Vitamin A-Fortified Milk Increases Total Body Vitamin A Stores in Mexican Preschoolers¹⁻³

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Abstract

Vitamin A (VA) deficiency (VAD) continues to be a major nutritional problem in developing countries, including Central America. In Mexico, milk is a well-accepted vehicle for the administration of micronutrients, including VA, to preschoolers. Thus, we conducted a randomized, controlled, clinical trial to investigate the efficacy of daily consumption of 250 mL of VA-fortified milk (which provided 196 retinol equivalents/d) for 3 mo on VA stores in mildly to moderately VAD preschoolers who were not enrolled in a food assistance program. Twenty-seven mildly to moderately VAD children were randomly assigned based on screening measurements to either the intervention (n = 14) or control group (n = 13) (children in the control group did not receive placebo). All children in the control group and 79% (n = 11) of the children in the intervention group completed the study. The total body VA (TBVA) pool size was estimated using the deuterated retinol dilution technique before and after the intervention. After 3 mo, median changes in the serum retinol concentration for the intervention and control groups were 0.13 and −0.21 μmol/L, respectively (P = 0.009). Median changes in the TBVA stores were 0.06 and 0.01 mmol, respectively (P = 0.006) and estimated median changes in the liver VA concentration were 0.09 and 0.01 μmol/g, respectively (P = 0.002). The VA-fortified milk was well accepted among preschoolers and significantly increased TBVA stores, liver VA stores, and serum retinol concentration, indicating that it may be an effective means to ameliorate VAD in young Mexican children. J. Nutr. 143: 221–226, 2013.

Introduction

Vitamin A (VA) deficiency (VAD) remains a public health problem in the developing world; it especially affects women of reproductive age and preschool-age children (1). In fact, it is estimated that >127 million preschool-age children worldwide have VAD (2). VAD may result in anemia, reduced immune function, xerophthalmia, blindness, and increased morbidity and mortality (1,3).

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³ Supplemental Figure 1 and Supplemental Tables 1 and 2 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
⁴ Abbreviations used: CIAD, Research Center For Food and Development; D4, tetra-deuterated retinyl acetate; D8, octa-deuterated retinyl acetate; RE, retinol equivalent; TBVA, total body vitamin A; VA, vitamin A; VAD, vitamin A deficiency/deficient.
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In a 1995 publication, the WHO identified severe subclinical VAD in Mexico (4), but it was not until 2001 that a national nutrition survey (5) reported moderate VAD in 26% of 3- to 6-yr-old children and severe VAD in 2% of 3–4-yr olds. VA supplements have been administered in Mexico since 1993 to children from 6 mo to 4 y [60 mg (210 μmol) of retinyl palmitate] during National Health Campaigns and, although they provide immediate improvement in VA status, their effect is limited with time (6,7). Food fortification, on the other hand, tends to be slower but has a much wider and more sustained impact (8) and previous studies have shown that VA-fortified food intake, such as sugar (9,10), cereals (11,12), salt (13), and milk (14), have a positive impact on VA status in different age groups.

Indeed, the Mexican national school breakfast program, which provides foods fortified with iron, zinc, and VA (preformed VA is added to ultra-high temperature processed milk and provides 35% of the preschoolers’ daily recommended intake), has been shown to be effective in reducing the prevalence of iron and zinc deficiencies, but not VAD (evaluated based on serum retinol concentrations) (15). In addition to the school breakfast program, a Mexican national food aid program has
distributed micronutrient-fortified Liconsa milk since 2001 to those whose nutritional status may be compromised due to low socioeconomic status or limited access to food. Consumption of Liconsa fortified milk has been associated by multiple linear and logistic regression models with a reduction in the prevalence of anemia in Mexican children (16), but its impact on the VA status of preschool-age children has not been determined. One cup (250 mL) of Liconsa VA-fortified milk provides 49% of the preschoolers’ daily recommended intake and might be an ideal vehicle for improving the VA status of children, especially in areas of Mexico such as the Northwest, where animal food sources (beef, eggs, and milk) are important parts of the diet (17) and consumption of fruits and vegetables is lower than in other regions of the country. Nevertheless, in developing countries, plant foods containing pro-VA carotenoids often are the primary sources of VA (18) and, aside from their role as VA precursors, they can also act as antioxidants in humans and animals (19).

