The efficacy of a local ascorbic acid–rich food in improving iron absorption from Mexican diets: a field study using stable isotopes[1–3]

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ABSTRACT

Background: One potentially sustainable approach to improving iron status at the community level is to encourage the consumption of local ascorbic acid–rich foods, in conjunction with meals high in nonheme iron.

Objective: The study, conducted in rural Mexico, measured stable isotopes of iron to evaluate the effect on iron absorption of the addition of 25 mg ascorbic acid as agua de limón (limeade) to 2 typical meals per day for 2 wk.

Design: Fifteen nonpregnant, nonlactating, iron-deficient (ferritin < 12 μg/L) women ($\bar{x} \pm$ SD age: 28.3 ± 7.7 y) fasted overnight and were brought to a community clinic. After an initial blood sample, subjects consumed 0.25 mg ^{57}Fe with both breakfast and lunch for 14 d. On day 29, another blood sample was taken, and a reference dose of 2.7 mg ^{58}Fe with 25 mg ascorbic acid was given. For the following 15 d, participants consumed 0.25 mg ^{57}Fe added to both breakfast and lunch with 25 mg ascorbic acid added to each meal as limeade. A final blood sample was taken on day 59.

Results: Iron absorption was calculated from recovery of isotopes in blood obtained 14 d after administration of each isotope. When 25 mg ascorbic acid as limeade was added to test meals twice a day for 2 wk, iron absorption increased significantly ($P < 0.001$) in every subject: the mean absorption rose from 6.6 ± 3.0% to 22.9 ± 12.6%.

Conclusions: The consumption of 25 mg ascorbic acid as limeade twice daily with meals substantially improved iron absorption and may improve the iron status of nonpregnant, nonlactating, iron-deficient women. *Am J Clin Nutr* 2003;78:436–40.

KEY WORDS Iron absorption, bioavailability, ascorbic acid, stable isotopes, Mexican diet

INTRODUCTION

Iron deficiency and anemia are recognized worldwide as a public health problem, especially in developing countries. Approximately 2.5 billion of the world's people have iron deficiency, and 1.2 billion have iron deficiency anemia (1, 2). In Mexico, anemia is present in 27% of children aged < 5 y, 40% of school-age children, and 29% of women of childbearing age (3, 4). The prevalence of iron deficiency in these same groups is 35%, 48%, and 30%, respectively (3, 4).

Populations such as those in rural Mexico, which have a high intake of cereals (particularly maize) and legumes as staples and a low intake of meat, poultry, and fish, may be iron deficient because of poor dietary iron bioavailability (5–7). Whereas the total iron content of the traditional Mexican diet is quite high, almost all of this iron is in the nonheme form and is predominantly from beans and maize (8), which contain large amounts of phytate. Intakes of nonheme-iron absorption enhancers such as ascorbic acid are low in Mexico (4, 9). Thus, increasing the intake of ascorbic acid from local foods may be a sustainable approach to improving iron absorption and status in rural Mexico.

Published studies showed that ascorbic acid increases iron absorption with a linear dose-related response of up ≤ 200 mg/d (10). Several investigators concluded that, in the long term, ascorbic acid–induced increases in nonheme-iron bioavailability from whole diets might be less than that observed from ascorbic acid–rich foods or from foods to which ascorbic acid was added. In iron-sufficient Mexican boys 12–15 y old, feeding ascorbic acid as 300 mL orange juice for 2 wk increased nonheme-iron bioavailability to a greater extent than did feeding a meal to which synthetic ascorbic acid had been added in the same amount (11). Likewise, when nonheme-iron absorption was measured from the whole diet over a 2-wk period, there was a 2.5-fold difference between a diet containing foods that enhance iron absorption and a diet containing foods that inhibit iron absorption, but there was a 5.9-fold difference in iron absorption from foods to which ascorbic acid was added and foods containing inhibitors (12).

Before starting a community trial of the effect on iron status of increasing the intake of ascorbic acid as agua de limón (limeade), the present study was designed to test the effect on iron absorption from typical Mexican diets of consuming limeade that provides 25 mg ascorbic acid with both breakfast and lunch over a period of 2 wk. The study was conducted with typical diets in the field on iron-deficient (ID) women. Iron absorption was assessed by measuring the stable isotopes of iron, which were previously used in the field (13). The hypothesis to be tested was that the consumption of habitual doses of limeade significantly increases iron absorption.

