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Effect of Increasing Concentrations of Zinc on the Absorption of Iron from Iron-Fortified Milk

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Abstract The cofortification of milk with iron (Fe) and zinc (Zn) is a strategy used to prevent these deficiencies during childhood. Given that Zn can negatively interact with iron in aqueous solutions, the objective of the present study was to determine the effect of Zn on Fe absorption of milk fortified with Fe and Zn. Twenty-eight women between 33 and 47 years of age, with contraception and a negative pregnancy test, participated in one of two absorption studies. They received on four different days, after an overnight fast, 200 mL of milk (26 % fat) fortified with 10 mg Fe/L, as (A) ferrous sulfate, or the same milk but with graded doses of added Zn, as Zn sulfate of (B) 5, (C) 10, and (D) 20 mg/L (study 1, n=15). In study 2 (n=13), subjects received the same milk formulations, but these were also fortified with ascorbic acid (70 mg/L). Milk was labeled with radioisotopes ^{59}Fe or ^{55}Fe, and the absorption of iron was measured by erythrocyte incorporation of radioactive Fe. The geometric mean and range of ±1 SD of Fe absorption in study 1 were as follows: formula A=6.0 % (2.8–13.0 %); B=6.7 % (3.3–13.6 %); C=5.4 % (2.2–13.2 %); and D=5.2 % (2.8–10.0 %) (ANOVA for repeated measures, not significant). For study 2, data are as follows: 8.2 % (3.6–18.7 %); B=6.4 % (2.5–16.4 %); C=7.7 % (3.2–18.9 %); and D=5.2 (1.8–14.8 %) (ANOVA for repeated measures, not significant). In conclusion, according to the results from this study, it appears that the addition of zinc up to 20 mg/L does not significantly inhibit iron absorption from milk fortified with 10 mg/L of iron.

Keywords Zinc · Iron · Iron absorption · Interaction · Milk · Humans

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Introduction

Iron (Fe) and zinc (Zn) deficiencies constitute one of the most important nutritional and public health problems affecting developing countries [1]. In these settings, these deficiencies often coexist [2]. Young children are one of the most susceptible age groups because of high requirements for growth and usually having a diet that is low in bioavailable Fe and Zn.

Food fortification is the most practical strategy to prevent these deficiencies in the population [3]. Because milk contributes an important proportion to the caloric intake of infants during the first year of life, milk formulas are usually fortified with both micronutrients. However, one of the most important factors that influence the overall success of fortification programs is the bioavailability of the mineral compound added to the fortified food [3]. A potential problem of fortifying a food with both Fe and Zn is a possible negative interaction between these minerals, in which addition of one mineral at high levels may interfere with the absorption of the other.

Several studies in humans have shown a negative effect of Zn on Fe absorption when both minerals are given simultaneously as a solution in fasting conditions [4]. However, the single study that has addressed this issue in fortified milk showed that Fe absorption was not inhibited when Zn was provided in a premature infant formula in a Fe/Zn wt/wt ratio 1:4 [5]. Usually, this ratio is between 1.5:1 and 2:1 in most infant formulas, but this proportion is reversed in human milk, since Zn concentration is higher than that of Fe [6].

The aim of the study was to determine, using a double radioisotopic technique in humans, the effect of adding graded concentrations of Zn on the absorption of iron from a powdered full-fat cow's milk fortified with Fe.

Subjects and Methods

Subjects

Twenty-eight women between 33 and 47 years of age were selected to participate in one of two iron absorption studies. None were pregnant, as confirmed by a negative test for human chorionic gonadotropin in urine, all were using a birth control method (e.g., intrauterine device, oral contraceptive, or tube ligation) at the time of the study, were in apparent good health, and none had consumed vitamin or mineral supplements 6 months prior to the study. A written, informed consent was obtained from all the volunteers before the studies began. The study protocol and consent form were approved by the institutional review board of the Ethics Committee of the Institute of Nutrition and Food Technology.

Test Meals

Powdered, with 26 % fat, cow's milk was reconstituted with distilled water at 10 % concentration (wt/vol), and 5 % of sucrose was added. The reconstituted milk was fortified with 10 mg/L of Fe, as ferrous sulfate. This iron-fortified milk was used to prepare four different formulations where different concentrations of Zn (5, 10, or 20 mg/L), as zinc sulfate, were added to each formulation (study 1). In the second study, ascorbic acid was also added to each of the four formulations used in the study 1 in a 2:1 molar ratio to iron (70 mg/L). The native Fe and Zn concentrations of the milk used in study 1 were 0.1 and 3.3 mg/L, respectively, and the corresponding figures for milk used in study 2 were 0.1 and 2.4 mg/L, respectively. The fortificants and isotopes were mixed with milk 1 h before administration.