Here, we studied the impact of VA-fortified milk on total body VA (TBVA) stores in mildly to moderately VAD children in northwestern Mexico. We used an isotope dilution method, the deuterated retinol isotope dilution technique, to assess the change in VA status between the intervention and control groups in preschoolers after 3 mo of daily consumption of VA-fortified milk. This analytical and mathematical methodology was previously shown to be sensitive to detect changes in status due to dietary or pharmacological VA supplementation and it is thus useful for evaluating the effectiveness of intervention programs (20) and randomized controlled trials as well.

**Participants and Methods**

**Participants and study design.** Preschool children (3–6 y old) from low socioeconomic areas of northwest Mexico were eligible to participate in this randomized, controlled, clinical trial. Screening measurements took place during a 2-mo period and included the evaluation of serum retinol (21) and hemoglobin concentrations (22), serum C reactive protein (CRP; EIA-3954 CRP; DRG Diagnostics), serum retinol binding protein (RBP; ELISA kit K6110; Immunodiagnostics), weight and height (23), dietary information (24), and stool samples for parasites (25). For the screening phase, we explained the study protocol to the children’s parents during a school meeting and informed consent was obtained from those willing to participate. The inclusion criteria were mild to moderate VA deficiency (moderate VAD serum retinol concentrations, 0.35–0.7 μmol/L and mild VA, 0.7–1.05 μmol/L) (1,26,27) and absence of subclinical inflammation (28). The presence of anemia (hemoglobin <110 g/L for children 5–11.9 g/L for children 5–11.9 g/L for children 5–11.9 g/L for children 5–11.9 g/L for children 5–11.9 g/L for children 5–11.9 g/L for children 5–11.9 g/L for children 5–11.176 C until analyzed. Blood sampling and storage. Fasting 5-mL samples of venous blood were drawn using a syringe or Safety Lok Vacutainer blood collection set and collected into no-additive vacutainers (Becton Dickinson) covered with aluminum foil to prevent light damage. Blood samples were placed on ice for transport to the Department of Nutrition at CIAD. Serum was obtained and stored at −70°C until analyzed at CIAD (screening) or shipped on dry ice to the USDA/Agricultural Research Service Human Nutrition Research Center on Aging at Tufts University, where it was stored at −70°C until analyzed.

**Analysis of serum samples.** Serum retinol and pro-VA carotenoids were extracted and analyzed by HPLC as previously described (32) by using retinyl acetate and echinenone as internal standards. HPLC-purified standards were used to calculate the retinol, α-carotene, all-trans-β-carotene, and cryptoxanthin concentrations by using an external calibration curve obtained from pure standards (Sigma-Aldrich). The inter-run variation was 6% for retinol and 11% for pro-VA carotenoids. Total serum retinol (labeled and unlabeled) was determined.

The isotopic ratio of labeled/unlabeled retinol in serum samples was determined by GC-MS analysis. Samples were extracted (32) and the retinol fraction was collected after HPLC separation on a C18 column. Solvent was evaporated under N2 and the residue was derivatized at 70°C C for 30 min with 10 μL of N,O-bis (trimethylsilyl) trifluoroacetamide with 10% trimethylchlorosilane (both from Pierce) and 1 μL was injected into the GC-MS system (33). Total enrichment of labeled retinol was determined by integrating the peaks under the reconstructed mass chromatograms of the negative ions at m/z 268 to 270 for H, m/z 271 to 273 for D4 retinol, and m/z 274 to 278 for D8 retinol. Thus, the calculation of percentage enrichment was (labeled retinol/unlabeled retinol) × 100 (33).

