

# Cofortification of iron-fortified flour with zinc sulfate, but not zinc oxide, decreases iron absorption in Indonesian children<sup>1-4</sup>

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## ABSTRACT

**Background:** Iron deficiency is a major nutritional concern in developing countries, and food fortification is a common strategy to treat it. In Indonesia wheat flour is fortified with 60 mg Fe/kg, but because of increasing concerns about marginal zinc status in at-risk populations, consideration is being given to cofortifying flour with zinc. However, little is known about the effect of zinc fortification of flour on iron bioavailability or about the optimum form of zinc supplementation.

**Objective:** We measured iron and zinc bioavailability from wheat-flour dumplings containing 25 g flour fortified with 60 mg Fe/kg, either alone or with 60 mg Zn/kg as zinc oxide or as zinc sulfate.

**Design:** Ninety children aged 4–8 y were recruited and assigned randomly to the 3 groups; 86 completed the study. Iron and zinc absorption were measured with established stable-isotope methods.

**Results:** Iron absorption from the flour fortified with iron only was good ( $15.9 \pm 6.8\%$ ), but when corrections were made for hemoglobin concentrations, it was significantly lower from the flour cofortified with zinc sulfate ( $11.5 \pm 4.9\%$ ;  $P < 0.05$ ) but not from the flour cofortified with zinc oxide ( $14.0 \pm 8.9\%$ ). Zinc absorption was not significantly different between the zinc oxide and zinc sulfate cofortified flours ( $24.1 \pm 8.2\%$  compared with  $23.7 \pm 11.2\%$ ;  $P = 0.87$ ).

**Conclusions:** Iron and zinc appear to be highly bioavailable from foods made from fortified flour, but zinc sulfate cofortification may have a detrimental effect on iron absorption. *Am J Clin Nutr* 2002;76:813–7.

**KEY WORDS** Food fortification, iron absorption, stable isotopes, zinc absorption, children, Indonesia

## INTRODUCTION

Iron deficiency is the most common micronutrient deficiency globally, affecting between 1.5 and 2 billion people, of whom 500 million have iron deficiency anemia (1, 2). Most of these people live in developing countries (1, 2). Food fortification is an attractive strategy to combat iron deficiency because it could increase the iron intake of the entire population at an annual cost of as little as \$0.20/person (3). Many countries fortify food staples, such as wheat or maize flour, sugar, or salt, with different iron sources (3). In Indonesia wheat flour is fortified with 60 mg elemental Fe/kg.

Studies over several years have shown that zinc supplementation of high-risk populations in developing countries leads to significant decreases in mortality and morbidity from diarrhea (4–6) and respiratory diseases (6) and may also improve growth

(7). This has led to the belief that subclinical zinc deficiency may be common in developing countries and has sparked interest in fortifying food staples with zinc. Zinc fortification is common in developed countries, where the most common forms of zinc used are zinc oxide and zinc sulfate (8). Wheat flour is generally a relatively poor source of iron and zinc (11.7 mg/kg and 7 mg/kg, respectively) (9), but it is commonly iron fortified. In Indonesia, consideration is being given to cofortifying iron-fortified flour with zinc. However, there is concern that zinc cofortification might reduce the absorption of iron from fortified flour. Some reviews considered the effect of iron on zinc absorption (8, 10, 11). It appears that iron may decrease zinc absorption, especially if the minerals are given in an aqueous form (in cola or water) to fasted individuals (8). There did not appear to be any detectable effect when the minerals were in complex foodstuffs such as bread rolls or infant cereal (12). Less research exists on the possible effect of zinc fortification on iron absorption (10). Some evidence, however, suggests that zinc may reduce iron absorption in adults from an aqueous solution (13) but not from complex foodstuffs (14). The objectives of the present study in Indonesian children were, therefore, to 1) measure iron absorption from food products made from iron-fortified wheat flour, 2) assess the effect of cofortification with zinc oxide or zinc sulfate on iron absorption, and 3) compare the zinc absorption from food products made with iron-fortified flour and cofortified with zinc oxide or zinc sulfate.

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## SUBJECTS AND METHODS

### Study population

Ninety healthy children, 45 boys and 45 girls, were recruited from a rural outreach clinic in Situ Udik, a small village outside Bogor,  $\approx 70$  km south of Jakarta, Java, Indonesia.