Isotopic Studies

Iron radioisotopes (^{59}Fe and ^{55}Fe) of high specific activity were used as tracers for the iron absorption studies (Du Pont de Nemours, Wilmington, DE). The radioisotope doses and administration protocols had been previously approved by the Chilean Commission of Nuclear Energy.

An experimental cross-over design was performed, where iron absorption was compared within the same subjects. The women participating in study 1 (n=15) received on four different days (days 1, 2, 14, and 15) 200 ml of milk fortified with 10 mg Fe/L or the same milk that also had either 5, 10, or 20 mg Zn/L. In study 2 (n=13), the subjects received the same four iron- and zinc-fortified milk formulas but which had also been fortified with 70 mg/L of ascorbic acid. In both studies, Fe was labeled with 111 kBq ^{55}Fe on days 1 and 14 and with 37 kBq ^{59}Fe on days 2 and 15. The specific activities of the isotopes were 18.5 kBq of ^{59}Fe and 55.5 kBq of ^{55}Fe/mg of elemental iron. All preparations were consumed after an overnight fast, and no food or beverages other than water were allowed during the following 4 h. On day 14, a venous blood sample (30 mL) was obtained to measure the circulating radioactivity from milk formulas consumed on days 1 and 2 and to assess the Fe status of the subjects. In addition, these blood samples also served as baseline values of the amount of ^{59}Fe and ^{55}Fe radioactivity in red blood cells for the next set of absorption studies (days 14–15). A final venous sample (20 mL) was obtained on day 28 to determine circulating radioactivity from milk formulas consumed on days 14 and 15.

The amount of milk ingested was calculated by differential weight of the quantity of milk offered minus the amount left-over. For the calculation of total radioactivity ingested, radioactivity was counted in sextuplicate from radiolabeled milk aliquots. Blood radioactivity was measured from duplicate venous samples. Measurements of radioactivity were performed according to the method by Eakins and Brown [7] using a liquid scintillation counter (Tri-Carb 1500TR, Packard Instruments Co., Downers Grove, IL, USA). Samples were counted a sufficient number of times to allow <3 % counting error. Radioactivity from labeled food aliquots and venous samples was counted simultaneously at the end of the study to avoid an error in the calculation of Fe absorption due to decay that has occurred between administration of the isotopes and the absorption measurement 14 days later. In addition, the absorption of Fe from milk provided on days 14 and 15 was corrected for the amount of isotope that had been administered on days 1 and 2 by subtracting the radioactivity of the blood sample of day 14 from red blood cell radioactivity of day 28. The percentage of Fe absorption was calculated on the basis of blood volumes estimated for differences in height and weight [8] and assuming 80 % incorporation of the radioisotope into the erythrocyte [9]. This method is reproducible in our laboratory with a coefficient of variation of 5 %.

Blood Analyses

Hemoglobin and mean cell volume (CELL-DYN 1700, Abbott Diagnostics, Abbott Park, IL), transferrin saturation [10], Zn-protoporphyrin (ZP Hematofluorometer model 206D, AVIV Biomedical Inc., Lakewood, NJ), and serum ferritin [11] were assessed to evaluate the Fe status of the subjects. Fe status was considered to be normal when all of these laboratory indexes were within the reference range; depleted Fe stores was defined as serum ferritin <12 μg/L, Fe deficiency without anemia was defined as having normal hemoglobin concentration with at least two abnormal laboratory results (transferrin saturation <15 %, Zn-protoporphyrin >70 μg/L RBC, serum ferritin <12 μg/L), and Fe deficiency anemia was defined as hemoglobin <120 g/L with ≥2 abnormal laboratory measurements.