**Estimation of TBVA and liver VA stores.** Data from blood samples collected on d 14 after the administration of each labeled dose were used for the estimation of TBVA stores according to the Olson equation (34)
using a half-life of 32 d for liver VA stores as has been estimated for preschoolers (35). We also estimated liver VA concentrations based on the assumptions that liver weight in children is ~3% of body weight and that ~90% of TBVA is stored in the liver in populations with adequate VA status (36).

Statistical analysis. Sample size was based on results by Ribaya-Mercado et al. (10) for the assessment of the change in TBVA stores in response to an intervention with VA-fortified sugar in Nicaraguan schoolchildren. Because our trial was shorter (3 vs. 12 mo), an estimation of the change in TBVA stores 3 mo after the intervention was made. Assuming a 2-tailed analysis with α set at 0.05 and an estimated SD of 0.365 mmol, 16 participants (8/group) would be needed to provide 80% power to detect a difference of 0.54 mmol in TBVA stores between groups. We increased the number of participants to 27 (n = 13 in control and n = 14 in the intervention group), assuming a potential dropout rate of 40%. All outcome variables were analyzed in participants who completed the study (control group, n = 13; intervention group, n = 11) (Fig. 1).

Data analysis was performed using the NCSS statistical software (NCSS 2007). Descriptive statistics were generated for the general characteristics of the study population. Values in the text are median (IQR) and differences were considered significant at \( P < 0.05 \). The Mann-Whitney U test was used to compare median TBVA stores, serum retinol, pro-VA carotenoids and estimated liver retinol stores between groups. We increased the number of participants to 27 (n = 13 in control and n = 14 in the intervention group), assuming a potential dropout rate of 40%. All outcome variables were analyzed in participants who completed the study (control group, n = 13; intervention group, n = 11) (Fig. 1).

Results

Participants. Twenty-five preschoolers with serum retinol concentrations ≥1.05 \( \mu \text{mol/L} \), 6 with positive subclinical inflammation, 1 possibly infected with hepatitis C virus, and 1 with chickenpox were excluded before randomization. Based on results of the screening’s determination of serum retinol concentration, 48% (n = 31) of the assessed preschoolers were mildly to moderately VAD; this prevalence is similar to that observed by previous studies conducted in the same region (37,38). Participants’ characteristics were randomly distributed between groups, even after 3 children dropped out of the intervention group before the final assessment (Table 1). Similar to predictions in the CDC reference growth charts for boys and girls aged 4–6 y (39), we observed a significant weight gain (1.15 kg) and an increase in height (3.4 cm) in the final assessment (\( P < 0.05 \)). No significant differences were found for other anthropometric indices in the pre- compared with postintervention measurements. Anemia and subclinical inflammation were not detected based on hemoglobin and serum CRP results, respectively. Based on an analysis of fecal samples for parasites, 81.5% (n = 22) of the children were not infected, 11.1% (n = 3) tested positively for nonpathogenic Entamoeba coli, and 7.4% (n = 2) for the pathogenic Giardia intestinalis (one infected child in each group). Children who were found to have giardiasis received treatment from a pediatrician after the study.

Study outcomes. Serum retinol, TBVA stores, and estimated liver stores increased in response to the daily consumption of VA-fortified milk for 3 mo in the intervention compared with the control group (Table 2).

Even when we selected only mildly to moderately VAD children during the screening phase based on serum retinol concentration, during the baseline determinations almost all participant preschoolers had serum retinol ≥1.05 \( \mu \text{mol/L} \) (Table 2), which can be possible because of the homeostatic regulation of VA (36). Based on baseline serum retinol concentrations, 15% (n = 2) of children in the control group had mild VAD and 85% (n = 11) were VA sufficient. Mild VAD increased to 38% (n = 5) for the control group and moderate VAD was found in 8% (n = 1) in the final assessment; the remaining 54% (n = 7) of the children in this group had a serum retinol concentration ≥1.05 \( \mu \text{mol/L} \).