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SUBJECTS AND METHODS

Preparation of isotopes

The stable isotopes ^{58}Fe and ^{57}Fe were obtained in the form of "iron wire" with 95.48% and 93.13% isotopic purity, respectively (Horizon Tech, Great Neck, NY). Both forms of metal iron were converted to ferrous sulfate with 0.5 mol H_2SO_4/L (14). The ^{58}Fe isotope was dissolved at room temperature in 9 mL of 7 mol HNO_3/L and 37.5 mL of 0.5 mol H_2SO_4/L, dried uncovered at 120 °C in a muffle furnace for 2–3 h, and then dried at 230 °C for 30 min and at 500 °C for 30 min. The whitish powder was then reconstituted in 50 mL of 0.2 mol H_2SO_4/L. The ferrous sulfate solution obtained was filtered through a 0.5-μm Millex filter (Millipore, New Bedford, MA) and brought to a final volume of 372 mL and a 0.75 mg/mL concentration, which was checked by atomic absorption spectrometry (Perkin-Elmer, Norwalk CT). The ^{57}Fe oxide as a wire was converted to a ferrous sulfate solution by the addition of 38 mL concentrated H_2SO_4 in a sealed vial with a nonreactive lid (polytetrafluoroethylene cap) at 110 °C until the solution was clear (≈2 h). This solution was transferred to a beaker, and the vial was rinsed with 41 mL of 0.5 mol H_2SO_4/L. The solution was dried in a muffle furnace at 120 °C for 2–3 h, at 230 °C for 30 min, and at 500 °C for 30 min. The powder obtained was reconstituted with 50 mL of 0.2 mol H_2SO_4/L, filtered through a 0.5-μm Millex filter (Millipore), and brought to a final volume of 308 mL. The final iron concentration was checked with the use of atomic absorption spectrometry. All acids used for the preparation of the isotope solutions were Ultrex II Ultrapure Reagent (JT Baker Inc, Pillsburg, NJ). Final solutions were stored with protection against light at 5 °C. ^{57}Fe was enriched to 95.7% ^{57}Fe and ^{58}Fe was enriched to 93% ^{58}Fe.

Subjects and location

The research was conducted in a rural area in Mexico, and a metabolic unit and community clinic were used for the study. Thirty-four nonpregnant, nonlactating (NPNL) women were evaluated for hemoglobin and plasma ferritin concentrations; from these 34, 16 ID (plasma ferritin concentration: < 12 μg/L) subjects were identified. One of the subjects became pregnant and was subsequently removed from the study. The purpose of the study was described to the 15 women ($\bar{x}$ ± SD age: 28.3 ± 7.7 y), who gave written informed consent. The project was approved by the Human Subjects Review Committee of the University of California, Davis. Results for 4 subjects were not included in the statistical analyses because of laboratory error in handling samples; the statistical analyses were based on data from the remaining 11 subjects.

Study design and test meal

The test meal, based on the model rural diet described by Rosado et al (5, 15), was maize tortillas and beans with vegetables and small amounts of salsa. Three vegetables [cactus (*Opuntia* sp), green snap beans, and squash] were selected to add variety to the meals. The amounts of each vegetable were equivalent in terms of their fiber content (16). Thus, 3 test meals were given on a 3-d rotating basis to the subjects, each served twice daily (**Table 1**). The WORLDFOOD software program from the University of California was used to calculate the energy, phytate, ascorbic acid, and iron content of the test meals (17). The molar ratio of phytate to iron for each meal was 21.9, 20.1, and 18.9, respectively.

TABLE 1
Energy, phytic acid, ascorbic acid (AA), and iron content of the 3 experimental meals[1]

Food	Amount	Energy	Phytic acid	AA	Total iron
	g	*kcal*	*mg*	*mg*	*mg*
Meal 1					
Maize tortillas	200	444	960	0	2.8
Refried beans	138	254	631	1.4	2.9
Onion	50	22	0	3	0.1
Cactus (*Opuntia* sp)[2]	60	16	0	22	0.3
Salsa	30	6.4	1.8	5.8	0.1
Total	478	742	1,593	32	6.2
Meal 2					
Maize tortillas	200	444	960	0	2.8
Refried beans	138	254	631	1.4	2.9
Onion	50	22	0	3	0.1
Squash[2]	190	38	0	4	0.2
Salsa	30	6.4	1.8	5.8	0.1
Total	608	764	1,593	14.2	6.1
Meal 3					
Maize tortillas	200	444	960	0	2.8
Refried beans	138	254	631	1.4	2.9
Onion	50	22	0	3	0.1
Green snap beans[2]	210	74	191	21	2.7
Salsa	30	6.4	1.8	5.8	0.1
Total	628	800	1784	31.2	8.6

[1] The 3 diets were served on a 3-d rotating basis.
[2] The equivalent amount of cactus, squash, and green snap beans was determined according to the fiber content of each food.

