Supplementation of a Cereal-Based Diet with Heme Iron: Interactions between Iron and Calcium, Phosphorus, and Magnesium in Rats

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We studied the influence of a commercial cereal-based diet supplemented with bovine blood on Fe, Ca, P, and Mg metabolism in control and Fe-deficient rats to investigate the interactions caused by high levels of dietary Fe and whether heme Fe supplementation could reduce these interactions. After feeding Fe-deficient rats with a diet that contained 100 mg of Fe/kg as elemental Fe, the digestive and metabolic utilization of Ca, P, and Mg decreased, whereas the sternum concentration of these minerals increased in comparison with control rats fed the same diet. Nevertheless, when cereal-based diet was supplemented with heme Fe, no decrease in Ca or Mg absorption was found in Fe-deficient rats.

Keywords: *Cereal; heme; iron; milk*

INTRODUCTION

Anemia has been estimated to affect approximately 30% of the human population (De Maeyer and Adiels-Tegman, 1985). Ferropenic anemia, the most widespread simple nutritional deficiency in the world, affects about 25% of all children (Pollit *et al.*, 1986). This disorder is especially common in children between the ages of 4 and 12 months, when Fe reserves are insufficient to cover the requirements for growth and basal losses (Dallman, 1988). Nevertheless, the chances of developing anemia during late infancy are lower in children fed milk formulas or Fe-fortified cereals than in children given only cow's milk (Calvo and Gnazzo, 1990).

The problems associated with Fe fortification, as well as the relative advantages of different fortifiers and vehicles, have been frequently studied (International Nutritional Anemia Consultative Group, 1979; Cook and Reusser, 1983; Hurrell, 1984; Barret and Ranum, 1985; Hallberg, 1985; MacPhail *et al.*, 1985; Patrick, 1985). Although much has been discovered with respect to suitable levels of Fe fortification that avoid interactions between this mineral and other trace elements such as Zn, Mn, Cu, Cd, and Co (Flanagan *et al.*, 1978; Solomons *et al.*, 1983; Dallman, 1989; Lönnerdal, 1989), interactions have also been found between Fe and Ca, Mg, and P (Snedeker *et al.*, 1982; Deehr *et al.*, 1990; Pallarés *et al.*, 1993). The beneficial effects of fortification of foods with heme Fe have also been well documented (Stekel, 1984; Calvo *et al.*, 1989; Pallarés *et al.*, 1993). We investigated the possible interactions of Fe with Ca, P, and Mg in control animals and in rats made Fe-deficient, during feeding with either a commercial cereal−milk formula that contained Fe in the form of elemental iron or this diet supplemented with heme Fe in the form of bovine blood. Our purpose was to investigate whether high levels of dietary Fe produced interactions and whether these interactions could be avoided by dietary supplementation with heme Fe.

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MATERIALS AND METHODS

Experimental Design. A commercial cereal-based diet with elemental iron (diet 100) and this diet supplemented (in our laboratory) with heme Fe (bovine blood) (diet 200) were assayed for a experimental period of 7 days, in control and iron-deficient rats. We studied the bioavailability and therapeutic effects of Fe and the interactions between this mineral nutrient and Ca, P, and Mg metabolism.

Prior to this study, the animals were made Fe-deficient by feeding for 40 days, with a diet lacking Fe supplementation (low-Fe diet). Control animals were fed for 40 days, with a normal-Fe diet (ferric citrate), containing amounts of Fe sufficient to satisfy the nutritional requirement of this species (American Institute of Nutrition, 1977). During the experimental period, we measured food intake, body weight, dietary concentration of Fe, Ca, P, and Mg, intake and fecal excretion of Fe, Ca, P, and Mg, hemoglobin concentration, serum Fe, P and Ca, and Fe, Ca, P, and Mg concentration in liver, femur, and sternum.

Diets. Table 1 summarizes the composition of the normal-Fe diet. The low-Fe diet was obtained by omitting Fe from the mineral supplement. Table 2 shows the composition of the experimental diets (diet 100 and diet 200).