The prevalence of VAD in the control group tended to increase from baseline to the final assessment (\( P = 0.10 \)). In the intervention group, 2 children (18%) had mild VAD and the remaining 82% of the children had a serum retinol concentration

### TABLE 1  Screening anthropometric and biochemical characteristics of participant preschoolers randomly allocated to control or intervention groups

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>Intervention</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>5.4 (4.2, 5.9)</td>
<td>5.5 (4.2, 5.9)</td>
<td>0.98</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>17.7 (15.3, 20.6)</td>
<td>18.7 (15.9, 20.1)</td>
<td>0.79</td>
</tr>
<tr>
<td>Height, cm</td>
<td>108.7 (102.7, 112.7)</td>
<td>107.6 (103.2, 112.1)</td>
<td>0.84</td>
</tr>
<tr>
<td>Height-for-age Z-score</td>
<td>-0.23 (-0.63, 0.45)</td>
<td>-0.45 (-0.9, 0.35)</td>
<td>0.62</td>
</tr>
<tr>
<td>Weight-for-age Z-score</td>
<td>-0.33 (-0.83, 0.39)</td>
<td>-0.54 (-0.85, 0.17)</td>
<td>0.75</td>
</tr>
<tr>
<td>BMI-for-age Z-score</td>
<td>-0.25 (-0.72, 0.26)</td>
<td>-0.18 (-0.27, 0.17)</td>
<td>0.39</td>
</tr>
<tr>
<td>Serum VA, ( \mu \text{mol/L} )</td>
<td>0.80 (0.72, 0.89)</td>
<td>0.82 (0.66, 0.91)</td>
<td>0.88</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>127 (125, 132)</td>
<td>126 (122, 134)</td>
<td>0.49</td>
</tr>
<tr>
<td>Serum CRP, mg/L</td>
<td>0.66 (0.04, 1.59)</td>
<td>0.60 (0.04, 1.59)</td>
<td>0.84</td>
</tr>
</tbody>
</table>

1 Values are median (IQR), n = 11 (intervention) or 13 (control). \( P \) (Mann-Whitney U test).
Table 2: Changes in VA-related variables in Mexican preschoolers who did or did not consume VA-fortified milk daily for 3 mo

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>3 mo</th>
<th>Change 3 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum retinol, µmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intervention</td>
<td>1.19 (1.11, 1.50)</td>
<td>1.38 (1.14, 1.46)</td>
<td>0.14 (0.07, 0.24)**</td>
</tr>
<tr>
<td>Control</td>
<td>1.29 (1.15, 1.50)</td>
<td>1.09 (0.97, 1.26)*</td>
<td>-0.21 (-0.40, -0.05)</td>
</tr>
<tr>
<td>Difference in changes³</td>
<td>0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBVA stores, mmol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intervention</td>
<td>0.18 (0.14, 0.22)</td>
<td>0.29 (0.17, 0.35)*</td>
<td>0.06 (0.04, 0.12)**</td>
</tr>
<tr>
<td>Control</td>
<td>0.16 (0.13, 0.22)</td>
<td>0.17 (0.12, 0.21)</td>
<td>0.01 (0.02, 0.04)</td>
</tr>
<tr>
<td>Difference in changes³</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VA liver stores, µmol/g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intervention</td>
<td>0.34 (0.23, 0.40)</td>
<td>0.47 (0.30, 0.52)*</td>
<td>0.09 (0.05, 0.20)**</td>
</tr>
<tr>
<td>Control</td>
<td>0.25 (0.20, 0.41)</td>
<td>0.28 (0.19, 0.34)</td>
<td>0.01 (-0.05, 0.03)</td>
</tr>
<tr>
<td>Difference in changes³</td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Values are median (IQR), n = 11 (intervention) or n = 13 (control). *Different from corresponding baseline value, P < 0.05 (Wilcoxon’s Signed Rank test); **Different from control group, P < 0.05 (Mann-Whitney U test). TBVA, total body vitamin A; VA, vitamin A.

³ Median difference of intervention minus control.