Iron absorption from the meals was measured with ^{58}Fe and ^{57}Fe isotopes, according to the approach traditionally used to measure iron absorption with radioisotopes (18). The 15 subjects were transported to the community clinic in the fasted state, and their weights and heights were measured. A reference dose of 2.7 mg ^{58}Fe with 25 mg ascorbic acid as limeade was given to the subjects in the absence of food. The following day, the women received 0.25 mg ^{57}Fe (added extrinsically) at both breakfast and lunch, but no ascorbic acid was added to the meals. This continued for the next 14 d, for a total dose of 6.9 mg ^{57}Fe. Fourteen days later, a second fasted blood sample was obtained, and the women were weighed again. A second reference dose containing 2.2 mg ^{58}Fe with 25 mg of ascorbic acid as limeade was administered. During the next 14 d, the women consumed 0.25 mg ^{57}Fe at both breakfast and lunch with 25 mg limeade added to both meals (total dose: 6.9 mg ^{57}Fe). Fourteen days later, a final blood sample was taken, and the final weight measurement was taken. After the first meal of each day, the subjects were kept under supervision in the clinic for ≥ 4 h to ensure that they did not consume other foods during this time. They then had the second meal at the community clinic and returned to their homes for the remainder of the day, having been instructed not to eat anything for the next 4 h.

Preparation of limeade and measurement of ascorbic acid content

The ascorbic acid content of freshly squeezed lime juice was measured by using the 2,6-dichlorophenolindophenol method twice every day immediately before each meal was served (19). The limeade was prepared by dissolving 1.1 kg sugar in 8 L water at breakfast and at lunch and pouring it into the subjects' glasses. Then, the amount of lime juice needed to provide 25 mg ascorbic acid per meal was added separately to each glass.

TABLE 2
Characteristics of the 11 subjects included in the study[1]

Characteristic	Value
Age (y)	28.5 ± 7.9
Height (cm)	154.6 ± 5.3
Weight (kg)	
Day 0	60.3 ± 9.8
Day 29	59.9 ± 9.8
Day 59	59.7 ± 10.1
Hemoglobin (g/L)	
Day 0	131 ± 22
Day 29	129 ± 22
Day 59	132 ± 18
Plasma ferritin (μg/L)	
Day 0	6.3 ± 3.3
Day 29	6.4 ± 2.3
Day 59	8.4 ± 3.9

[1] $\bar{x}$ ± SD.

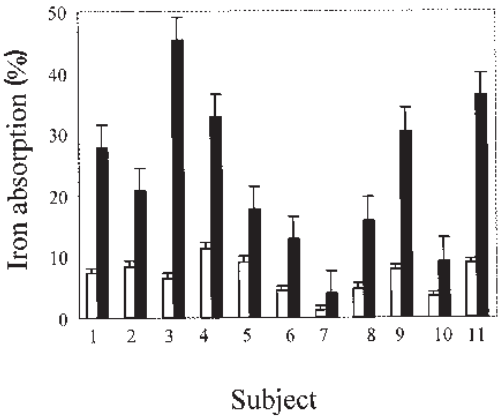

FIGURE 1. Percentage iron absorption in 11 nonpregnant, nonlactating, iron-deficient women from rural Mexico for whom 25 mg ascorbic acid was (■) or was not (□) added as limeade to test meals given twice a day over a 2-wk period.

Biochemical measurements

Hemoglobin and plasma ferritin concentrations were measured in the blood samples taken at 0, 29, and 59 d. The hemoglobin concentration was measured in one drop of venous blood by using the HemoCue (HemoCue Inc, Mission Viejo, CA). Plasma ferritin concentrations were measured in duplicate by immunoradiometric assay (Coat-A-Count Ferritin IRMA; Diagnostic Products Corp, Los Angeles). Cutoffs for anemia and iron deficiency were a hemoglobin concentration of ≤ 120 g/L (adjusted for the altitude of 2300 m) (20) and a plasma ferritin concentration of ≤ 12 μg/L (21).

Estimation of iron absorption

The stable isotopes of iron were measured in blood by using a magnetic-sector thermal-ionization mass spectrometer (22). Each blood sample (0.1–0.5 mL) was digested in 2–10 mL concentrated HNO_3 in a titration flask on a hot plate at a subboiling temperature for 24 h. Once dried, the sample was redissolved in 1–2 mL of a 6-mol HCl/L solution. A polyethylene column 0.4 cm in diameter and 8 cm in length, with a 4-mL reservoir on top, was filled with anion exchange resin and cleaned with 4 mL of an ultraclean 6-mol HCl/L solution and 4 mL ultrapure H_2O. The column was then reconditioned in a 6-mol HCl/L solution, and each sample solution was loaded. After the sample solution had passed through, the column was washed with 6 mL of a 6-mol HCl/L solution, and then iron was extracted from the column by using 1 mL of a 0.5-mol HCl/L solution. The collected solution was dried, resuspended in 30–50 mL 3% HNO_3, and loaded onto the filament for mass spectrometric analysis.