Subjects were considered eligible for the study if they were aged between 4.0 and 8.0 y, had a height and weight greater than the third centile for age, and had had no infectious diseases, respiratory tract infections, or diarrhea within the preceding 2 wk. Subjects were ineligible if they had any known chronic medical condition, were taking any medications, or were taking vitamin or mineral supplements. The nature of the study and its potential risks and discomforts were explained to each subject's parents by native Indonesian study personnel, and written, informed consent was obtained from the subject's parents. The study received ethical approval from the Ethical Committee of the National Institute for Health Research and Development, Bogor, Indonesia, and from the Institutional Review Board of Baylor College of Medicine, Houston. Subjects received antihelminthic treatment with 500 mg mebendazole as a single oral dose  $\approx 2$  wk before the start of the study proper.

Subjects were randomly assigned to 3 groups with the use of a random number table generated with the RAND function in MICROSOFT EXCEL 98 for Macintosh (Richmond, VA). Randomization was stratified by sex. Group 1 (Fe) consumed a meal containing 25 g flour fortified with 60 mg Fe/kg, as iron sulfate; group 2 (Fe+ZnO) consumed a meal containing 25 g flour fortified with 60 mg Fe/kg as iron sulfate and 60 mg Zn/kg as zinc oxide; group 3 (Fe+ZnSO<sub>4</sub>) consumed a meal containing 25 g flour fortified with 60 mg Fe/kg as iron sulfate and 60 mg Zn/kg as zinc sulfate.

### Isotope preparation

<sup>58</sup>Fe, <sup>67</sup>Zn, and <sup>70</sup>Zn, produced in the former Soviet Union, were obtained from Trace Sciences International (Toronto). <sup>58</sup>Fe (95% enrichment by mass) was obtained as the sulfate, and <sup>67</sup>Zn (74% enrichment by mass) was obtained as zinc sulfate and zinc oxide. <sup>70</sup>Zn sulfate (90% enrichment by mass) was made into an aqueous solution by the Investigational Pharmacy of Texas Children's Hospital, Houston, and tested for sterility and pyrogenicity before intravenous administration.

### Food preparation

For each of the 3 labeled foods, 900 g white flour (which was not iron or zinc fortified) was weighed out and seasoned with 4 cloves crushed garlic, 5 mL salt, and 2.5 mL black pepper. Then, 54 mg <sup>58</sup>Fe, as the sulfate, was added to each of the 3 batches of flour, and 54 mg <sup>67</sup>Zn was added to the flour as the sulfate (Fe+ZnSO<sub>4</sub>) or oxide (Fe+ZnO). The final group (Fe) had no additional zinc added. The isotopes were mixed in a small volume of doubly distilled water before being added to the flour. The isotope container was rinsed several times to ensure that all of the isotope was added to the dough mixture. Additional doubly distilled water was added as required and the mixture was kneaded for 5–10 min until a smooth, pliable dough was produced. The total mass of the dough was measured and divided into 36 equal portions. These were weighed out on calibrated scales to within  $\pm 0.1$  g of the desired weight. The portion size (one thirty-sixth of the total dough produced for the group) for the 3 different groups ranged between 38 and 42 g. Each portion contained 25 g flour, 1.5 mg <sup>58</sup>Fe (Fe, Fe+ZnO, and Fe+ZnSO<sub>4</sub> groups), and 1.5 mg <sup>67</sup>Zn as zinc oxide (Fe+ZnO group) or zinc sulfate (Fe+ZnSO<sub>4</sub> group).

The portion of dough was divided into 4 equal parts and rolled into balls, which were placed in a steaming bag and securely knotted at the top. Each batch of food was steamed for 15 min in individual bags. After this the dumplings were frozen in plastic containers until needed. Each subject received a single portion of the flour dumplings (4 dumplings = 25 g flour) and was observed to ensure that the entire portion was consumed.