Table 1 General characteristics and iron nutritional status of subjects

	Study 1 (n=15)	Study 2 (n=13)	p[a]
Age (years)	38.1±3.6	38.8±4.2	NS
Weight (kg)	65.5±11.0	61.7±10.1	NS
Height (m)	1.57±0.1	1.58±0.1	NS
Hemoglobin (g/L)	139±14	143±10	NS
Mean cell volumen (fL)	88.4±5.3	85.8±2.6	NS
Zn-protoporphyrin (μg/dL RBC)	69.7±12.5	58.7±9.7	<0.05
Transferrin saturation (%)	28.4±7.5	17.6±5.6	<0.001
Serum ferritin[b]	13.8 (7.1–26.9)	27.4 (15.7–48.0)	<0.01

NS not significant

[a]Student's *t* test

[b]Geometric mean (range of ±1 SD)

Sample Size and Statistical Methods

A sample of 13 subjects per group was estimated sufficient to detect one standard deviation difference in Fe absorption within the same subject, with a standard error α equal to 0.05 and 90 % power.

Because the percentages of Fe absorption and serum ferritin have skewed distributions, the values were first converted to their logarithms. The results were then retransformed to their antilogarithms to recover the original units and then expressed as geometric means and ±1 SD ranges. A Student's *t* test was used to compare the age, weight, height, and biochemical iron status indicators of subjects in study 1 versus those in study 2 (Statistica for Windows, release 4.5, StatSoft Inc., Tulsa, OK). An ANOVA for repeated measures was used to compare the absorption of Fe from the four milk formulations administered within each study. All comparisons were done at the 5 % level of significance.

Results

No significant differences in age, weight, and height were observed between the two groups (Table 1). The Fe nutritional status of 75 % of the subjects who participated in these studies was normal; however, subjects of study 2 have a better Fe nutritional status than those of study 1. Zn-protoporphyrin and transferrin saturation were lower (p <0.05 and <0.001, respectively) and serum ferritin was higher (p <0.01) in subjects who participated in study 2. One woman in study 1 presented Fe deficiency anemia, two had Fe deficiency without anemia, and three only had depleted Fe stores, compared with one woman that had Fe deficiency anemia in study 2.

The Fe absorption from milk fortified with iron alone or in combination with zinc at different Zn/Fe ratios is shown in Table 2 (study 1). Zn in a concentration of up to 23.3 mg/L did not significantly inhibit Fe absorption from milk containing 10.1 mg/L of Fe. The geometric mean (range±1 SD) absorption of milk fortified with Fe alone (0.3:1 Zn/Fe wt/wt ratio) was 6.0 % (2.8–13.0 %), and at 0.8:1, 1.3:1, and 2.3:1 Zn/Fe wt/wt ratios, it was 6.7 % (3.3–13.6 %), 5.4 % (2.2–13.2 %), and 5.2 % (2.8–10.0 %), respectively (ANOVA for repeated measures, p=0.28). In study 2 (Table 3), the Fe absorption values from milk fortified with Fe, ascorbic acid, and graded concentrations of Zn was 9.1 % (4.2–19.5 %) at 0.2:1 Zn/Fe wt/wt ratio and 8.6 % (3.8–19.7 %), 6.2 % (2.7–14.4 %), and 7.2 % (3.1–17.0 %) at 0.7:1, 1.2:1 and 2.1:1 Zn/Fe wt/wt ratios, respectively (ANOVA for repeated measures, p=0.07).

Table 2 Effect of increasing zinc concentration on the iron absorption of milk fortified with 10 mg/l of iron (study 1, n=15)

Minerals	Concentration (mg/L)[a]			
Zinc	3.3	8.3	13.3	23.3
Iron	10.1	10.1	10.1	10.1
Iron absorption (%)[b,c]	6.0 (2.8–13.0)	6.7 (3.3–13.6)	5.4 (2.2–13.2)	5.2 (2.8–10.0)

[a] Native plus added minerals

[b] Geometric mean (range ±1 SD)

[c] One-way repeated measures ANOVA, F=1.31, p=0.28

Discussion

When breast feeding is not possible, infant formulas are used to meet nutritional requirements of infants. There is wide evidence that Fe-fortified cow's milk with minimal modifications and infant formulas are successful in preventing iron

Table 3 Effect of increasing zinc concentration on the iron absorption of milk fortified with 10 mg/l of iron and 70 mg/l ascorbic acid (study 2, n=13)

Micronutrients	Concentration (mg/L)			
Zinc[a]	2.4	7.4	12.4	22.4
Iron[a]	10.1	10.1	10.1	10.1
Ascorbic acid	70.0	70.0	70.0	70.0
Iron absorption (%)[b,c]	9.1 (4.2–19.5)	8.6 (3.8–19.7)	6.2 (2.7–14.4)	7.2 (3.1–17.0)

[a] Native plus added micromineral

[b] Geometric mean (range ±1 SD)

[c] One-way repeated measures ANOVA, F=2.57, p=0.07

deficiency anemia in infancy. The majority of infant formulas in Europe and in the USA are fortified with 5–8 and 11–12 mg/L of Fe, respectively [12, 13]. These formulas also are fortified with 4–6 mg/L of Zn. However, one of the constraints that limit the amount of Fe and Zn that can be added to a milk formula is the possibility of a negative interaction between both minerals during absorption.

