Ascorbic acid from lime juice does not improve the iron status of iron-deficient women in rural Mexico[1–3]

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ABSTRACT

Background: Although ascorbic acid (AA) increases dietary iron bioavailability, there has been no food-based community trial of its efficacy in improving iron status.

Objective: The objective was to assess the efficacy of 25 mg AA as agua de limón (limeade), consumed with each of 2 daily meals, in improving the iron status of iron-deficient women.

Design: Two rural Mexican populations were randomly assigned to an AA or a placebo group, each with 18 iron-deficient women. The AA group was given 500 mL limeade containing 25 mg AA twice a day, 6 d/wk, for 8 mo. The placebo group was given a lime-flavored beverage free of AA or citric acid. Beverages were consumed within 30 min of 2 main daily meals. Data were collected on morbidity (3 times/wk), dietary intake (on 6 d), socioeconomic status, parasites (twice), medical history, and response to treatment. Blood samples at 0, 2, 4, 6, and 8 mo were analyzed for hemoglobin, plasma AA, plasma ferritin, transferrin receptors, and C-reactive protein.

Results: AA intake was significantly ($P < 0.0001$) higher in the AA group, but nonheme iron, heme iron, and phytic acid intakes did not differ significantly. Plasma AA was significantly ($P < 0.01$) higher in the AA group at 2, 4, 6, and 8 mo. There were no final differences between groups in hemoglobin, plasma ferritin, or transferrin receptor concentrations or in the ratio of transferrin receptors to plasma ferritin after control for initial concentrations.

Conclusion: Increasing dietary AA by 25 mg at each of 2 meals/d did not improve iron status in iron-deficient women consuming diets high in phytate and nonheme iron. *Am J Clin Nutr* 2003;78:267–73.

KEY WORDS Iron deficiency, bioavailability, ascorbic acid, ferritin, transferrin receptors, women, community trial, rural Mexico

INTRODUCTION

In poor rural Mexican communities, 80–85% of the population consumes a diet based on maize tortillas, beans, green vegetables, and fruit (1). In the typical Mexican rural diet, < 64% of the total energy comes from carbohydrates, maize provides 40% of the total protein and 45% of the energy, and the intake of meat, fish, and poultry is very low. This type of diet is associated with poor iron bioavailability (2), anemia, and deficiencies of iron and other micronutrients (3). In rural Mexico, iron deficiency affects 10–70% of individuals in different age and population groups, although iron intake overall is higher than recommended (127–193% of recommendations) (4, 5). A potential approach to improving iron status is to make this iron more available for absorption.

Ascorbic acid (AA) is the main dietary enhancer of iron absorption, apart from meat, fish, and poultry, but the intake of AA-rich foods is low in rural Mexico. The median AA intake of 33 mg/d in nonpregnant, nonlactating (NPNL) women is consumed as the locally produced alcoholic beverage pulque, a lime beverage known as agua de limón (or limeade; prepared with water, sugar, and lime juice), red and green tomatoes, potatoes, chili peppers, wild greens, cactus, other citrus fruit, and mangoes in season (2). Pulque is the main source of AA for NPNL rural Mexican women in the present study, as well as the third most important source of nonheme iron, and the amount of pulque consumed is the main predictor of a lower risk of anemia and iron deficiency (5).

The enhancing effect of AA on the absorption of iron from Latin American foods has been well documented in meal feeding studies (6–9). Iron absorption was tripled by the consumption of 300 mL orange juice with beans, tortillas, and coffee (9). There is a relatively consistent improvement in iron absorption when AA is added to meals, but it is more difficult to show that AA improves iron status. For example, there was no significant improvement in iron-status indicators when menstruating women in the United States were given 100 mg AA/d for 9 mo (10), 2000 mg AA/d for ≤ 2 y (11), or 1500 mg AA/d for 10 wk (12).