### Study overview

The subjects were seen at the rural health clinic. After fasting overnight they received an intravenous infusion of 0.2 mg <sup>70</sup>Zn (Fe+ZnO and Fe+ZnSO<sub>4</sub> groups only), and 5 mL blood was drawn. Then the subjects received a meal consisting of the steamed dough balls, which were reheated immediately before they were served by steaming for  $\approx 5$  min. In addition they had a small amount (30 mL) of a seasoned tomato purée and 100 mL water. The subjects fasted for an additional 2 h before being discharged to their homes. The composition of the meal was not analyzed, but it would be expected to contain  $\approx 100$  kcal energy, 3 g vegetable protein, 4 mg vitamin C, 0.53 mg Fe, 0.25 mg Zn, and 29 mg phytic acid (Nutrition Data Systems for Research v4.03\_31, University of Minnesota, Minneapolis), excluding added iron and zinc isotopes.

Approximately 48 and 72 h after discharge subjects in the Fe+ZnO and Fe+ZnSO<sub>4</sub> groups collected  $\approx 50$  mL urine by "clean catch" into a plastic bowl before transferring it to a screw-top polypropylene container. Fourteen days after the isotope was administered, the subjects returned to the rural health clinic and 5 mL blood was collected by venipuncture. At the end of the study all subjects with anemia were treated according to local clinical guidelines at no cost to them or their families.

### Isotope ratio measurements

Iron isotope ratios were measured in the red blood cells at 14 d, as previously described (15). Briefly, 0.3–0.5 mL red blood cells were digested with 10 mL of 15 mol nitric acid on a hot plate overnight. The digest was allowed to cool and redissolved in 0.6 mL of 6 mol ultrapure hydrochloric acid before being separated by anion exchange. An 8-cm-long, 0.4-cm-diameter column was loaded with anion-exchange resin (AG 1-X8; Bio-Rad laboratories, Hercules, CA) and prewashed with 4 mL deionized water and 1 mL of 6 mol ultrapure hydrochloric acid. The sample was then loaded onto the column, followed by 6 mL of 6 mol ultrapure hydrochloric acid and 0.5 mL of 0.5 mol ultrapure hydrochloric acid. The sample was eluted from the column with 1 mL of 0.5 mol ultrapure hydrochloric acid into a teflon vial, dried on a hot plate, and dissolved in 40  $\mu$ L 3% ultrapure hydrochloric acid. Then 10  $\mu$ L of the sample was loaded onto rhenium filaments with 2  $\mu$ L of 0.7 mol phosphoric acid and 6  $\mu$ L silica gel, and the iron isotope ratio was measured with a thermal ionization magnetic sector mass spectrometer (MAT 261; Finnigan, Bremen, Germany). Replicate blocks of 10 scans each were performed until the required degree of precision ( $< 0.2\%$ ) was achieved. The results were expressed as the ratio of <sup>58</sup>iron to <sup>56</sup>iron. The ratio of the 2 nonadministered isotopes (<sup>56</sup>iron and <sup>54</sup>iron) was used to correct for temperature-specific differences in fractionation. Trace element-free reagents and disposables were used throughout.

Urinary zinc isotope ratios were measured after acid digestion and anion exchange (16). Aliquots of 12 mL of urine were digested with 10 mL of 15 mol nitric acid overnight on a hot plate. The dried sample was dissolved in 1 mL of 6 mol

**TABLE 1**Demographic data for the 3 groups of subjects<sup>1</sup>

Characteristic	Fe group (n = 14 M, 15 F)	Fe+ZnO group (n = 14 M, 14 F)	Fe+ZnSO <sub>4</sub> group (n = 14 M, 15 F)	P
Age (y)	5.83 ± 1.08 <sup>2</sup>	5.83 ± 0.94	5.63 ± 1.0	0.69 <sup>3</sup>
Weight (kg)	17.4 ± 2.3	17.5 ± 2.3	17.3 ± 2.4	0.91 <sup>3</sup>
Height (cm)	108.4 ± 7.6	107.3 ± 6.9	105.0 ± 6.6	0.20 <sup>3</sup>
Hemoglobin (g/L)	117 ± 8	113 ± 9	117 ± 10	0.18 <sup>3</sup>
Anemia [n (%)] <sup>4</sup>	8 (28)	16 (57)	11 (38)	0.07 <sup>5</sup>
Ferritin (mg/L) <sup>6</sup>	33.8 ± 21.3 (26.3)	30.3 ± 18.2 (24.4)	28.3 ± 19.0 (21.1)	0.57 <sup>5</sup>
Iron deficiency [n (%)] <sup>7</sup>	2 (7)	2 (7)	8 (28)	0.03 <sup>5</sup>

