Short Communication

A single dose of vitamin A improves haemoglobin concentration, retinol status and phagocytic function of neutrophils in preschool children

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Since there is a reported interrelationship between vitamin A and Fe metabolism, and with immunological response, the objective was to evaluate the effect of a single dose of vitamin A administered to preschool children, on Fe and vitamin A nutritional status, anaemia and phagocytic function of neutrophils, 30 d after supplementation. A total of eighty children (sixty-eight supplemented and twelve controls) were supplemented orally with 200 000 IU (60 mg) vitamin A, and evaluated for nutritional, haematological and immunological responses at the beginning of the study and 30 d after supplementation. Parameters studied included Hb, serum ferritin, retinol and Fe concentrations, transferrin saturation, IL-4, interferon-γ and phagocityc capacity of neutrophils using non-fluorescent latex microbeads. After supplementation there was a significant increase in Hb concentration ($P=0.03$), mean corpuscular Hb concentration ($P=0.001$) and serum retinol ($P=0.0078$). Prevalences of anaemia and vitamin A deficiency decreased significantly from 17·6 % to 13·2 % and from 25 % to 13·2 %, respectively. Regarding phagocytic function, there was a significant increase in the number of microbeads engulfed by neutrophils ($P<0.05$) and no significant changes in cytokine concentrations at 1 month after treatment. A single dose of 200 000 IU (60 mg) vitamin A administered orally to a group of preschool children with a high prevalence of vitamin A deficiency enhanced serum retinol and Hb concentrations, decreased the prevalence of anaemia and vitamin A deficiency and improved the constitutive phagocytic capacity of neutrophils. Vitamin A supplementation could help to decrease vitamin A deficiency, anaemia prevalence and to improve the innate immunity response in preschool children. The effects were obtained without Fe supplementation.

Vitamin A: Iron: Supplementation: Phagocytic function: Preschool children

Vitamin A and Fe deficiencies are important public health problems worldwide, affecting especially children and women[1,2]. Supplementation and food fortification programmes with these two nutrients have been successful in reducing the risk of morbidity and mortality. Several reports indicate an effect of vitamin A increasing Fe mobilisation from the liver and Fe absorption from enterocytes[3–5]. There are also reports in experimental animals, indicating that vitamin A deficiency affects the function of the neutrophils, specifically chemotaxis, adherence, phagocytosis and oxidant generation, which could impair phagocytic activity and elimination of pathogenic agents[6].

Since there are no reports about the dose and duration of the effect of vitamin A on Fe metabolism as well as on immunological function in humans, and due to worldwide implementation of single-dose vitamin A supplementation programmes during vaccination campaigns, the objective of the present study was to evaluate the effect of a single dose of 200 000 IU (60 mg) vitamin A, administered orally to preschool children, on Fe and vitamin A nutritional status, anaemia, phagocytic function of neutrophils and cytokine production, 30 d after supplementation.

Experimental methods

Subjects

The sample consisted of eighty children aged from 2 to 6 years, apparently in good health, appearing for routine nutritional check ups at the Nutrition Consult at the Hospital Victorino Santaella in Los Teques, capital city of Miranda State in Venezuela, between February and December 2007. Exclusion criteria for the study included: age < 2 or > 6 years, intake of vitamin A, Fe or multivitamin supplements during the month before the interview, Hb concentrations below 70 g/l or above 140 g/l, serum retinol concentration below 0·35 μmol/l (100 μg/l) and reported respiratory or intestinal infections during the month prior to the interview.

Each parent or child's guardian was interviewed to explain the objective and methodology of the study, and for a socio-economic classification according to a modification of the Graffar method[4]. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethics committees of the Venezuelan Institute for Scientific Research and the Hospital Victorino Santaella. Written informed consent was obtained from all parents.

Vitamin A supplementation

After having been randomly assigned to the experimental or control group, a 7 ml venous sample was taken from the antecubital vein of the arm of each child who had been fasting for at least 8 h. To the experimental group, a single dose of 200 000 IU (60 mg) vitamin A was administered orally[7] in gel caplets (Vivax Laboratories, Caracas Venezuela) with 110 ml of pasteurised complete milk or with 110 ml of pasteurised apple juice for children with allergy or intolerance to cows' milk. The control group only received 110 ml of pasteurised complete milk or pasteurised apple juice. At 30 d after supplementation, a second blood sample was taken to perform haematological and immunological determinations.