There are only 2 reports from outside the United States on the effect on iron status of providing AA in community-based trials. In India, treatment with AA (100 mg synthetic AA/d provided as a supplement) for 2 mo improved hemoglobin concentrations significantly in anemic preschool children, compared with a placebo (13). Ferritin response was not analyzed. In China, 50 mg synthetic AA/d, given as a supplement for 8 wk, improved the iron status (serum ferritin concentration) of 65 mildly anemic children

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(14). There are no reported long-term, community-level efficacy trials of the effect of adding AA from local food sources to meals high in poorly available, nonheme iron.

The current study reports the long-term (8 mo) effect of increasing AA intake from agua de limón on the iron status of iron-deficient, rural Mexican women by using a dose that had first been proven, with the use of iron isotopes, to double iron absorption in similar women.

SUBJECTS AND METHODS

Subjects and location

The research was conducted in the Solís Valley, a rural area 170 km northwest of Mexico City. The sample size (n = 18 per group) was established with the assumption of a biologically significant increase of 5 μg/L in plasma ferritin (PF), and the calculation was based on the mean and SD of PF in NPNL women in previous studies in this area (5). To identify eligible participants, hemoglobin and PF concentrations were measured in < 150 NPNL women. Among them, 42 iron-deficient (PF < 12 μg/L) subjects in 2 communities were identified. The communities, located < 10 km apart and very similar, were randomly assigned to the experimental or the control group. The randomization was done at a community level because, although the placebo and the limeade looked very similar, the taste and volume of the 2 beverages were slightly different. Only 9 of the 42 iron-deficient subjects were anemic (hemoglobin < 130 g/L); of these 9 subjects, 5 were in the experimental (AA) group and 4 were in the control (placebo) group. Of the original 21 women from the experimental group and 21 from the control group who agreed to participate, 3 in each group were subsequently excluded because of pregnancy. The purpose of the study was described to the women, who gave written informed consent. The project was approved by the Human Subjects Review Committee of the University of California, Davis, and the Committee on Biomedical Research on Humans of the National Institute of Nutrition of Mexico. All women who were anemic at the end of the study were provided with iron supplements for 3 mo.

Selection of food source and dose of ascorbic acid

Before the present community trial, the best potential food source of AA, agua de limón, was selected by using existing dietary data from the community. Then, to ensure that the amount of agua de limón consumed would increase iron absorption, we tested the effect of adding different amounts of AA as agua de limón on the absorption of iron from typical Mexican diets. In the first of these studies, in which we used an extrinsic radiolabeled iron, the addition of 25 mg AA to one daily meal of subjects at the University of California, Davis, did not improve iron absorption, but the addition of 25 mg AA to each of 2 daily meals increased iron absorption significantly (P < 0.05), from 3.1 ± 6.2% to 7.4 ± 8.2% (15). This was confirmed in a second study, in which iron absorption from single meals was measured by using stable iron isotopes in iron-deficient women from the same Mexican communities in which the present study was located (16). In a third study, stable isotopes of iron were distributed among the meals for 14 d in rural Mexico (16). The limeade was given to NPNL iron-deficient women at the frequency and in the amount determined to be effective in the first 2 studies. The results of both of the stable isotope studies agreed with those of the radiolabeled iron study: when 2 daily meals were served with 25 mg of AA as limeade, iron absorption was doubled.

Study design

The AA content of a batch of freshly squeezed lime juice was determined by the 2,6-dichloroindophenol titration method (17). The limeade was prepared twice a day by dissolving 1.2 kg sugar in 8.4 L water and pouring the mixture into the single-serving containers provided to each subject. Then the amount of lime juice needed to provide 25 mg AA per meal was added to each container. The placebo was prepared from a commercial powdered beverage modified especially for this study. The powder contained sugar, lime flavor, and lime color, but no AA or citric acid. To mimic the acidic taste of lime juice, a small amount (0.5% by wt) of fumaric acid was added. The placebo drink was prepared twice a day before delivery by dissolving 540 g of the powder in 5.4 L water. Then 300 mL of the prepared placebo was poured into each subject's container.