All samples were analyzed for isotopic enrichment by using a thermal-ionization mass spectrometer (model 261; Finnigan MAT, Bremen, Germany). Each sample was manually heated for 10–15 min to ≈3 A to obtain a total ion current of 5–8 × 10^{-11} of the iron isotopes on the axial Faraday detector.

Red blood cell iron incorporation was determined by evaluating the recovery of the orally administered isotopes in blood obtained 14 d after isotope administration, as previously described (22). Circulating iron was calculated with the use of a mean blood volume of 65 mL/kg, the measured hemoglobin concentration, and the concentration of iron in hemoglobin (3.47 mg/g).

Statistical analysis

It was estimated that 10 subjects would be a large enough sample for detection of a 2-fold expected difference in iron absorption between treatments with and without ascorbic acid, on the basis of an α of 0.05 and a β of 0.20 and a variability in iron absorption reported in previous studies (12).

Statistical analyses were performed by using SAS software (release 6.04; SAS Institute Inc, Cary, NC). Values for age, weight, hemoglobin, and plasma ferritin are expressed as means ± SDs and were examined for differences at baseline and after 29 and 59 d. Plasma ferritin concentrations were transformed to their log form to conform to normal distribution. Each subject served as her own control. Differences in iron absorption among time points were tested for significance with the use of a repeated-measures analysis of covariance. Tukey's test was used to compare treatments; $P < 0.05$ was used to denote significance. Linear regression was calculated for the relation between the percentage change in iron absorption and the ferritin concentration.

RESULTS

Weight changes were not significant after 8 wk of study, nor were there significant changes in hemoglobin or plasma ferritin values (**Table 2**). The final ferritin concentration for one of the subjects was assumed to be an outlier because it was much higher than the previous values, possibly because of an infection. When the outlier was removed from the analysis, there was no significant difference between the initial mean plasma ferritin concentration (6.3 ± 3.3 μg/L) and the final value (8.4 ± 3.9 μg/L). Only 3 subjects were anemic at baseline, and 2 remained anemic at the end of the study. All the subjects received iron supplements after the study was concluded.

The absorption of the 2 reference doses was 30.7 ± 14.1% on day 1 and 35.3 ± 17.2% on day 29. Absorption data are shown in **Figure 1**. Iron absorption was significantly higher in every subject during the 2 wk when the limeade was provided with breakfast and lunch. Overall, absorption increased more than 3-fold, from 6.6 ± 3.0% to 22.9 ± 12.6% ($P < 0.001$).

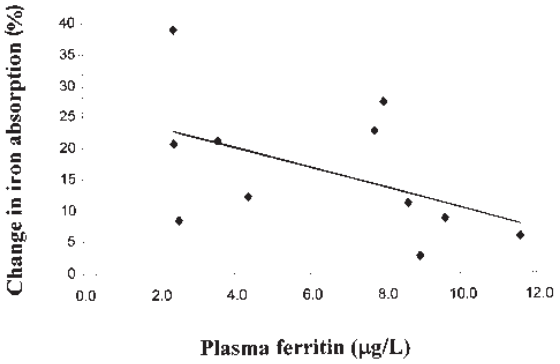

FIGURE 2. Linear relation between plasma ferritin concentration and percentage change in iron absorption. ◆, actual; —, predicted.

The percentage change in iron absorption according to initial plasma ferritin concentrations is shown in **Figure 2.** As expected, there was a significant inverse relation (R^2 = 0.59, $P < 0.006$) between the percentage change in iron absorption and the plasma ferritin concentration.

