1= height for age Z scores at baseline.
*$p<0.1$

of B cells, part of Th2 response.[1]

However, we showed an interaction effect in children <3.5 years, evidence for zinc modifying the vitamin A effects on IgG in this age group. This may be due to younger children having a less developed immune system[22] or differences in zinc status. A study of 4-5 year-old children in Indonesia showed increased IgG levels after consuming fish biscuits fortified with iron and zinc.[50] We would expect fish biscuits to also contain some vitamin A, supporting the case that serum IgG level increase can be expected with nutritional changes, including after increasing zinc status.

Zinc and vitamin A supplementation had neither significant main effects nor interaction effects on salivary IgA levels, consistent with a Gambian zinc supplementation study showing no effect on generally healthy 7-30 month old children.[51] As with serum IgG, salivary IgA is a part of the Th2 immune response, and so zinc effect is not expected.[1]

Further, salivary IgA is a measure of mucosal IgA. These results are consistent with animal studies that showed vitamin A deficient mice had higher total salivary IgA than control mice, but lower influenza-specific salivary IgA levels[52] and that zinc and vitamin A deficiency resulted in decreased IgA levels but with mucosa IgA still higher compared to serum IgA.[53] Thus, supplementation may affect serum IgA and antigen-specific salivary IgA but not total salivary IgA. Salivary IgA measurement is also subject to a large measurement error as the protein saliva has to be measured to control the salivary IgA level and this measurement also carries some error. A larger sample size may be needed to detect any significant difference between groups.

In this trial zinc and placebo supplementation were randomly assigned, but all participants received routine vitamin A supplementation 2 months after commencement of their zinc or placebo supplementation. The study had a very high compliance and low dropout rate. In terms of potential limitations, sample size was calculated to allow estimation of main and interaction effects. Consequently, the stratified analysis is relatively underpowered.

We have reported elsewhere that zinc combined with vitamin A supplementation reduced the percentage of days with Upper Respiratory Tract Infection (URTI) by 30%, and reduced URTI episodes by 34%.[11] Consequently, we might expect to observe effects on immune response as overall main and interaction effects where they exist, but only identify strong effects in stratified analyses. Zinc is known to assist the body in making protein and DNA, which is important for infant and childhood development. In addition, some trials indicate that zinc supplementation promotes linear growth and weight gain.[54] In the present study, the effect of supplementation on growth was not clear as there were no significant differences in WAZ, WHZ, HAZ between the groups at two and four months after the intervention. Within the groups, WAZ increased in the first two months but then increased in the next two months in both groups, WHZ increased only in the zinc group but then decreased in the next two months in both groups. On the other hand, HAZ increased in both groups in the first two months, higher in the zinc group, and continued to increase in the control group but not in the zinc group. Further studies with larger samples are required to confirm the findings.

The fact that the children in this study were generally in marginal nutritional status, can explain why they received an effect from zinc and vitamin A supplementation on immune response and morbidity but not in their growth. That is, a growth response was limited by other nutritional factors.

The serum retinol levels were higher at 2 months after the supplementation in zinc (37.2±7.63 µg/dL compared to 34.8±10.3 µg/dL) and control groups (36.0±8.76 µg/dL compared to 34.4 ±9.01 µg/dL). Thus, the increase in immune response is in accordance with the level of serum retinol and URTI morbidity.

We have shown benefits for immune response in a population who had low serum retinol and with mild zinc deficiency. Further research is warranted to examine its impact on morbidity and treatment.

Conclusion

The study provides evidence that zinc supplementation increases cellular immune responses (ex-vivo IFN-γ), and modifies the effect of vitamin A supplementation on immune responses in at least some sub-groups for humoral (serum IgG and salivary IgA) and local mucosal (salivary secretory IgA) immunity. This potentially explains some inconsistencies in results of vitamin A supplementation on morbidity, adding evidence that responses can be influenced by zinc status and pathogen, with age, gender, and nutritional status also potentially influencing results. Outcomes were consistently best in the group receiving both zinc and vitamin A supplementation.

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AUTHOR DISCLOSURES

The authors declare no conflict of interest.

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