Twice a day, 6 d/wk, for 8 mo, the women in the experimental group received the agua de limón containing 25 mg AA, and those in the control group received the placebo. Field workers delivered the freshly prepared beverages to the women's homes in the early morning and later in the day. The women were instructed to drink their beverages either with the first and second main meals of the day or not later than 30 min after eating those meals. These meals contained more total nonheme iron than did the other meals of the day. During each subsequent visit, the field workers asked the women the actual time of consumption of the limeade or placebo with each meal.

Biochemical determinations

Blood samples were taken at baseline and at 2, 4, 6, and 8 mo. Hemoglobin concentration was determined in one drop of venous blood by using the HemoCue hemoglobin analyzer (HemoCue Inc, Mission Viejo, CA). Plasma AA was determined by the 2,4-dinitrophenylhydrazine method (18). C-reactive protein was assessed qualitatively by a latex slide test (Immunex CRP; Wampole Laboratories, Cranbury, NJ). PF concentrations were determined in duplicate by using an immunoradiometric assay (Coat-A-Count Ferritin IRMA; Diagnostic Products Corp, Los Angeles). Plasma transferrin receptors (TfR) were measured by using an enzyme-linked immunoassay (Ramco Laboratories Inc, Houston). Cutoffs for anemia and iron deficiency were as follows: hemoglobin < 130 g/L (adjusted for the altitude of 2300 m) (19), PF concentration < 12 μg/L (19), and plasma TfR concentration > 8 mg/L (20). The cutoff for plasma AA that indicated depletion was < 10 μmol/L (21).

Fecal parasites were analyzed at 1 and at 6 mo. Morbidity questionnaires were administered by the field workers 3 times a week during the 8-mo study to allow for the potential confounding effect of illnesses on iron status. We collected information on 4 main categories of illnesses and symptoms, ie, acute upper and lower respiratory illnesses, gastrointestinal illnesses (eg, diarrhea), fever, and general symptoms (eg, headache, malaise, and dizziness).

Collection and analysis of food intake data

Food intake was assessed by evaluating three 24-h recalls on each of 2 occasions (ie, data from a total of 6 d)—once during the planting season (April–May) and once during the preharvest

TABLE 1
Body weight, plasma ascorbic acid, and iron-status indicators in 36 iron-deficient women during the 8-mo intervention[1]

Indicator	Baseline	2 mo	4 mo	6 mo	8 mo
Weight (kg)[2]					
Experimental	60.1 ± 10.8	61.3 ± 10.2	60.7 ± 9.5	61.1 ± 9.7	61.3 ± 9.7
Control	58.9 ± 13.2	59.3 ± 13.1	61.4 ± 13.6	62.1 ± 13.0	62.2 ± 13.9
Plasma ascorbic acid (μmol/L)[3]					
Experimental	34.2 ± 8.0	51.3 ± 10.3[4]	47.3 ± 9.7[4]	47.3 ± 8.0[4]	51.3 ± 10.3[4]
Control	28.5 ± 13.7	31.4 ± 16.0	31.9 ± 14.3	34.8 ± 15.4	31.4 ± 15.7
Hemoglobin (g/L)					
Experimental	137 ± 15	138 ± 16	137 ± 15	137 ± 13	140 ± 13
Control	139 ± 14	133 ± 14	133 ± 14	134 ± 15	137 ± 17
Plasma ferritin (μg/L)					
Experimental	6.4 ± 3.3	7.3 ± 4.1	10.7 ± 7.4	10.4 ± 6.2	9.0 ± 5.2
Control	6.2 ± 2.8	6.4 ± 3.7	7.3 ± 5.4	9.3 ± 6.3	8.7 ± 6.1
Plasma transferrin receptors (mg/L)					
Experimental	12.6 ± 9.2	8.9 ± 6.0	4.9 ± 4.5	10.0 ± 8.9	8.1 ± 5.8
Control	8.7 ± 7.6	7.0 ± 5.3	5.9 ± 4.4	8.5 ± 5.8	7.0 ± 4.7
Plasma transferrin receptors:ferritin[5]					
Experimental	3183 ± 3171	1418 ± 1773	1903 ± 2176[6]	2403 ± 3500	888 ± 1353
Control	1936 ± 2488	1962 ± 2763	1529 ± 1577	1911 ± 1812	1134 ± 1333