<sup>1</sup>Fe group, iron sulfate group; Fe+ZnO group, iron sulfate and zinc oxide group; Fe+ZnSO<sub>4</sub> group, iron sulfate and zinc sulfate group.<sup>2</sup> $\bar{x} \pm SD$ .<sup>3</sup>ANOVA.<sup>4</sup>Defined as a hemoglobin concentration <110 g/L in children aged 6 mo to 6 y or <120 g/L in children aged >6 y.<sup>5</sup>Chi-square test.<sup>6</sup>Geometric  $\bar{x}$  in parentheses.<sup>7</sup>Defined as a serum ferritin concentration <12 µg/L.

hydrochloric acid and loaded onto an anion-exchange resin column (AG 1-X8 resin; Bio-Rad Laboratories, Hercules, CA) that had been prewashed with 10 mL double-distilled water and 5 mL of 6 mol hydrochloric acid. The column was washed with serial 5-mL aliquots of 6, 3, 2, 1, and 0.5 mol hydrochloric acid, and the samples were eluted with 6 mL double-distilled water. Then 10 µL of 0.7 mol phosphoric acid was added, and the sample was dried on a hot plate overnight in a teflon vial before being resuspended in 40 µL double-distilled water. Finally, 10–20 µL of this solution, 2 µL of 0.7 mol phosphoric acid, and 6 µL silica suspension were loaded onto rhenium filaments. Isotope enrichments were measured by magnetic sector thermal ionization mass spectrometer (Finnigan MAT 261; Bremen, Germany). Isotope ratios were expressed with respect to the nonadministered isotope, <sup>66</sup>Zn, and corrected for differences in fractionation with the use of the <sup>64</sup>Zn-<sup>66</sup>Zn ratio. Replicate blocks of 10 scans were performed until the desired degree of precision was obtained.

### Iron and zinc absorption calculations

Iron absorption was measured from the incorporation of <sup>58</sup>Fe into red blood cells at 14 d as previously described (17). Zinc absorption was calculated from the fractional excretion of the oral and intravenous isotope in the 48- and 72-h urine samples (17). The 2 estimates of zinc absorption were averaged to give a final value.

### Definitions of anemia and iron deficiency

Anemia was defined as recommended by the World Health Organization as a hemoglobin concentration <110 g/L in children aged 6 mo–6 y or <120 g/L in children aged >6 y (18). Iron deficiency was defined as a plasma ferritin concentration <12 µg/L.

### Other methods

The hemoglobin concentration of EDTA anticoagulated blood was measured photometrically with a Humalyzer 2000 (Human, Taunusstein, Germany) at the Nutrition Research and Development Center, Bogor, Indonesia. Hemoglobin reacts with potassium cyanide and hexacyanoferrate-III to form a hemoglobin-cyanide complex that absorbs maximally at 540 nm and that can be measured photometrically. Known hemoglobin cyanide standards solutions were used as a quality control (Merck KgaA, Darmstadt, Germany).

Serum ferritin was measured by enzyme-linked immunosorbent assay (Ferritin FS; DiaSys International, Holzheim, Germany) calibrated with TruCal Ferritin standards (DiaSys International).

Hemoglobin and plasma ferritin concentrations were measured from the blood drawn on day 14, except for one subject who had clinical signs and symptoms of respiratory illness on day 14. For this subject stored plasma from the first day of the study was used to measure plasma ferritin. In 34 cases duplicate measurements of serum ferritin were made on day 1 and day 14. The mean difference was  $-1.4 \pm 6.8$  µg/L (median: 0.8 µg/L).

### Statistical analysis

Differences in iron and zinc absorption between the groups were assessed by using analysis of variance or in the case of iron absorption, analysis of covariance with indexes of iron status (hemoglobin concentration or serum ferritin) as covariants. Post hoc testing was by Tukey-Kramer's test. Results were considered statistically significant at a P value of <0.05. A sample size of 30 was selected because it has an 80% power ( $\beta = 20\%$ ) of detecting a difference of two-thirds of an SD between the groups at a P value of 0.05 ( $\alpha = 5\%$ ). Statistical analysis was carried out with the use of STATVIEW, version 5.01 (SAS Institute, Cary, NC), and power calculations were made by using DSTPLAN, version 4.2 (University of Texas MD Anderson Cancer Center, Houston).