DISCUSSION

Most of the research on iron bioavailability was performed with the use of radioactive iron isotopes (10, 23). For ethical reasons, this method is not suitable for use in pregnant or lactating women, women who may become pregnant in the near future, or children. Thus, stable isotopes are needed to investigate iron bioavailability in such groups. An initial limitation of stable isotopes was the low sensitivity of their measurement, which required that relatively large doses of the isotope be provided, which in itself affected iron absorption. The use of cation exchange systems to isolate iron from blood and of better instrumentation permitted greater precision in the measurement of stable isotopes (^{58}Fe and ^{57}Fe) with the use of thermal-ionization mass spectrometry (22, 24). Stable-isotope methods were validated against radioisotopic methods for the measurement of iron absorption in healthy women and were used to study iron absorption during pregnancy (25, 26). Studies also showed that stable isotopes are useful for measuring the bioavailability of iron from cereals in infants (27, 28). The effect of adding ascorbic acid on iron absorption from these infant cereals was also studied with the use of a double stable-isotope technique (28). In Jamaica, iron absorption was measured by using stable isotopes from an iron-fortified, chocolate-flavored milk drink with a high content of polyphenols, phytate, and calcium (28). Two doses of synthetic ascorbic acid, 25 and 50 mg, were given, and iron absorption increased from 1.6% to 5.1% and 7.7%, respectively. The present results indicate that stable isotopes can be used to measure iron absorption at the community level in rural Mexico. This enabled us to measure the effect of ascorbic acid on iron absorption in ID women living in their normal environment and accustomed to consuming diets similar to the test meal.

Before conducting this long-term study with stable isotopes in the field, we used radioactive iron isotopes to show that ascorbic acid from limeade with a Mexican meal was effective at increasing iron absorption in iron-replete adult men. Adding 25 mg ascorbic acid once a day did not improve iron absorption significantly, but adding 25 mg ascorbic acid to each of 2 meals per day increased iron absorption from 3.1 ± 6.2% to 7.4 ± 8.2% ($P < 0.05$) (O García, LH Allen, JL Rosado, unpublished observations, 2001). In the present study, the addition of 2 daily doses of ascorbic acid as limeade to typical rural Mexican meals for 2 wk increased iron absorption in ID women from 6.6 ± 3.0% to 22.9 ± 12.6% ($P < 0.001$). The percentage change in iron absorption and the initial plasma ferritin values showed, as expected, that subjects with a low initial concentration of ferritin had a better response to ascorbic acid concentrations. Thus, the effect of ascorbic acid from limeade was stronger in the subjects with more severe iron deficiency and also when the limeade was given for 2 wk rather than in a single meal ($P < 0.05$). The long-term administration of ascorbic acid as limeade tripled iron absorption, whereas only a 2-fold increase was found in the 2 short-term studies. Our results do not agree with those of Cook et al (29), who found less effect after 2 wk. The meal in their study had a lower content of inhibitors (< 500 mg) than the meal used in the present study, and their subjects were not iron deficient.

Although ascorbic acid improves iron bioavailability, the effectiveness of increasing ascorbic acid intake to improve iron status has not been shown. Cook et al (29) found that providing 2 g synthetic ascorbic acid/d with meals for 16 d did not increase serum ferritin in non-ID volunteers eating self-selected diets. The lack of response was not shown to be due to an adaptation to the high ascorbic acid intake, because iron absorption from single meals could still be stimulated by ascorbic acid at the end of the 16 wk. Similarly, the consumption for 8 wk of 100 mg synthetic ascorbic acid/d, divided among 3 meals per day, by healthy female volunteers failed to increase serum ferritin (30). It is probable that the effect of ascorbic acid may be more evident in ID populations, such as the population of rural Mexico, who consume a diet high in iron absorption inhibitors and low in ascorbic acid (12). However, supplementation with 500 mg ascorbic acid, divided into 3 doses per day, for 5 wk failed to improve iron status in women with low iron stores as assessed by serum ferritin (31). Thus, the effectiveness of ascorbic acid in improving iron status over the long term is uncertain. The long-term effect of ascorbic acid on iron status should be more evident with the consumption of diets with a high content of inhibitors (10). We did see an effect of ascorbic acid on iron absorption, although we did not show an increase on plasma ferritin concentrations at the end of the 2 wk, probably because the study cases were too short for significant changes to develop. According to the percentage iron absorption determined in the present study, a span of ≈2 mo would be needed to show an increase in plasma ferritin.

In conclusion, the intake of 25 mg ascorbic acid as limeade twice a day for 2 wk more than doubles iron absorption from typical Mexican meals. The increase in the efficiency of iron absorption, combined with the high content of iron and iron inhibitors present in rural Mexican diets, justifies testing the effectiveness of increasing ascorbic acid intake in the form of limeade as a practical approach for improving iron status in ID populations, such as the population of rural Mexico.

MD contributed to data collection and to the performance of field activities. JLR contributed to the design of the experiment, collection and analysis of data, and writing the manuscript. LHA contributed to the design of the experiment, analysis of data, and writing the manuscript. SAA contributed to isotope analysis, the design of the experiment, and writing the manuscript. OPG contributed to data collection and analysis and writing the manuscript. None of the authors had any financial or personal relation with the organization sponsoring the research.

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