≥1.05 µmol/L at both baseline and final assessments. The serum retinol concentration significantly decreased for preschoolers in the control group (P = 0.016). Median serum RBP concentrations during the screening did not differ between the control (0.83 µmol/L) and intervention (0.79 µmol/L) groups (data not shown) and there was a positive correlation between serum retinol and RBP in this population (r = 0.83; P = 0.45; P = 0.03).

The percentage enrichment of VA in serum was measured at baseline after an oral dose of 5.08 mg of [2H4]-retinyl acetate was administered and it was 34% (31, 39%) and 2% (1.6, 2.7%) 24 h and on d 14 post-dose, respectively. In the final evaluation, the isotopic ratio of labeled:unlabeled VA in serum was 1.2% (1, 1.6%) on d 14 after an oral dose of 4.01 mg of [2H8]-retinyl acetate.

TBVA stores increased by a median of 40% (0.060 mmol; P = 0.02) in children who consumed VA-fortified milk for 3 mo (Table 2). When we excluded one outlier whose TBVA stores increased >100%, the median increase was 37% (0.056 mmol; P = 0.037). All preschoolers in the intervention group except one had a significant increase in TBVA stores over the 3-mo intervention (that child had a decrease of −0.112 mmol). In the control group, median TBVA stores did not change.

Relative to the estimated baseline liver VA concentrations, there was no change in the control group. The intervention group had a significant increase in VA liver stores of 28% (0.087 µmol/g; P = 0.026) [or 27% (0.075 µmol/g) when the outlier is excluded; P = 0.047] over the course of the study (Table 2). Liver VA concentrations increased in all children from the intervention group except one; this child had a reduction in TBVA stores as well as in VA liver concentration (−0.191 µmol/g). During the course of the study, VA-fortified milk provided a total of 17.6 mg (61.6 µmol) VA to preschoolers in the intervention group. This group had a 12.4-mg (43.3 µmol) VA increase in liver stores; therefore, 70% of the extra VA that preschoolers in the intervention group received during the study was absorbed and retained.

After 3 mo, the median changes in serum concentrations of β-carotene, α-carotene, and cryptoxanthin did not differ between groups (Supplemental Table 1).

No significant differences were found in dietary intake of macronutrients, V, iron, and zinc between the control and intervention groups, excluding the additional fortified milk intake (Supplemental Table 2). Participant preschoolers’ median VA intake was 530 RE/d. The VA mean adequacy percentage (217 ± 120%; range, 19–644%) was high, but when individual VA intakes were analyzed, 3 children (12%) did not attain the Estimated Average Requirement (31). The main dietary sources of VA were mangos, carrots (cooked and raw), milk, eggs, and fortified cereals. More than one-half of the daily VA intake (53%) came from fortified foods such as cereals, milk, and nondairy fortified beverages. The TBVA stores had a positive and significant correlation with dietary VA intake in participant preschoolers (r = 0.49; P = 0.018).

Discussion

We found a significant improvement (40% increase) in TBVA stores in preschoolers who received 250 mL/d of VA-fortified milk (providing 196 RE/d) for 3 mo compared with the control group (P < 0.05). A limitation of the present study could be the fact that we did not provide a placebo milk to the children in the control group; nevertheless, dietary analysis showed that preschoolers consumed ~440 mL/d of commercially available milk (40). Our interviews revealed no consumption of VA supplements during the study, so we feel confident in attributing this change to the consumption of VA-fortified milk. In northwest Mexico, not all commercially available milk is fortified with VA and those fortified provide only one-third of VA per cup compared with Liconsa milk. The level of fortification used in our study was slightly higher than that for milk in industrialized countries (150 RE/d) (41) but much lower than the 492 RE/d provided through VA-fortified sugar to Nicaraguan schoolchildren (10). In that study, fortified sugar consumption for 1 y was associated with a 112% increase in TBVA stores. The difference between our 40% increase after 3 mo and the Nicaraguan results may be related to VA stability. Specifically, it has been estimated that after 9 mo of shipment and storage, only 40–70% of the added VA remains in the sugar (42), perhaps due to loss by air and light exposure. In contrast, VA in dried milk has been shown to be highly stable (68–100%) (43). Based on our calculation of liver VA concentration (Table 2), all participants were above the established cutoff for deficiency (0.07 µmol retinol/g of liver) (36) both at baseline and the final
assessment. Furthermore, it was estimated that 70% of the VA provided through fortified milk was effectively absorbed and stored after 3 mo.

