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Effect of Zinc Sulfate Fortificant on Iron Absorption from Low Extraction Wheat Flour Co-Fortified with Ferrous Sulfate

Manuel Olivares · Fernando Pizarro · Daniel López de Romaña

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Abstract The co-fortification of wheat flour with iron (Fe) and zinc (Zn) is a strategy used to prevent these deficiencies in the population. Given that Zn could interact negatively with Fe, the objective was to assess the effect of Zn on Fe absorption from bread prepared with wheat flour fortified with Fe and graded levels of Zn fortificant. Twelve women aged 30–43 years, with contraception and a negative pregnancy test, participated in the study. They received on four different days, after an overnight fast, 100 g of bread made with wheat flour (70 % extraction) fortified with 30 mg Fe/kg as ferrous sulfate (A) or prepared with the same Fe-fortified flour but with graded levels of Zn, as zinc sulfate: 30 mg/kg (B), 60 mg/kg (C), or 90 mg/kg (D). Fe radioisotopes (^{59}Fe and ^{55}Fe) of high specific activity were used as tracers and Fe absorption iron was measured by the incorporation of radioactive Fe into erythrocytes. *Results:* The geometric mean and range of ±1 SD of Fe absorption were: A=19.8 % (10.5–37.2 %), B=18.5 % (10.2–33.4 %), C=17.7 % (7.7–38.7 %), and D=11.2 % (6.2–20.3 %), respectively; ANOVA for repeated measures $F=5.14$, $p<0.01$ (Scheffè's post hoc test: A vs D and B vs D, $p<0.05$). We can conclude that Fe is well absorbed from low extraction flour fortified with 30 mg/kg of Fe, as ferrous sulfate, and up to 60 mg/kg of Zn, as Zn sulfate. A statistically significant reduction of Fe absorption was observed at a Zn fortification level of 90 mg Zn/kg.

Keywords Wheat flour · Food fortification · Iron absorption · Iron · Zinc · Humans

M. Olivares (✉) · F. Pizarro · D. López de Romaña
Institute of Nutrition and Food Technology, University of Chile, Santiago, Chile
e-mail: molivare@inta.uchile.cl

Introduction

Iron (Fe) deficiency is the single most prevalent nutritional disorder worldwide, mainly affecting underdeveloped countries, where it usually coexists with zinc (Zn) deficiency [1]. It is estimated that the magnitude of Zn deficiency might not be different from that for iron, given that both minerals have similar distributions in the food supply and the usual diets of these populations are low in food products of animal origin [2].

Fortification of wheat flour with Fe is one of the strategies commonly used worldwide to prevent Fe deficiency. Currently, over 70 countries compulsorily or voluntarily fortify wheat flour with Fe [3]. These flours are commonly co-fortified with niacin, riboflavin, thiamin, and folic acid. However, a growing number of countries are also adding Zn [3]. Ferrous sulfate is one of the Fe compounds generally recognized as safe (GRAS) for consumption that is recommended for fortification of low extraction flour, while Zn oxide and Zn sulfate are the most commonly used GRAS Zn fortificants [4–6].

There is a potential risk for negative interactions when minerals that share absorptive pathways are added to food. Several studies have shown that there is a mutual inhibition in absorption when Fe and Zn are consumed together [7, 8]. This negative interaction is greater when these elements are given in a solution than when they are added to food matrixes [7, 8]. Information regarding the effects of Zn on Fe absorption of flour fortified with Fe is insufficient and controversial [9, 10]. Therefore, the purpose of this study was to determine the effect of increasing levels of Zn fortificant, as Zn sulfate, on the absorption of Fe from low extraction flour fortified with Fe 30 mg/kg, as ferrous sulfate.

Subjects and Methods

Subjects

Twelve women between 30 and 43 years of age were selected to participate in the study. 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 in the previous 6 months. A written informed consent was obtained from each volunteer before participation in the study. The protocol was reviewed by and was in accordance with the standards set by the Institute of Nutrition and Food Technology's Ethics Committee on Human Research, the Chilean Commission of Nuclear Energy approved radioactive doses.