[1] $\bar{x}$ ± SD; n = 18 in each group. Means were compared with the use of repeated-measures ANCOVA ($P < 0.05$).
[2,3,5] Group × time interaction: [2] $P = 0.35$, [3] $P < 0.001$, [5] $P = 0.021$.
[4,6] Significantly different from control (t test with control for baseline): [4] $P \leq 0.01$, [6] $P < 0.02$.

season (September–October). The food intake data were used to compare the intakes of AA, iron, phytate, energy, protein, and fat in the AA and placebo groups. The WORLDFOOD PROGRAM (University of California, Berkeley, CA) was used to calculate the energy, protein, fat, dietary phytate, AA, and iron (heme and nonheme) intake per day and per meal (22). The meals were defined, according to the time of day, as breakfast, lunch, late lunch, early dinner, dinner, and snacks (between meals). Nutrient intake per meal was analyzed to determine whether the limeade and the placebo were in fact consumed with the daily meals that were highest in iron and phytic acid. Bioavailable iron was estimated by using the algorithm of Murphy et al (23). The probability of inadequacy of intake of other nutrients was also calculated.

Questionnaires

A socioeconomic status questionnaire was completed at the beginning of the study to ensure that there was no socioeconomic difference between groups (ie, communities) that could cause differences in food intake and health and to account for the potential confounding effect of socioeconomic status on a change in iron status. The subjects' perceptions of the intervention were evaluated at the end of the study by administering a response-to-treatment questionnaire that asked about the subjects' perceptions of the placebo or limeade and their reporting of their behavior in terms of substituting other beverages during the intervention. A medical history questionnaire was completed at the end of the study to account for nonnutritional factors that could affect iron status and iron absorption, ie, parity, type of birth control, previous diagnosis and treatment of anemia, and duration and heaviness of menstruation.

Statistical analysis

Statistical analyses were performed by using SAS software, version 8 (24). Continuous variables were examined for conformity to the normal distribution, and, if appropriate, they were logarithmically transformed. Treatment groups (n = 36 total) were compared at baseline with respect to age, weight, hemoglobin, plasma AA, PF, plasma TfR, and TfR:PF by using Student's t test. All variables except age were examined with the use of analysis of covariance, which included the initial value of the response variable, and we set significance at $P < 0.05$. Ferritin values were not included in the statistical analysis for samples with a positive C-reactive protein result (n = 0–2 samples at any time point).

Morbidity data, for which no suitable transformation was found, were examined with the Wilcoxon rank sum test to detect any differences between groups during the 8 mo. The frequency of positive responses in the questionnaires was analyzed with the use of the chi-square test to determine any differences between groups. Dietary data were analyzed with the use of the one-way ANOVA.

RESULTS

Anthropometric measurements and indicators of iron status are summarized for both groups in **Table 1**. The mean age of the subjects in the experimental and control groups did not differ significantly (28.2 ± 9.6 and 28.3 ± 10.7 y, respectively). There were 4 anemic women in the experimental group and 5 in the control group. Three of the women in each group were still anemic at the end of the study. Plasma AA was significantly ($P < 0.01$) higher in the experimental group at 2, 4, 6, and 8 mo, which confirmed that subjects in that group were consuming the limeade and had a higher AA intake and that the AA was absorbed.