Animals. The experimental animals were 46 recently weaned male white Wistar rats with an initial body weight of 50−65 g each, obtained from the Laboratory Animal Service of the University of Granada. We divided the rats into four groups of 11 (experiments C-100 and D-100) or 12 (experiments C-200 and D-200) animals each. All animals were housed from day 0 of the experiment in individual metabolic cages to facilitate the separate collection of urine and feces. The cages were kept in a well-ventilated, temperature-controlled room (21 °C) with a controlled 12-h light/dark period.

Experiment C-100. After feeding with the normal-Fe diet (ferric citrate) for 40 days, the rats were fed with diet 100 (elemental Fe) during the experimental period (7 days).

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Table 1. Composition of the Normal Diet

component	g/kg of dry weight
protein (casein + DL-methionine/g)	120
fiber (micronized cellulose)	80
fat (olive oil)	40
mineral supplement[a]	35
vitamin supplement	10
choline chloride	2
equal parts of saccharose and wheat starch to 1 kg	

[a] The mineral supplement contained the following (g/kg): calcium phosphate dibasic ($CaHPO_4$), 500.0; sodium chloride (NaCl), 74.0; potassium citrate, monohydrate ($K_3C_6H_5O_7.H_2O$), 220.0; potassium sulfate (K_2SO_4), 52.0; magnesium oxide (MgO), 24.0; manganous carbonate (43−48% Mn), 3.5; ferric citrate (16−17% Fe), 6.0; zinc carbonate (70% ZnO), 1.6; cupric carbonate (53−55% Cu), 0.3; potassium iodate (KIO_4), 0.01; sodium selenite ($Na_2SeO_3.5H_2O$), 0.01; chromium potassium sulfate [$CrK(SO_4)_2 \cdot 12H_2O$], 0.55; and finely powdered sucrose to make up to 1000 g (American Institute of Nutrition, 1977).

Experiment D-100. After feeding with the low-Fe diet (no Fe supplement) for 40 days, the rats were fed with diet 100 during the experimental period (7 days).

Experiment C-200. After feeding with the normal-Fe diet for 40 days, the rats were fed with diet 200 (elemental iron + heme Fe) during the experimental period (7 days).

Experiment D-200. After feeding with the low-Fe diet for 40 days, the rats were fed with diet 200 during the experimental period (7 days).

In all experiments we used the following biological technique: After feeding for 40 days, the control groups (C) with normal-Fe diet or the iron-deficient groups (D) with low-Fe diet, 3 days were allowed for adaptation to each experimental diet followed by a 7-day experimental period during which all feces were collected. At the beginning and the end of each experimental period (days 43 and 50 in all experiments), body weight, food intake, and output of feces were recorded. Food intake by each rat was determined by weighing the amounts of food provided, refused, and spilled. Throughout the study all animals were given double-distilled water *ad libitum*. On days 0, 40, and 50 of each experiment, blood was collected from a tail vein after an overnight period of food deprivation (12 h). The blood was immediately placed in tubes containing EDTA and used for hematological analyses. On day 50 of each experiment, the animals were anesthetized with pentobarbital sodium (5 mg/100 g of body weight) and completely bled through an abdominal aorta cannula. The entire volume of blood was centrifuged to separate the serum, which was frozen at −30 °C until biochemical analysis. The liver, one femur, and the sternum were also removed and frozen for later determinations of mineral content.

Biological Indices. Percentage apparent digestibility coefficient (ADC) was calculated with the formula

$$\text{percentage ADC} = \frac{\text{absorbed}}{\text{intake}} \times 100 \quad (1)$$

where nutrient absorption = intake − fecal excretion.