Biochemical analyses

From the 7 ml blood samples obtained at the beginning of the study (T_0) and 30 d after supplementation (T_{30}), 4 ml were used to obtain serum and 3 ml were treated with EDTA for haematological, cytokine production and phagocytosis tests. Haematological determinations included Hb, packed cell volume and mean corpuscular Hb concentration[4]. Anaemia was defined as mild when Hb concentrations ranged between 100 and 109 g/l for children from 2 to 4·9 years of age and between 100 and 114 g/l for children aged 5–6 years. For the same age groups, anaemia was classified as moderate when Hb concentration varied between 70 and 99 g/l, and as severe if the Hb was less than 70 g/l[2]. In the present study, severe anaemia was an exclusion criterion.

Serum Fe and total Fe-binding capacity were determined according to the method established by the International Committee for Standardization in Haematology[4]. Unsaturated Fe-binding capacity was calculated by subtracting serum Fe concentration from total Fe-binding capacity, and the percentage of transferrin saturation was calculated from serum Fe concentration and total Fe-binding capacity. Serum ferritin was determined by ELISA with monoclonal antibodies[2].

For serum retinol determination, blood samples were processed to obtain serum (centrifuged at 1060 g for 10 min at 4°C), protected from light and frozen until analysed by HPLC[8]. Briefly, 100 µl serum were extracted with heptane containing butylhydroxytoluene, dried under a N_2 stream and suspended in methanol for separation in a HPLC system (Waters Corp., Milford, MA, USA) with a Bondapack C18 column (3·9 × 300 mm), using 100 % methanol as mobile phase at 0·8 ml/min. Detection was carried out at 322 nm and retinol concentrations were calculated from a standard curve using Millennium PDA software (Waters Corp.). A serum retinol concentration below 0·70 µmol/l (20 µg/l) was considered vitamin A deficiency[1].

Evaluation of phagocytosis

Neutrophils were obtained from a subsample of six children, and processed by a modification of the method reported by Dunn & Tyrer[9]. Briefly, 500 µl blood were carefully layered on top of 800 µl Ficoll-Hypaque 1077 (Pharmacia Biotech, Uppsala, Sweden), previously placed in a 1·5 ml conical plastic tube and incubated for exactly 20 min at room temperature. Without centrifugation, the top layer was removed and washed twice at 55 g for 5 min, with a PBS solution containing 0·1 % fetal bovine serum (Gibco, Grand Island, NY, USA). Cells were re-suspended in the same buffer for a total and differential cell counting, and after an additional wash, suspended in Roswell Park Memorial Institute (RPMI) 1640 media with 15 % fluorescence activated cell sorting (FACS)-staining buffer to a final concentration of $2\cdot5 \times 10^6$ neutrophils/ml.

Phagocytosis was studied mixing 500 µl of the cell suspension ($1\cdot25 \times 10^6$ cells) with 0·8 µm diameter non-fluorescent latex beads (Sigma, St Louis, MO, USA), at a proportion of fifty beads per cell. After incubation at 37°C for 1 h, cells were washed with PBS and diluted in 200 µl PBS–fetal bovine serum (0·1 %). Paraformaldehyde (Merck, Darmstadt, Germany) was added drop by drop, to a final concentration of 16 mg/ml.

The cells were smeared on glass slides by cytocentrifugation (Fisher Scientific, Pittsburgh, PA, USA) and stained with Wright–Giemsa solution. Then two counting procedures were performed in each cytospin: the number of neutrophils ingesting beads from a total of twenty neutrophils, as well as the number of latex microbeads engulfed by ten neutrophils. All the microscopic evaluation and counting were performed by three independent observers.

Cytokine production

Production of cytokines IL-4 and interferon-γ was measured in whole blood stimulated with lipopolysaccharide and phytohaemagglutinin (Phaseolus vulgaris) as previously reported[10,11]. Briefly, to 2 ml blood samples, phytohaemagglutinin and lipopolysaccharide (Sigma, St Louis, MO, USA) were added to final concentrations of 10 µg/ml and 10 ng/ml, respectively, and incubated at 37°C for 8 h. After centrifugation at 4000 g for 10 min, supernatant fractions were centrifuged again at 5000 g for 10 min, to obtain platelet-poor plasma. Samples were stored at −70°C until assayed. The cytokines' concentrations were measured by ELISA (IL-4, BD OptEIATM kit II and interferon-γ, BD OptEIATM kit II), following the manufacturer's instructions (Becton Dickinson, San Diego, CA, USA).