Test Meals

All breads were prepared by study personnel at the Micronutrient Laboratory of the Institute of Nutrition and Food Technology. Unfortified wheat flour of 70 % extraction, produced by an industrial mill, was used to prepare the breads used for the iron absorption tests (Granotec SA, Santiago, Chile). Four batches of iron-fortified bread dough of ~2 kg each were prepared by mixing 1.26 kg flour, 17.64 g yeast, 9.24 g sugar, 12.6 g salt, and 188.2 mg $FeSO_4{\cdot}7H_2O$ (30 mg Fe/kg; Merck, Darmstadt, Germany) without or with either 166.3, 332.5, or 498.8 mg of $ZnSO_4{\cdot}7H_2O$ (30, 60 and 90 mg Zn/kg; Merck, Darmstadt, Germany) per batch. The ferrous sulfate and zinc sulfate were added first to the water to allow for homogenization. All ingredients were mixed by hand in polyethylene recipients during 30 min, and the mixture was then fermented at 22 °C for 60 min. Bread dough was shaped into buns and left to ferment for 30 more minutes. Finally, the buns were baked at 230 °C for 25 min. The final product offered to the subjects was French-type bread buns of ~100 g each. All breads were prepared the day before administration.

Isotopic Studies

Fe radioisotopes (^{59}Fe and ^{55}Fe) of high specific activity were used as tracers for the Fe bioavailability studies (Du Pont de Nemours, Wilmington, DE). Solutions of ^{59}Fe or ^{55}Fe were prepared in deionized distilled water and served with the test meals. The radioactivity doses of these aqueous solutions were 37 kBq of ^{59}Fe and 111 kBq of ^{55}Fe per 100 ml.

Bread and the isotopically labeled solution were consumed after an overnight fast, and no food or beverages other than water were allowed for the following 4 h. The subjects were asked to chew the bread pieces together with a sip of the labeled drink. The amount of labeled solution intake was calculated by differential weight of the glass before and after the intake. For the calculation of total radioactivity ingested, radioactivity from solution aliquots was counted in sextuplicate as standards. Measurement of blood radioactivity was performed from duplicate venous samples following the method by Eakins and Brown [11]. Samples were counted as the sufficient number of times to ensure <3 % counting error. A liquid scintillation counter (TRICarb 2000, Canberra Packard, Downers Grove, IL, USA) was used for all the isotope measurements. Percentage of absorption was calculated based on the blood volume, as estimated from height and weight [12], and assuming 80 % red cell use of radioactive iron [13].

Study Protocol

An experimental crossover design was performed, where iron absorption was compared within the same subjects. The sequence of administration of the breads made with wheat flour with different concentrations of zinc was randomly assigned to the group. On day 1, the subjects received ~100 g bread (~70 g of wheat flour) fortified with 30 mg of Fe, 30 mg of Zn/kg of wheat flour (Zn/Fe weight ratio 1:1), and 100 ml of a solution labeled with 111 kBq ^{55}Fe, and on day 2, they received 100 g bread fortified only with iron (control bread; Zn/Fe weight ratio 0:1) and 100 ml of a solution labeled with 37 kBq of ^{59}Fe. A venous blood sample was obtained on day 14 to measure circulating radioactivity and to determine the iron status of the subjects. Subjects were then given 100 g bread fortified with 30 mg of Fe, 90 mg of Zn/kg wheat flour (Zn/Fe weight ratio 3:1), and 100 ml of a solution labeled with 111 kBq of ^{55}Fe. The following day (day 15), they received 100 g bread fortified with 30 mg Fe, 60 mg of Zn/kg wheat flour (Zn/Fe weight ratio 2:1), and 100 ml of a solution labeled with 37 kBq of ^{59}Fe. A final venous sample was obtained on day 28 to measure the increase in red blood cell radioactivity.

Radioactivity from labeled solution 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 administered on days 14 and 15 was corrected for the 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.

Blood Analyses

Hemoglobin and mean cell volume (CELL-DYN 1700, ABBOTT Diagnostics, Abbott Park, IL), serum iron and total iron binding capacity [14], Zn protoporphyrin (ZP Hematofluorometer model 206D, AVIV Biomedical Inc., Lakewood, NJ), and serum ferritin [15] were measured to evaluate the iron status of the subjects. Fe status was considered to be normal when all of these laboratory indexes were within the reference range. Depleted iron stores were defined as serum ferritin <12 μg/L, Fe deficiency without anemia was defined as normal hemoglobin 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 as hemoglobin <120 g/L with 2 ≥abnormal other iron status measurements.