Hemoglobin regeneration efficiency (HRE) was calculated as follows (Mahoney, 1974):

$$\text{hemoglobin-Fe (mg)} = \text{body wt (g)} \times \frac{\text{mL of blood}}{\text{g of body wt}}(\text{assumed to be 0.067 mL}) \times \frac{\text{g of hemoglobin}}{\text{mL of blood}} \times \frac{\text{mg of Fe}}{\text{g of hemoglobin}} (\text{assumed to be 3.35 mg}) \quad (2)$$

$$\text{percentage HRE} = \frac{\text{mg of hemoglobin−Fe(final)} - \text{mg of hemoglobin−Fe(initial)}}{\text{mg of Fe consumed}} \times 100 \quad (3)$$

Analytical Methods. Water contents of the diet, feces, liver, femur, and sternum were determined by drying the materials at 105 + 2 °C until no further weight change was observed. An appropriate amount of the resulting material was ashed at 450 °C, and the residue was extracted with 5 M HCl and brought up to an appropriate volume with double-distilled water for Fe and P analyses or with lanthanum chloride solution (10 g/L) for Ca and Mg analyses to avoid possible interference by P. To determine Fe, Ca, and Mg contents, we used atomic absorption spectrophotometry (Perkin-Elmer 1100B); P content was measured with the technique of Fiske and Subbarow (1925).

Hemoglobin concentration was determined with a Symex CC-130 automatic cell counter. Serum concentrations of Fe, Ca, and P were measured by colorimetry (Trinder, 1956; Sarkar and Chauhan, 1967; Drewes, 1972).

Statistical Treatment. All values are expressed as the mean ± standard error. The results from all experiments and analyses were tested statistically by analysis of variance with the one-way procedure of the SPSS/PC software package. Means were compared with Duncan's test for all four means. *P* values below 0.05 were considered significant.

Table 2. Composition of the Commercial Cereal-Based Diet (Diet 100),[a,b] Average Analysis

component	g/kg
protein	125
fat	90
carbohydrates	725
mineral salts	30
moisture	30

[a] Diet 200 was made in our laboratory by adding 0.21 g of bovine blood (Hb = 140 g/L) to diet 100. Both diets were adjusted to 80 g/kg of diet (dry weight) with micronized cellulose. [b] Ingredients: wheat flour, dried skim milk, sucrose, corn flour, maltodextrines, animal edible fat, vegetal edible fat, vegetable oils, honey, minerals, rice flour, barley flour, rye flour, tapioca, vitamins, vanilla, and cinnamon. Enriched with (per kg of diet): calcium, 280 mg; vitamin C, 900 mg; niacin, 120 mg; iron, 100 mg; vitamin E, 100 mg; pantothenic acid, 60 mg; vitamin A, 10 000 IU; vitamin B_2, 12 mg; vitamin B_1, 10 mg; vitamin B_6, 10 mg; biotin, 400 μg; folic acid, 300 μg; vitamin D, 3000 IU; vitamin B_{12}, 12 μg.

Table 3. Body Weight and Food Intake in Control and Iron-Deficient Rats Fed Diets with Different Sources of Fe (Mean Values with Standard Errors)

		body wt (g) at			
group	*n*	day 43 (initial wt)	day 50 (final wt)	wt change (g/rat/day)	food intake (g of dry wt/rat/day)
C-100	11	253.6 ± 4.4	278.7 ± 4.1[c]	3.6 ± 0.2[a,c]	15.6 ± 0.3[a,c]
D-100	11	240.3 ± 5.8	260.0 ± 5.9	2.8 ± 0.3	18.9 ± 0.4[d]
C-200	12	261.2 ± 2.8	297.6 ± 4.1[b]	5.2 ± 0.3[b]	20.9 ± 0.4
D-200	12	250.1 ± 9.7	266.6 ± 9.3	2.4 ± 0.3	20.7 ± 0.5

[a] Significant difference between C-100 and D-100. [b] Significant difference between C-200 and D-200. [c] Significant difference between C-100 and C-200. [d] Significant difference between D-100 and D-200.