Statistical analysis

Results were expressed as mean values and standard deviations. The statistical significance of differences between the means at T_0 and T_{30} was assessed by the two-tailed Student's t parametric test for paired data, for a significance level of $P < 0\cdot05$. For evaluation of phagocytic activity, the Wilcoxon–Mann–Whitney test range was used with a significance level of $P \leq 0\cdot05$.

Table 1. Hb, packed cell volume, mean corpuscular Hb concentration (MCHC), serum Fe, unsaturated Fe-binding capacity (UIBC), total Fe-binding capacity (TIBC), transferrin saturation (TS), serum ferritin, serum retinol, and prevalence of anaemia and vitamin A deficiency in preschool children 30 d after receiving or not a single dose of 200 000 IU (60 mg) vitamin A

(Mean values and standard deviations)

| | Supplement (n 68) | | | | Control (n 12) | | | |
| | T_0 | | T_{30} | | T_0 | | T_{30} | |
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Hb (g/l)	114	8	117*	8	117	8	117	1
Packed cell volume (%)	37·1	2·1	37·1	1·9	37·2	1·9	36·6	2·0
MCHC (%)	31·7	1·2	32·2*	1·0	31·8	0·9	32·0	1·4
Serum Fe (μmol/l)	17·7	0·6	15·1*	0·7	12·8	0·9	18·0*	2·0
UIBC (μmol/l)	44·1	1·2	47·9*	1·5	51·0	2·9	45·7	3·9
TIBC (μmol/l)	61·8	1·0	62·7	1·1	63·8	2·5	63·7	2·3
TS (%)	29·1	1·2	24·9*	1·3	20·6	2·3	29·3*	4·1
Ferritin (μg/l)	13·2	9·3	12·6	9·9	12·6	6·7	10·3	4·7
Retinol (μmol/l)	0·86	0·20	0·96*	0·24	0·87	0·25	0·88	0·33
Anaemia (%)†	17·6		13·2		16·2		15·9	
Vitamin A deficiency (%)‡	25		13·2		25		25	

T_0, beginning of the study; T_{30}, 30 d after supplementation.
* Mean value was significantly different from that at T_0 for the same study group ($P < 0.05$).
† For definitions of anaemia, see the Biochemical analyses section.
‡ Vitamin A deficiency = serum retinol concentration < 0.70 μmol/l.

Results

Impact of vitamin A supplementation

The experimental group consisted of thirty-six boys and thirty-two girls, with a mean age of 4·0 (SD 1·3) years and the control group included five boys and seven girls and a mean age of 4·0 (SD 0·9) years. The small control group was included for comparison of potential unsupplemented changes during the trial. Socio-economic classification of all children was in the labour (IV) and low (V) socio-economic levels. There was only one case of lactose intolerance.

The effect of vitamin A on haematological parameters is summarised in Table 1. Hb concentration and mean corpuscular Hb concentration increased significantly in the supplemented group ($P = 0.03$ and $P = 0.001$, respectively), 30 d after a single dose of vitamin A, compared with the control group. For the supplemented group, the CV for Hb measurements was 6·6 and 6·5 % at T_0 and T_{30}, respectively. Regarding haematological parameters reflecting Fe metabolism, all the parameters studied, except for ferritin total Fe-binding capacity and packed cell volume, were affected at 1 month after receiving one dose of 200 000 IU (60 mg) vitamin A. Serum Fe and the percentage of transferrin saturation were significantly reduced ($P = 0.003$ and $P = 0.008$, respectively), while unsaturated Fe-binding capacity increased significantly ($P = 0.026$).

Prevalence of anaemia was significantly reduced from 17·6 to 13·2 % in the experimental group 30 d after supplementation, and the effect was evident for both mild and moderate cases of anaemia. There were no changes in anaemia prevalence for the control group.

The mean serum retinol concentration was significantly increased in the supplemented group after 1 month of a single vitamin A dose ($P = 0.0078$), compared with the beginning of the study and with the control group (Table 1). In the present study, the prevalence of vitamin A deficiency at the beginning of the study was 25 % for both groups and was reduced to 13·2 % after supplementation.

Phagocytic capacity

There was a statistically significant increase in the number of neutrophils ingesting beads after vitamin A supplementation. The number increased from four to eight neutrophils per twenty neutrophils counted at T_0 and T_{30}, respectively ($P < 0.05$) (data not shown).