Statistical Methods

Because 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 were then expressed as geometric means and ±1 SD ranges. An ANOVA for repeated measures (Statistica for Windows, release 4.5, StatSoft Inc., Tulsa, OK) was used to compare the absorption of iron from the four bread formulations administered. All comparisons were done at the 5 % level of significance.

Results

The general characteristics and iron biomarkers of the volunteers are shown in Table 1. One woman presented Fe deficiency without anemia and three women had depleted Fe stores.

The Fe absorption of bread fortified with Fe (30 mg/kg) alone or in combination with Zn at different Zn/Fe ratios is shown in Table 2. Zn fortification levels of 30 and 60 mg/kg did not inhibit Fe absorption; however, a statistically significant decrease was observed at 90 mg Zn/kg wheat flour. The geometric mean (range±SD) absorption of bread fortified with Fe alone (A) was 19.8 % (10.5–37.2 %), and at 0:1 (B), 1:1 (C) 2:1, and (D) 3:1 Zn/Fe weight ratios, it was 18.5 % (10.2–33.4 %), 17.7 % (7.7–38.7 %), and 11.2 % (6.2–20.3 %), respectively (ANOVA for repeated measures, $F=5.14$, $p<0.01$; Scheffè's post hoc test: A vs D and B vs D, $p<0.05$).

Table 1 General characteristics and iron nutritional status of subjects

Age (years)	38.6±4.5
Weight (kg)	70.8±9.5
Height (m)	1.60±0.1
Hemoglobin (g/L)	143±9
Mean cell volumen (fL)	88.4±3.4
Zn protoporphyrin (μg/dL RBC)	63.1±12.6
Transferrin saturation (%)	27.9±10.0
Serum ferritin (μ/L)[a]	16.3 (10.4–25.6)

[a] Geometric mean and range of ±1 SD

Discussion

A large number of countries are currently fortifying wheat flour with iron. Unfortunately, in most cases, the information on the iron compound that is used is not available [3]. It is estimated that H-reduced iron or atomized iron is frequently utilized because of their low cost and low reactivity; despite they are poorly absorbed [4]. Ferrous sulfate is less used; notwithstanding, it has a higher absorption and is one of the compounds recommended for fortification of low extraction flour [4–6]. With regard to zinc, compounds recommended for fortification are zinc oxide and zinc sulfate, being zinc oxide the most widely used because of its lower cost [5]. Many studies have shown that the absorption of zinc added to wheat flour, as zinc oxide or zinc sulfate, is comparable [9, 16, 17].

There is abundant evidence that when zinc and iron are given in an aqueous solution, zinc inhibits the absorption of iron in a dose-dependent fashion. At a high dose of both minerals, inhibition occurs at a 1:1 Zn/Fe molar ratio, while at lower dosages, this inhibition occurs at a ≥4:1 molar ratio [18, 19]. By contrast, studies have shown that this inhibitory effect is not observed when both minerals are provided in complex food matrices such as milk formulas, composite hamburger meal with vegetables, and a precooked ready-to-eat cereal-based food [8].

Scarce studies have been performed on the effects of Zn on Fe absorption in wheat flour co-fortified with both elements. We found that that up to 60 mg Zn/kg did not significantly decreased Fe absorption, but that a statistically significant inhibition of Fe absorption was observed at a zinc fortification level of 90 mg Zn/kg. In a study performed by Herman et al. [9] in 86 Indonesian children (4–6 years old) randomly assigned to consume wheat flour dumplings containing 25 g flour fortified with either 60 mg Fe/kg alone or with the same amount of Fe and 60 mg Zn/kg, as zinc oxide or as zinc sulfate, Fe absorption was significantly lower from the flour co-fortified with $ZnSO_4$, but this inhibitory effect was not observed when the flour was co-fortified with zinc oxide, which is much less soluble in water than is zinc sulfate. On the contrary, López de

Table 2 Effect of increasing levels of zinc fortificant on the iron absorption of bread fortified with iron and zinc (n=12)