Hemoglobin concentrations were not significantly different from baseline values at the end of the 8-mo intervention. After control for initial concentrations, there were no significant differences between groups in PF and TfR concentrations at the end of the study. The TfR:PF was significantly ($P < 0.02$) different between groups at 4 mo, but not at the end of the study, after control for the initial ratio. There was a significant negative correlation ($r = -0.54$, $P < 0.006$) between PF and TfR that did not differ between groups.

There were significantly ($P = 0.022$) more diarrhea episodes in the experimental group than in the control group during the

TABLE 2
Morbidity episodes in the 36 subjects during the 8-mo intervention[1]

Sickness or symptom[2]	Experimental group	Control group
Acute upper respiratory illness or symptoms	2 (0–10)	2 (0–7)
Gastrointestinal symptoms	2 (0–5)	1 (0–7)
Diarrhea	0 (0–4)[3]	0 (0–1)
General symptoms	3 (0–10)	2 (0–17)
Fever + acute upper respiratory illness	0 (0–2)	0 (0–2)
Fever + gastrointestinal symptoms	0 (0–2)	0 (0–0)
Fever + general symptoms	0 (0–3)	0 (0–1)

[1] Median; range in parentheses. $n = 18$ in each group. Medians were compared with the use of the Wilcoxon rank-sum test ($P < 0.05$).

[2] Acute upper respiratory illness included otitis, influenza, sore throat, aphonia, cough, rhinorrhea, nasal congestion, and sinusitis. Gastrointestinal symptoms included stomach ache, vomiting, nausea, and anorexia. Diarrhea was defined as the presence of watery stools for ≥3 d. General symptoms included headache, malaise, dizziness, and menstrual pain.

[3] Significantly different from control group, $P = 0.022$.

study (**Table 2**). No episodes of lower respiratory illnesses were reported. There were no significant differences between groups in episodes of acute upper respiratory illness or general symptoms; 80% of the general symptoms included headaches and malaise. The qualitative test for C-reactive protein showed that 90% of the subjects were free of infection at the time of each phlebotomy.

Most of the parasites detected in the feces (ie, *Entamoeba histolytica, Endolimax nana*, and *Entamoeba coli*) are endemic in the area and are not known to affect iron absorption. No hookworm was found in the fecal samples. There was no difference in the presence of parasites between the experimental and control groups at 1 or at 6 mo.

The daily mean nutrient intakes of the 2 groups are summarized in **Table 3**. The experimental group consumed less protein on average. The average daily AA intake in the experimental group was almost twice that in the control group ($P < 0.0001$). There was no significant difference between the mean intakes of nonheme iron, heme iron, and available iron in the 2 groups. In both groups, the same 3 daily eating occasions (out of 6 possible occasions) were the major contributors to the intakes of energy, phytate, and iron. At breakfast, early lunch, and late lunch, AA intakes were significantly ($P < 0.003$) higher in the experimental group, which showed that, as intended, the limeade was consumed with the meals that were the highest in iron and phytate. The 25 mg AA given in the morning was consumed with either of the 2 first meals of the day, and the second dose of 25 mg AA was consumed mainly with the third meal, which had the highest content of energy, nonheme and total iron, and several other nutrients.

TABLE 3
Daily nutrient intakes during the 8-mo intervention[1]

Nutrient	Experimental group	Control group
Energy (kcal)	2462 ± 565	2472 ± 601
Protein (g)	58.8 ± 14.9	69.5 ± 17.1
Fat (g)	44.3 ± 21.0[2]	54.4 ± 15.8
Ascorbic acid (mg)[3]	112.9 ± 41.3[4]	56.0 ± 35.9
Phytate (mg)	2885 ± 994	2967 ± 1095
Nonheme iron (mg)	11.1 ± 3.5	11.4 ± 3.3
Heme iron (mg)	0.8 ± 1.7	1.0 ± 1.6
Bioavailable iron (mg)	1.0 ± 0.3	1.0 ± 0.4

[1] $\bar{x}$ ± SD; $n = 18$ in each group. Means were compared with the use of one-way ANOVA ($P < 0.05$).