RESULTS

Chemical Analyses. The contents of the low-Fe diet after analysis were as follows (mg/kg of diet): Fe, 6.3; Ca, 5411; P, 4534; Mg, 516. The mineral contents in the normal-Fe diet were as follows (mg/kg of diet): Fe (ferric citrate), 43.5; Ca, 5250; P, 4500; Mg, 540. The mineral contents of diet 100 were as follows (mg/kg of diet): Fe (elemental Fe), 90.7; Ca, 4626; P, 2511; Mg, 493. In diet 200, the mineral contents were (mg/kg of diet) as follows: Fe (elemental Fe + heme Fe), 215.5; Ca, 4852; P, 2970; Mg, 541.

Biological Analyses. Fe-deficient rats gained less weight than controls, regardless of whether diet 100 or diet 200 was consumed, and Fe-deficient animals given

Table 4. Digestive Utilization of Fe, Ca, P, and Mg in Control and Iron-Deficient Rats Fed Diets with Different Sources of Fe (Mean Values with Standard Errors)

group	n	absorbed Fe (μg/rat/day)	ADC[e] Fe (%)	absorbed Ca (mg/rat/day)	ADC Ca (%)	absorbed P (mg/rat/day)	ADC P (%)	absorbed Mg (mg/rat/day)	ADC Mg (%)
C-100	11	251 ± 12[a,c]	17.7 ± 0.7[a,c]	42 ± 2[a,c]	57.8 ± 2.1[a,c]	29 ± 1[c]	75.0 ± 0.9[a]	3.8 ± 0.2[a,c]	49.9 ± 2.5[a,c]
D-100	11	727 ± 23[d]	42.5 ± 1.6[d]	20 ± 2[d]	22.7 ± 2.3[d]	27 ± 1[d]	55.8 ± 1.6[d]	1.7 ± 0.2[d]	18.0 ± 2.3[d]
C-200	12	1571 ± 73	34.9 ± 1.6	69 ± 4	68.0 ± 3.0	47 ± 1	76.3 ± 0.8	9.1 ± 0.3	80.1 ± 2.0
D-200	12	1660 ± 64	37.3 ± 1.4	61 ± 5	60.2 ± 4.5	45 ± 1	73.5 ± 0.8	8.6 ± 0.4	79.0 ± 2.5

[a] Significant difference between C-100 and D-100. [b] Significant difference between C-200 and D-200. [c] Significant difference between C-100 and C-200. [d] Significant difference between D-100 and D-200. [e] ADC, apparent digestibility coefficient.

Table 5. Hemoglobin (Hb) Values, Serum Values of Iron, and Hemoglobin Regeneration Efficiency (HRE) in Control and Iron-Deficient Rats Fed Diets with Different Sources of Fe (Mean Values with Standard Errors)

group	n	Hb (g/L) initial	Hb (g/L) final	HRE (%)	serum Fe (μg/L)
C-100	11	158 ± 2[a]	161 ± 1[a]	10.6 ± 0.6[a,c]	1070 ± 30[a]
D-100	11	81 ± 1	131 ± 2	27.2 ± 0.9[d]	240 ± 40[d]
C-200	12	156 ± 1[b]	159 ± 3[d]	4.7 ± 0.5[b]	1120 ± 10[b]
D-200	12	84 ± 2	133 ± 3	11.4 ± 0.9	670 ± 50

[a] Significant difference between C-100 and D-100. [b] Significant difference between C-200 and D-200. [c] Significant difference between C-100 and C-200. [d] Significant difference between D-100 and D-200.

Table 6. Iron Concentration in Several Organs in Control and Iron-Deficient Rats Fed Diets with Different Sources of Fe (Mean Values with Standard Errors)

group	n	liver (μg/g of dry wt)	femur (μg/g of dry wt)	sternum (μg/g of dry wt)
C-100	11	248 ± 10[a,c]	63 ± 2	79 ± 2[a,c]
D-100	11	143 ± 5	57 ± 2[d]	167 ± 14
C-200	12	290 ± 12[b]	57 ± 2[b]	115 ± 4[b]
D-200	12	146 ± 5	67 ± 2	177 ± 8

[a] Significant difference between C-100 and D-100. [b] Significant difference between C-200 and D-200. [c] Significant difference between C-100 and C-200. [d] Significant difference between D-100 and D-200.

diet 100 consumed more food than controls. Weight gain and food intake during the experimental period were higher in group C-200 than in group C-100 (Table 3).