Fe absorption is impaired by Zn when both minerals are given together in aqueous or saline solutions [4]. We have previously shown that Zn inhibits Fe absorption at a Zn/Fe wt/wt ratio ≥5.9:1 when the Fe dose is low (0.5 mg) and at a wt/wt ratio 1.2:1 when the dose of Fe is higher (10 mg) [14, 15]. Only one previous study has analyzed this interaction in milk. In this study performed in nine premature infants, Fe absorption was the same when either high (1,200 μg/kg) or low (300 μg/kg) doses of Zn were given mixed with premature milk formula or human milk [5]. In our current study, we found that Zn addition to milk fortified with 10 mg/L of Fe did not inhibit Fe absorption at a Zn/Fe wt/wt ratio up to 2.3:1 and when the same Fe-fortified milk was enriched with 70 mg/L of ascorbic acid, a well known enhancer of Fe absorption, a nonstatistically significant drop in Fe absorption was observed. Fe absorption decreased by 5, 32, and 21 % at Zn/Fe wt/wt ratios of 0.7:1, 1.2:1, and 2.2:1, respectively. It is not possible to discard that if one increased the statistical power by increasing the number of studied subjects, the decrease in Fe absorption observed at Zn/Fe ratios 1.2:1 and 2.2:1 could became statistically significant. We do not have an explanation why the effect of Zn on Fe absorption was more pronounced when ascorbic acid was added to the fortified milk. Theoretically, ascorbic acid should favor the absorption of Fe without affecting the absorption of Zn, thus decreasing the possible inhibitory effect of Zn on Fe absorption. The biological significance of this difference is questionable because there is evidence that short-term absorption studies overestimate the effect of enhancers and inhibitors on Fe absorption [16, 17]. However, it should be noted that despite the presence of Zn, the consumption of 750 mL of the Fe and ascorbic acid-fortified milk would cover a high proportion of the 95th percentile of the absorbed Fe requirements (0.93 mg/day) for infants between 6 and 12 months of age [18]. This coverage is 74, 70, 51, and 59 % at Zn/Fe wt/wt ratios of 0.7:1, 1.2:1, and 2.2:1, respectively.

For ethical reasons in our study, we use women as a substitute for the infants. Hurrell et al. [19] have demonstrated that results obtained in adults on Fe absorption from infant formulas can be extrapolated to infants. The percentages of Fe absorption of the milk fortified with Fe and with or without ascorbic acid, after adjusting for differences in Fe stores, are comparable with values previously published by us on infants [20].

The mechanisms implicated in the interaction between Fe and Zn have not been fully elucidated. It has been postulated that Fe and Zn compete for shared transporters at the enterocyte. This negative interaction has been attributed to a competitive binding to divalent metal transporter 1 (DMT1), a proton-coupled transporter of numerous divalent cations, including Zn [21]. However, some studies have questioned the physiological function of DMT1 on Zn uptake [21–23], and it has been hypothesized that both minerals could also compete for a transporter different from DMT1 located in the apical membrane of the enterocyte [24, 25]. On the other hand, kinetics studies on Fe and Zn transport performed in Caco-2 cells have provided evidences that the inhibitory effect of Zn on Fe uptake could be due to a noncompetitive inhibition [26].

The lack of inhibitory effect of Zn on Fe absorption observed when both minerals were added to milk could be explained by the fact that shared transporter molecules do not remain fully occupied because the amount of Fe and Zn ionic species available to be uptaken by the enterocyte are reduced due to their interaction with inhibitory ligands present in milk [27].

Conclusion

According to the results from this study, it appears that the addition up to 20 mg/L of Zn does not significantly inhibit Fe absorption from milk fortified with 10 mg/L of iron. This information should be considered for milk fortification with both Fe and Zn.

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