[2,4] Significantly different from control group: [2] $P = 0.05$, [4] $P < 0.0001$.

[3] In the experimental group, includes ascorbic acid from the supplemental agua de limón.

TABLE 4
Predicted percentages of subjects with inadequate nutrient intakes as determined from the mean of data for 6 d during the study[1]

Nutrient	Experimental group	Control group
	%	
Protein	12.3 ± 22.8	21.9 ± 32.1
Vitamin A[2]		
Basal	8.3 ± 16.4	11.2 ± 24.6
Normative	39.9 ± 37.9	29.0 ± 36.5
Vitamin D[3]	88.9 ± 27.2	92.1 ± 20.3
Vitamin E	94.5 ± 13.0	94.8 ± 13.1
Ascorbic acid[4]	0.6 ± 2.4[5]	25.0 ± 33.7
Thiamine	12.8 ± 21.3[6]	4.6 ± 14.6
Riboflavin	56.5 ± 40.1[6]	29.2 ± 36.3
Niacin[7]	77.1 ± 28.7[6]	59.7 ± 34.5
Vitamin B-6	0.6 ± 3.4	0.2 ± 1.2
Folate	22.9 ± 33.4	15.9 ± 22.7
Vitamin B-12	36.3 ± 41.4[6]	20.3 ± 34.0
Calcium	2.4 ± 5.8	5.2 ± 17.2
Iron[2]		
Basal	88.7 ± 18.9	88.6 ± 18.2
Anemia-preventive[8]	61.4 ± 33.4	64.4 ± 32.5
Zinc[2]		
Basal	26.3 ± 33.5	11.8 ± 21.2
Normative	55.7 ± 42.3[6]	34.3 ± 35.7
Copper[2]		
Basal	2.0 ± 6.8	1.8 ± 4.5
Normative	5.3 ± 13.8	4.4 ± 10.2

[1] x ± SD; $n = 18$ in each group. Differences between groups were analyzed with the use of the Wilcoxon rank-sum test ($P < 0.05$).

[2] Basal = the amount of nutrient necessary to prevent clinical deficiency; normative = the amount of nutrient necessary to meet essential needs without impairing function.

[3] Does not consider synthesis of vitamin D from ultraviolet exposure.

[4] In the experimental group, includes ascorbic acid from the supplemental agua de limón.

[5,6] Significantly different from control group: [5] $P = 0.001$, [6] $P < 0.05$.

[7] Does not consider synthesis of niacin from tryptophan.

[8] The amount of iron necessary to prevent a decrease in hemoglobin concentration below the World Health Organization cutoff that indicates anemia.

The predicted prevalence of inadequate nutrient intakes in both groups, for the 6 d of food intake data combined, is shown in **Table 4**. Basal and normative iron intakes (defined as the amount of iron in the diet needed to prevent clinical iron deficiency and to maintain a normal supply of tissue iron, respectively) and anemia-preventive iron intakes (defined by the World Health Organization as the amount of dietary iron required to prevent anemia, ie, a drop in hemoglobin concentration below the World Health Organization's cutoffs) were low in both groups (4). Although the daily iron intakes in both groups were high, the estimated intakes of available iron were inadequate, mainly because of both the low amount of meat, fish, and poultry in the diet and the consumption of tea and coffee with meals. As expected, the percentage of subjects with inadequate AA intake was virtually zero in the

experimental group ($P < 0.0001$). In the control group, 25% of the subjects had predicted inadequate intakes of AA.

No significant differences in socioeconomic status variables were found between groups at baseline. From the information collected with the medical history questionnaire, there were no differences between groups in variables that might affect iron status, such as number of pregnancies, type of birth control, prior diagnosis of anemia, prior treatment with iron and/or vitamin supplements, or reported days and heaviness of menstruation. In addition, none of these variables was significantly related to any iron-status indicator.