After giving diet 100 to both control and Fe-deficient groups, we found that the Fe apparent digestibility coefficient (ADC) and hemoglobin regeneration efficiency (HRE) were higher in group D-100, although serum Fe concentration was lower in comparison with their respective control group (Tables 4 and 5). Nevertheless, the ADC values of Ca, P, and Mg were lower in group D-100 than in group C-100 (Table 4). In the liver, the concentrations of Fe and Mg were lower in group D-100 than in group C-100 (no changes in Ca or P content) (Tables 6 and 7). Fe deficiency lowered the concentrations of Ca and P in the femur (no significant change in Fe or Mg content) (Tables 6 and 7) and led to an increase in Fe, Ca, and P concentrations in the sternum (Tables 6 and 7).

When we compared Fe-deficient versus control rats fed diet 200, HRE was greater in D-200 and serum Fe concentration was lower than in the control group (Table 5).

Liver concentrations of Fe, P, and Mg were lower in group D-200 than in group C-200 (Tables 6 and 7). In the femur of group D-200, Fe concentrations were higher, but Ca and P were lower than in group C-200 (Tables 6 and 7). In the sternum, Fe-deficient rats fed diet 200 showed a significant increase in the concentrations of Fe, Ca, and P (Tables 6 and 7).

When we investigated the effect of dietary Fe on the digestive and metabolic utilization of Fe, Ca, P, and Mg of control rats (C-100 vs C-200), the ADC values of Fe, Ca, and Mg were found to be greater with diet 200 than with diet 100 (Table 4). In the liver of group C-200 animals, the concentrations of Fe and Mg were higher, whereas that of P was lower, in comparison with group C-100 (Tables 6 and 7). Moreover sternum concentrations of Fe, P, and Mg were also higher than in group C-100 (Tables 6 and 7).

Comparisons between Fe-deficient rats fed diet 100 or diet 200 (D-100 vs D-200) showed that group D-200 had a lower Fe ADC and HRE but a higher concentration of serum Fe than group D-100 (Tables 4 and 5). Moreover, in group D-200, the ADC values of Ca, P, and Mg were higher than in group D-100 (Table 4).

In Fe-deficient rats given diet 200, we found a lower P content in the liver, a greater Fe content in the femur, and higher concentrations of Ca, P, and Mg in the sternum, in comparison with group D-100 (Tables 6 and 7).

DISCUSSION

The lower weight gain during the experimental period in both groups of Fe-deficient rats (D-100 and D-200) shows that Fe deficiency reduced the effect of feeding on weight gain, as was also noted by Beard (1987), Greger and Lyle (1988), and Moratalla (1994).

Among the animals given diet 100, the greater ADC of Fe and HRE in Fe-deficient animals was coincident with earlier findings (Thannoun *et al.*, 1987, 1988; Huebers *et al.*, 1990; Schümann *et al.*, 1990; Roberts *et al.*, 1993). The concentration of Fe in serum and liver was lower in Fe-deficient rats than in controls but higher in the sternum, which is a site of erythropoiesis.

Table 7. Ca, P, and Mg Concentrations in Several Organs in Control and Iron-Deficient Rats Fed Diets with Different Sources of Fe (Mean Values with Standard Errors)