## RESULTS

### Subject demographics

Ninety subjects (45 boys and 45 girls) were recruited. Four subjects failed to return for their 14-d visit, so 86 subjects completed the study (96%). Overall 35 (41%) of the subjects met the World Health Organization criteria for anemia (18). The proportion of anemic subjects tended to be highest in the Fe+ZnO group (Table 1). Despite this, only 14% of the subjects had a ferritin concentration <12 µg/L, and this was significantly more common in the Fe+ZnSO<sub>4</sub> group. Otherwise, the 3 groups were well matched in baseline demographic characteristics (Table 1).

### Iron and zinc absorption

Iron absorption was  $15.9 \pm 6.8\%$  in the Fe group,  $14.0 \pm 8.9\%$  in the Fe+ZnO group, and  $11.5 \pm 4.9\%$  in the Fe+ZnSO<sub>4</sub> group.

There was no significant relation between iron absorption and serum ferritin ( $r^2 = 0.003$ ,  $P = 0.62$ ). There was no significant difference in iron absorption by analysis of variance ( $P = 0.068$ ) or by analysis of covariance with ferritin as a covariant ( $P = 0.058$ ). When the data were analyzed by using analysis of covariance with hemoglobin concentration as a covariant, iron absorption did differ significantly between groups ( $P = 0.038$ ). Tukey-Kramer's post hoc test showed that iron absorption was significantly greater in the Fe group than in the Fe+ZnSO<sub>4</sub> group ( $P < 0.05$ ), but no statistically significant differences were seen between the Fe and Fe+ZnO groups or the Fe+ZnSO<sub>4</sub> and Fe+ZnO groups. There was no statistically significant difference in zinc absorption between the Fe+ZnO ( $24.1 \pm 8.2\%$ ) and Fe+ZnSO<sub>4</sub> ( $23.7 \pm 11.2\%$ ) groups ( $P = 0.87$ ).

The total iron tracer absorbed from the dumplings (25 g flour) was  $0.238 \pm 0.102$  mg (Fe group),  $0.210 \pm 0.134$  mg (Fe+ZnO group), and  $0.173 \pm 0.074$  mg (Fe+ZnSO<sub>4</sub> group). If intrinsic iron naturally present in the flour (11.7 mg/kg) (9) was as well absorbed as the tracer, the total absorption would have been  $0.285 \pm 0.122$  mg (Fe group),  $0.251 \pm 0.160$  mg (Fe+ZnO group), and  $0.207 \pm 0.089$  mg (Fe+ZnSO<sub>4</sub> group). The total zinc tracer absorbed from the dumplings was  $0.361 \pm 0.123$  mg (Fe+ZnO group) and  $0.355 \pm 0.167$  mg (Fe+ZnSO<sub>4</sub> group). If intrinsic zinc in the flour (7 mg/kg) (9) was as well absorbed as the tracer, zinc total absorption would have been  $0.403 \pm 0.137$  mg (Fe+ZnO group) and  $0.396 \pm 0.187$  mg (Fe+ZnSO<sub>4</sub> group).

## DISCUSSION

We studied the absorption of iron and zinc from wheat-flour dumplings fortified with iron sulfate, with or without zinc fortification. Absorption of iron and zinc was good, but zinc sulfate fortification appeared to reduce iron absorption from the wheat-flour dumplings. No such inhibition was noted in dumplings fortified with zinc oxide. The difference in iron absorption (4.4%) represents a relative change of 27%. This is likely to be highly clinically significant in populations that receive a substantial portion of their dietary iron from fortified flour.

The study population was selected to include only subjects with a weight or height above the third centile for age to exclude subjects who may have had acute or chronic medical conditions. Despite this, 41% of the subjects were anemic (18). Although we did not screen for another causes, most of these individuals would be expected to be iron deficient with or without hookworm infestation (1, 2). However, the number of subjects with serum ferritin concentrations suggestive of iron deficiency was low. Presumably, this reflects subclinical infections or other causes of an acute-phase response. There was no relation between serum ferritin and iron absorption, suggesting that serum ferritin concentration was not a good measure of underlying iron status in this population. A similar finding was described previously in rural villages in Bogor District, where  $\approx 50\%$  of infants aged  $< 12$  mo were anemic, although very few had low plasma ferritin concentrations (19).