When asked about their perception of the intervention, at the beginning of the study, 72% of the subjects in the experimental group said they liked the beverage, compared with 100% of the subjects in the control group ($P < 0.016$). The perception of the beverages' acidity differed significantly ($P < 0.016$) between groups at baseline: almost 40% of the experimental group thought the limeade was too acidic. Forty percent of the experimental group thought the amount of limeade was too large, but only 17% of the control group thought so. At the end of the study, there were no differences between groups in any of these responses, which suggests that the experimental group grew accustomed to the agua de limón. Most of the subjects in both groups said they did not substitute the agua de limón for any of their usual beverages, but, of those who did do so, most stopped drinking sodas and coffee. There was a significant ($P < 0.0001$) difference between the experimental group and the control group in terms of the proportions of subjects stating that they would not consume the limeade with the same frequency after the study ended (< 80% compared with 17%, respectively).

DISCUSSION

Our analyses of existing food intake data revealed that no other single food source of AA could be as effective in improving iron status in these communities (15, 16). Most of the foods with a high AA content are seasonal, in particular, fruit such as oranges and guava. The amounts of most of these foods needed to provide enough ascorbic acid to enhance iron absorption (25–50 mg/meal) would be considerably more than the amounts normally consumed by this population. In addition, sources of AA such as cactus, chili peppers, and tomatoes are cooked, and thus the AA content may vary considerably. Pulque, an alcoholic beverage made from the sap of the maguey cactus, is an important source of the vitamin for consumers of this beverage, but it was inappropriate for use in this intervention. Therefore, it was difficult to identify a suitable, locally available, frequently consumed food source of AA. Limeade was the best option, considering that it is available year-round, is not considered expensive, is already consumed by women in these communities and throughout rural Mexico, and is consumed more widely than any other fruit or source of AA. Just as important, it could also be consumed with meals that are high in nonheme iron.

These iron-deficient women were given 25 mg AA twice a day, 6 d/wk, for 8 mo. On the basis of the isotope studies, the amount of iron absorbed was expected to increase to ≥ 1 mg/d above the requirement of 1.5 mg/d. After 8 mo, an increment in PF concentration of ≥ 15 μg/L was expected, based on the amount of iron absorbed (calculated from the actual absorption studies and dietary iron intake) and assuming that each 8–10-mg increment in stored iron will increase the PF concentration by 1 μg/L (25). However, there was no effect on PF concentrations at the end of the 8-mo intervention.

Even though the PF concentration is one of the most widely used indicators of iron status, it is known to be affected by several factors, including infection and inflammation. In contrast, the TfR concentration is a sensitive indicator of tissue iron deficiency and is not affected by infection, inflammation, or mild malnutrition (26, 27). In addition, the total day-to-day variation is lower for TfR than it is for PF (28). TfR concentrations fell after 4 mo but increased again at 6 and 8 mo. At the end of the study, the mean concentration of TfR in the experimental group was marginally above the cutoff for iron deficiency (> 8 mg/L) but did not differ significantly from that in the control group.

Although all of these women had a low PF concentration at baseline, only 18 had an initial TfR concentration > 8 mg/L. However, those women who had the lowest PF concentrations had the highest TfR concentrations, as shown by the significant negative correlation between these iron-status indicators. Because TfR is not affected during the depletion of iron stores, whereas PF drops, and because TfR increases progressively during functional iron depletion, whereas PF remains the same, the ratio of these measurements is a more sensitive and specific indicator of iron status than is either measurement alone (29, 30). Adequate iron stores are reflected by a mean TfR:PF < 100, storage depletion by a median ratio of 500, and significant functional depletion by a ratio > 2000 (27). The mean ratio at baseline was 3000 in the experimental group and nearly 2000 in the control group, and all of the subjects had both storage and functional depletion at the beginning of the study. Although the ratio changed over time for both groups, it never fell to < 1400, which means that the subjects remained iron-depleted throughout the study. After 8 mo, TfR:PF had decreased significantly but only in the experimental group; most of the subjects reached their highest PF and lowest TfR concentrations at the end of the study but remained iron-deficient.