Minerals	Fortification level (mg/kg wheat flour)			
Zinc	0	30	60	90
Iron	30	30	30	30
Zn/Fe ratio	0:1	1:1	2:1	3:1
Iron absorption (%)[a]	19.8 a (10.5–37.2)	18.5 a (10.2–33.4)	17.7 a, b (7.7–38.7)	11.2 b (6.2–20.3)

One-way repeated measures ANOVA, F=5.14, p<0.01. Scheffè's post hoc test: different letters indicate statistically significant difference (p<0.05)

[a] Geometric mean (range±1 SD)

Romaña et al. found no harmful effect of Zn on Fe absorption in 54 anemic Peruvian children (3–4 years old) randomly assigned to receive at breakfast and at lunch 100 g of wheat products fortified with either 30 mg/kg Fe alone, as ferrous sulfate, or the same amount of Fe plus 30 or 90 mg of Zn/kg flour, as Zn sulfate [10].

The discrepancies between our results and those previously reported regarding the effect of zinc on iron absorption from refined wheat flour could be explained by one of several issues. First, in our study, bread was made from yeast-leavened dough prepared from low extraction (70 %) wheat flour. It is well-known that fermentation reduces the phytic acid content of the flour [20]. Previous studies performed in Indonesia and Peru used non-fermented white flour whose percentage of extraction was not specified. Second, the possible interaction of zinc and iron with other dietary components was different among the studies. Co-fortified bread was eaten only with water in our study, while Indonesian children ate dumplings, seasoned with garlic and black pepper, together with 30 ml of tomato puree [9] and Peruvian children ate a fortified biscuit at breakfast together with an egg omelet and milk with or without chocolate, and at lunch, they received a fortified noodle and chicken soup that also included yellow potato, quinoa, carrot, squash, celery leek, vegetable oil, and bouillon cube [10]. Third, there could be differences in the inflammatory status of the subjects of these three studies. A high proportion of Indonesian children (41 %) were anemic, but only 14 % of children had iron-depleted stores suggesting that Fe deficiency was not the only cause of anemia and that inflammatory processes could have been a contributory factor. In the Peruvian study, all children were anemic and the reported mean serum ferritin concentration was low. However, the percentage of iron deficiency anemia was not specified. In the present study, none of the 12 women were anemic, only 1 had iron deficiency without anemia, and 3 had depleted iron stores. Fourth, the differences of results obtained among studies cannot be attributed to the fact that our study was conducted in adult women and the other two in children, as there is evidence that the results of Fe absorption measurements performed in adults are comparable to those obtained in children [21]. Furthermore, the percentages of Fe absorption obtained in the current study are comparable with values previously published by us in adult women [22]. Finally, despite the reduced number of subjects assessed in our study, it was estimated that the sample size would be sufficient to detect a difference of five percentage points with an α error of 0.05 and an 80 % power. Furthermore, the strength of our study is that as each subject was his/her own control, so there were no other factors that could have affected the observed results other than the administered dose of zinc.

The mechanism by which Zn and Fe compete for their intestinal absorption is not entirely clarified. It has been postulated that both minerals compete for DMT1, the principal transporter of nonheme Fe that is able to transport other divalent cations, including zinc [23]. However, it has recently been questioned whether DMT1 has a significant role in this negative mutual interaction [24]. Furthermore, there is evidence that this inhibitory effect would occur by a noncompetitive or a mixed mechanism [25]. The ratio of Zn/Fe that inhibits Fe absorption would depend on the concentration given of both cations and their interactions with dietary ligands that can decrease or increase the amount of available Zn and Fe cations interacting at the basolateral levels of the enterocyte during uptake. Phytic acid is the most potent inhibitor of the absorption of iron and zinc in the wheat flour [26]. On the other hand, the nutritional status of iron and zinc can modify the expression of transporters involved in the uptake and efflux of these elements at the enterocyte.

Conclusion

Fe is well absorbed from low extraction flour fortified with 30 mg/kg of Fe, as ferrous sulfate, and co-fortified with up to 60 mg/kg of Zn, as Zn sulfate. A fortification level of 90 mg Zn/kg flour significantly reduces Fe absorption. This information may be useful for the improvement of programs of low extraction wheat flour fortification.

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