Although efforts have been made to improve VA status in Mexican children, more attention must be given to this public health problem (44,45). UNICEF reported that 50–79% of children receive 2 VA doses/y in Mexico (44) and 2 national programs provide micronutrients to selected beneficiaries (the school breakfast and provision of fortified Liconsa milk). Unfortunately, the school breakfast program has not been found to significantly reduce the prevalence of VAD (15), although benefits may have been masked by the use of serum retinol concentrations to detect changes in VA status, the amount of VA provided is 40% less than that of Liconsa fortified milk and, more importantly, the study design of the assessment could have been a limiting factor. Thus, analyzing our results, we can suggest that the amount of fortification in the school breakfast program’s milk should be increased to improve the VA status of the beneficiary population. Fortified milk is an excellent vehicle to provide needed micronutrients to children in northwestern Mexico, because milk is a well-accepted part of their typical diet.

In this study, the participation criteria were very stringent, excluding conditions that could be present in the general population. Nevertheless, preschoolers from the northern region of Mexico have the lowest prevalence of malnutrition and anemia compared with the rest of the country (46). Furthermore, previous research has shown that Liconsa milk is effective in improving the nutritional status of its beneficiaries according to anthropometric indices (47). Therefore, our results can be used in the decision making of existing or new food policies for the Mexican population.

In the present study, preschoolers had a median VA intake of 530 RE/d (Table 2). This value is higher than that published by the Mexican national nutrition survey for children 3–6 y in northern Mexico (276–331 RE/d) (5) but similar to that observed for schoolchildren from the same region (661 RE/d) (48). In our participants, over one-half (53%) of the VA in the diet was provided by fortified foods (including fortified milk), similar to what has been observed for poor, urban, Guatemalan toddlers (55%) (9). Carotenoids were an important source of VA in this study, but because the available database used REs instead of retinol activity equivalents, our values provided an overestimate of VA intake from carotenoids (by using conversion factor of 6:1 by weight for plant pro-VA β-carotene to retinol) (31), although we did observe that 18% of the VA dietary intake came from fruits and vegetables. Even when the inadequacy prevalence for VA intake was low among preschoolers, the results are not conclusive because of the high variability of this nutrient in the diet; only three 24-h recalls were applied and according to Willet (49), this provides an accuracy of 40% in the assessment of dietary intake of VA 95% of the time.

Coproparasitological results showed that 2 children (one in each group) were infected with *Giardia intestinalis*. This parasite has been associated with lower VA status in Mexican school children from the same area as our study participants (37,38). In the infected child in the control group, the serum retinol concentration was lower at the final assessment than at baseline and there was no change in TBVA stores. TBVA stores of the infected child in the intervention group increased during the study period, suggesting that VA status can be improved using fortified milk, even in children infected with *G. intestinalis*.

In summary, daily consumption of 250 mL of VA-fortified milk for a 3-mo period was associated with significant increases in TBVA stores, liver VA concentration, and serum retinol concentration in preschool children. We suggest that VA-fortified milk is an effective and well-accepted vehicle for improving VA status in young children; thus, it should be provided to all preschool-age children in high risk areas of the country. Furthermore, fortification with adequate amounts of VA of all commercially available milk according to Mexican Official Normativity 086-SSA1–1994 (5–100% of the daily recommended intake) should be considered. Countries under similar nutritional conditions interested in using VA-fortified milk to reduce VAD in their population should revise the optimal fortification levels prior to implementation.

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**Literature Cited**