The number of latex microbeads engulfed by individual neutrophils also increased significantly ($P < 0.005$) in four of the five cases studied, 30 d after vitamin A supplementation, while in the randomly selected control case, there was no significant changes in the number of ingested beads (Table 2).

Cytokine measurements

There were no significant differences in IL-4 and interferon-γ production when comparing the T_0 and T_{30} in both the

Table 2. Phagocytic capacity, measured as microbeads engulfed by neutrophils, obtained from a randomly selected subsample of preschool children, before and 30 d after receiving or not a single dose of 200 000 IU (60 mg) vitamin A†

(Mean values and standard deviations)

| | Number of microbeads counted inside neutrophils | | | |
| | T_0 | | T_{30} | |
Sample	Mean	SD	Mean	SD
1	2·9	2·6	3·2	2·6
3	3·8	2·9	4·9*	1·8
4	3·4	1·7	4·8*	2·0
5	1·9	0·9	11·4*	6·8
6	3·4	1·9	7·4*	7·9
Mean	3·1	0·7	6·3*	2·9
Control	3·4	1·0	4·0	1·9

T_0, beginning of the study; T_{30}, 30 d after supplementation.
* Mean value was significantly different from that at T_0 ($P < 0.05$; Wilcoxon–Mann–Whitney for no paired samples).
† Mean values of the counted microbeads inside ten neutrophils from each sample. Cells were counted by three independent observers.

supplemented and control groups, nor between the supplemented and control groups (data not shown).

Discussion

Vitamin A supplementation protocols for children usually recommend one or two oral doses of 50 000 to 200 000 IU (15 to 60 mg) every 4–6 months up to the age of 6 years[7]. In the present study we found a significant increase in serum retinol with only one dose of 200 000 IU (60 mg) vitamin A, and this effect persisted for at least 1 month, significantly reducing the prevalence of vitamin A deficiency from 25 to 13 %. Food consumption patterns of the participants were assessed twice during the study, and did not indicate changes over the course of the study (data not shown).

The prevalence of vitamin A deficiency at the beginning of the study was 25 % for both groups, which, according to the criteria established by the International Vitamin A Consultative Group[12], if extrapolated to the general population, indicates a public health problem. Two studies performed in Venezuela with preschool children reported prevalences of vitamin A deficiency of 27 and 22 %, similar to the values reported in the present study[13,14].

In the present study we also observed a favourable effect of vitamin A supplementation on Hb concentration, with a significant increase of 3 g/l at 1 month after supplementation. It is important to point out that this increase was still present at 1 month after supplementation and was achieved without Fe administration. Mejia & Chew reported an increase of Hb concentration of 9 g/l in anaemic children supplemented daily with 10 000 IU (3 mg) vitamin A during 2 months[3]. Also, a study in Morocco, in vitamin A-deficient and anaemic children who were supplemented with two doses of 200 000 IU (60 mg) at 5-month intervals, reported a significant increase in Hb concentration of 7 g/l, 10 months after the first dose[15]. The present study found an increase in Hb concentration and a decrease in anaemia prevalence in a group that included both anaemic and non-anaemic children.

There were no variations in ferritin concentrations at 1 month after supplementation, but it is possible that changes might have occurred earlier after vitamin A administration. Mejia & Chew reported similar findings in ferritin concentration 2 months after daily vitamin A supplementation to anaemic children[3]. The role of vitamin A in improving Fe metabolism and diminishing anaemia can be explained by at least three pathways: increased erythropoiesis[16], Fe mobilisation from hepatic deposits[3], and increased intestinal non-haeme Fe absorption[4]. It would be interesting to investigate the exact moment in which each event happens in time, to study the acute effect of vitamin A supplementation on Fe metabolism-related proteins and for how long these effects last.

Phagocytosis results suggest a favourable effect of vitamin A on the constitutive phagocytic capacity of neutrophils, evaluated by the increase in the number of phagocyting neutrophils and also by the increase in the number of microbeads engulfed by each neutrophil, without cell stimulation. Under these experimental conditions, vitamin A had no effect on IL-4 and interferon-γ production.

The results from the present study show that the supplementation of preschool children with 200 000 IU (60 mg) vitamin A as a single oral dose increased serum retinol and Hb concentrations and could help to decrease vitamin A deficiency and anaemia prevalence in this vulnerable population group. The effect lasted for at least 1 month after vitamin A supplementation and required no Fe supplementation. Vitamin A also improved the constitutive phagocytic function of neutrophils of the innate immunity, needed as the first line of defence against pathogenic agents, especially for this age group.

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