The possibility that iron can inhibit zinc absorption through competition for a shared absorptive pathway has been considered (20), and there are several possible sites of inhibition (21). One intriguing possible site of interaction between iron and zinc is at the duodenal transport protein DCT-1 (divalent cation transporter 1) (22). DCT-1 appears to be important in iron absorption but can also transport many other metals, including zinc (22). If iron and

zinc can inhibit each other's absorption by competition for DCT-1, the effects would be expected to be most noticeable when one metal is in relative excess compared with the other. This is consistent with previous observations that iron has little effect on zinc absorption when zinc-iron ratios are 1:1 but an inhibitory effect on zinc absorption when zinc-iron ratios are 1:2 or less (23).

Although several studies examined the effect of iron supplementation on zinc absorption, few considered the effect of zinc supplementation on iron absorption. One study suggested that zinc could inhibit radioiron absorption when the zinc-iron ratio was 1.14:1 (molar ratio: 1:1) but not when it was 0.36:1 (molar ratio: 0.4:1) (13). A second study showed that a zinc-iron ratio of 5:1 did significantly reduce iron absorption from an aqueous solution but did not affect heme iron absorption from a hamburger meal (14). In the present study the zinc-iron ratio was 1:1, and a reduction in iron absorption was noted if zinc sulfate was the fortificant but not if zinc oxide was. The reason for the difference between zinc oxide and zinc sulfate is not immediately clear. Zinc sulfate is, however, much more soluble in water than is zinc oxide (24), and it is possible that not all the zinc oxide dissolved in the dough. If this was the case, the zinc-iron ratio in the aqueous phase might be  $< 1:1$  when zinc oxide was the fortificant. At this lower ratio, zinc might have no effect on iron absorption, or the effect might be sufficiently reduced to make it undetectable with a sample size of 30/group. Indeed, a sample size of 274/group would have been required to have an 80% power of showing that the observed difference in iron absorption between the Fe and Fe+ZnO groups was significant at  $P < 0.05$ . Although the concept that in vitro solubility is directly related to mineral absorption is untested (25), the lower solubility of zinc oxide might be expected to reduce zinc absorption compared with zinc sulfate. We found no significant difference in zinc absorption from the 2 zinc salts. An alternative possibility is, therefore, that zinc oxide dissolves more slowly in the gut and so has higher lumen concentrations in the proximal small intestine, where the interaction with iron absorption might occur, but ultimately dissolves more in the more distal parts of the gut, allowing zinc absorption to be similar in the Fe+ZnO and Fe+ZnSO<sub>4</sub> groups.

Several studies have measured iron absorption from bread and other flour products. It is not easy to compare our results to previous studies because iron absorption is affected by differences in the dose of iron, the form in which it is added, the amount of flour, the iron status of the population, the bran (26), and the phytic acid content of the dough (27). Estimates vary from as low as 2.2% to as high as 65% (28–31). The study most similar to the present study is that of Brune et al (26), who examined iron absorption from rolls made from 80 g white flour fortified with ferrous sulfate and who found that iron absorption averaged 20%, although it fell to nearer 4% when bran was added. This figure is higher than in our study, perhaps because our dumplings were unleavened and therefore may have had a higher phytic acid content (26). Flour is usually fortified with relatively insoluble forms of elemental iron powder, so our study, which used ferrous sulfate as a fortificant, may have overestimated iron absorption. However, our main objective was to investigate the possibility of an interaction between zinc fortification and iron absorption, and to do this, we needed to use a form of iron fortification that was likely to be well absorbed, such as ferrous sulfate.

Similarly, comparison between zinc absorption in our study and the literature is complicated by differences in the phytate content of flour (27). However, estimates between 10% and 40% are typical (29, 32, 33) and broadly in line with our findings.

In conclusion, we measured iron and zinc absorption from flour dumplings cofortified with iron and zinc. Iron and zinc were well absorbed; however, the addition of zinc sulfate to the dumplings significantly decreased the amount of iron absorbed. No similar effect was seen for dumplings cofortified with zinc oxide. The results suggest that caution should be exercised when considering whether to fortify iron-fortified flour, or foods made from iron-fortified flour, with zinc, especially with zinc sulfate. 

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