The lack of improvement in iron stores was not due to noncompliance or failure of the intended intake of AA. Women in both communities had a high intake of nonheme iron and phytate, and the subjects consumed the limeade with the meals that were highest in iron and phytate. No parasites were detected that could interfere with iron absorption. Although one study reported that AA may have less effect on iron absorption when consumed with multiple meals per day over 5 d than when consumed at a single meal (31), we confirmed that AA fed either at single meals or at 2 meals/d for 2 wk doubled iron absorption in iron-deficient women in the same communities (16).

It is therefore probable that other factors had a stronger influence on iron status than did AA intake. Although there was no significant difference in the measured intakes of nonheme or heme iron between the groups, we had no control over the subjects' diets except for the dose of AA. Parity, reported duration and intensity of menstruation, and previous anemia were not related to iron status, but menstrual blood losses were not actually measured. Because these women were selected on the basis of their iron-deficient status, they may have had above-average menstrual blood loss, which would make it unlikely that the small improvement in iron absorption would be able to increase iron stores. Another consideration is that, as iron absorption and status improve, not only is the efficiency of absorption down-regulated over time (32), but the iron losses in menstrual blood will increase (33). Thus new steady states are successively obtained, in which losses of iron again tend to equal absorption. Although an improvement in iron

stores may have been detectable in a larger group of subjects and with a longer intervention, this still would not argue against the poor efficacy of AA for improving iron status in these women.

The results of this study do not necessarily mean that increased AA intake should not be encouraged. There are several situations in which higher intakes of the vitamin would be more likely to improve iron status. One is a situation in which meals contain substantial amounts of fortificant iron, at least in the form of ferrous sulfate (34). It has been estimated that a 6:1 (by wt) ratio of AA to iron is required to usefully increase the absorption of soluble nonheme iron, whereas a ratio closer to 12:1 may be needed if the foods are high in phytic or phenolic acid, as they are in rural Mexico (34). The ratios in this study were ≈ 10:1 in the AA group and 5:1 in the control group. The effect of AA on iron absorption is dose related (7). It may be possible in some situations, eg, when other citrus or AA-rich foods are available, for people to consume even larger amounts of the vitamin, although this was not feasible in the Solís Valley. Intakes were already > 100 mg/d in the supplemented group, and the women said that they would not sustain that amount of consumption after the study ended. In an epidemiologic study of elderly in the United States whose average intake of AA from foods was ≈150 mg/d, a 10-mg difference in AA intake predicted a 1% greater serum ferritin concentration, and fruit and meat intakes were the positive predictors of ferritin concentration (35). In the elderly, men, and children, daily iron losses are much smaller because of the absence of menstruation, so that improvements in absorption are more likely to result in an improvement in stores.

Another factor in this community, as in many developing countries, is that other micronutrient deficiencies, such as those of vitamins A and B-12, may have confounded the enhancing effect of AA on iron status. According to the estimated prevalence of inadequacy, many of the subjects had an inadequate intake of nutrients that could affect iron absorption or utilization, including riboflavin, vitamin A, and vitamin B-12.

In conclusion, increasing AA intake as limeade did not improve the iron status of iron-deficient women consuming diets high in phytate and nonheme iron in rural Mexico. Because of the lack of efficacy and the paucity of local food sources that can adequately increase AA intake, we conclude that this food-based intervention is not practical or effective for improving the iron status of women in these communities, at least once they have already become iron depleted. Iron fortification or supplementation is required. It is possible, however, that AA could produce a detectable increase in iron stores in some other situations.

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