group	n	liver (per g of dry wet) Ca (μg)	liver (per g of dry wet) P (mg)	liver (per g of dry wet) Mg (mg)	femur (per g of dry wt) Ca (mg)	femur (per g of dry wt) P (mg)	femur (per g of dry wt) Mg (mg)	sternum (per g of dry wt) Ca (mg)	sternum (per g of dry wt) P (mg)	sternum (per g of dry wt) Mg (mg)
C-100	11	78 ± 1	9.8 ± 0.1[c]	0.68 ± 0.01[a,c]	224 ± 7[a]	101 ± 1[a]	3.8 ± 0.1	106 ± 5[a]	49 ± 1[a,c]	2.2 ± 0.1[c]
D-100	11	76 ± 4	9.7 ± 0.3[d]	0.61 ± 0.02	197 ± 4	95 ± 1	3.9 ± 0.1	151 ± 4[d]	56 ± 2[d]	2.2 ± 0.1[d]
C-200	12	88 ± 2	7.7 ± 0.2[b]	0.73 ± 0.02[b]	229 ± 4[b]	103 ± 2[b]	4.0 ± 0.1	111 ± 2[b]	53 ± 2[b]	2.4 ± 0.05
D-200	12	84 ± 8	6.9 ± 0.1	0.64 ± 0.02	202 ± 1	97 ± 1	4.0 ± 0.2	175 ± 3	60 ± 1	2.5 ± 0.04

[a] Significant difference between C-100 and D-100. [b] Significant difference between C-200 and D-200. [c] Significant difference between C-100 and C-200. [d] Significant difference between D-100 and D-200.

These findings show that while dietary Fe was supplied after chronic experimental Fe deficiency, Fe metabolism was devoted preferentially toward the formation of hemoglobin, in accordance with the findings of Milne *et al.* (1990). These authors found that when hematopoiesis increased, Fe repletion in hemopoietic organs (including, preeminently, the sternum) took priority over the conservation of Fe reserves.

The ADC of Ca and Mg in group D-100 was lower than in group C-100. These results found in Fe-deficient rats may have occurred because Fe deficiency decreased the activity of enterocyte receptors as a consequence of curtailed cellular oxygen supply (Henderson *et al.*, 1986; Beutler, 1988; Hercberg and Galan, 1988; Scrimshaw, 1991; Campos Guerra, 1992). Because part of the Ca, P, and Mg is absorbed through an active mechanism (Schachter and Kowarski, 1982; Desplan *et al.*, 1983; Hardwick *et al.*, 1991), net absorption of these minerals would be expected to decline in Fe-deficient animals. To maintain Ca and P homeostasis, serum concentrations are kept stable, while concentrations in the femur, which normally acts as a Ca and P reservoir, decreased in Fe-deficient rats. The apparently paradoxical finding of higher sternum Ca and P concentrations in Fe-deficient rats can be explained by the increased Ca and P requirements for hematopoiesis. This hypothesis is supported by the finding of greatly increased numbers of platelets in Fe-deficient rats, which implies a notable increase in Ca and P requirements for erythropoiesis and thrombopoiesis (Moratalla, 1994).

In Fe-deficient rats given diet 200 (equal parts of Fe supplied as elemental Fe and heme Fe), we also found an increase in HRE and a decrease in Fe content in serum and liver in comparison with respective control rats, although we found no decrease in Ca or Mg absorption (as seen with diet 100); moreover, despite the significant decrease in P ADC in group D-200 versus group C-200, P absorption remained high. This result may have been due to the improved mineral absorption in response to supplementation with heme Fe or to a faster recovery of intestinal Fe receptors due to increased oxygenation, which probably greatly enhanced absorption.

The enhancing effect of diet 200 versus diet 100 was clear because the ADC values of all minerals were not decreased in group D-200 in comparison with group C-200. Moreover, Fe, Ca, P, and Mg absorptions were higher in the Fe-deficient group fed with diet 200 versus diet 100.

Our results show that doubling the dietary Fe supply by adding heme Fe to a cereal-based diet not only increased Fe absorption but also palliated the adverse effects of Fe deficiency on Ca, P, and Mg metabolism. Therefore, supplementation of a cereal-based diet with heme iron does not produce interactions between iron and calcium, phosphorus, and magnesium metabolism in rats.

The findings in rats might be carefully extrapolated to humans, as indicated by Gordon and Godber (1989) and Roberts *et al.* (1993). A cereal-based diet supplemented with heme Fe may thus be useful in the treatment and prevention of ferropenic anemia in children.

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