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Effect of various calcium salts on non-heme iron bioavailability in fasted women of childbearing age

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ABSTRACT

Introduction: Micronutrient deficiencies are one of the most important public health issues worldwide and iron (Fe) deficiency anemia is the most prevalent micronutrient deficiency. Iron deficiency often coexists with calcium deficiency and iron and calcium supplementation often overlap. This has led to investigations into the interaction between these two minerals, and whether calcium may inhibit iron absorption in the gut.

Objective: To determine the effect of various calcium salts on non-heme iron bioavailability in fasted women of childbearing age.

Methods: A randomized and single blinded trial was conducted on 27 women of childbearing age (35–45 years old) divided into 2 groups (n1 = 13 and n2 = 14, respectively). On four different days, after an overnight fast, they received 5 mg of Fe as $FeSO_4$ (labeled with ^{55}Fe or ^{59}Fe) with 800 mg of elemental calcium in the form of either calcium chloride, calcium gluconate, calcium citrate, calcium carbonate, calcium lactate, calcium sulfate or calcium phosphate. Calcium chloride was used as the control salt in both groups. Iron was labeled with the radioisotopes ^{59}Fe or ^{55}Fe, and the absorption of iron was measured by erythrocyte incorporation of radioactive Fe

Results: 800 mg of elemental calcium as calcium citrate produced a significant decrease in non-heme iron bioavailability (repeated measures ANOVA, F = 3.79, p = 0.018).

Conclusion: Of the various calcium salts tested, calcium citrate was the only salt that decreased non-heme iron bioavailability relative to the calcium chloride control when taken on an empty stomach. These results suggest that inhibition of non-heme iron absorption in fasted individuals is dependent upon the calcium salt in question and not solely dependent on the presence of calcium.

1. Introduction

Micronutrient deficiencies are the most common public health problem worldwide, affecting more than 33% of the world's population particularly in low and middle income countries [1]. Poor diet and low food variety are important factors contributing to micronutrient deficiencies. Micronutrient deficiencies increase the risk of various infections, cause stunting and impaired cognitive development, among other complications [2].Vulnerable groups for micronutrient deficiencies include pregnant and lactating women, infants, children under five years, and adolescents [3–5].

Among micronutrient deficiencies, iron and calcium deficiencies are the most prevalent and cause anemia and osteoporosis, respectively [5]. Treatment of these deficiencies involves prophylactic supplementation with both iron and calcium salts. This poses a problem as an acute inhibitory effect of calcium on non-heme iron absorption has been demonstrated. The effects of specific calcium salts have not been well studied, although calcium, in general, has been shown to be an important negative factor in non-heme iron absorption [6–10].

Both iron and calcium salts seem to be potential therapeutic micronutrient supplements for anemia and osteoporosis. Their interaction has been studied when ingested together with food but not when ingested on an empty stomach. The present study evaluated the effect of various calcium salts on non-heme iron bioavailability in fasting non-pregnant women of childbearing age.

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2. Subjects and methods

2.1. Trial design

A prospective and single blinded trial to determine the effect of various calcium salts on non-heme iron bioavailability in non-pregnant women of childbearing age (35–49 years) was performed. This study was conducted at the Micronutrients Laboratory of the Institute of Nutrition and Food Technologies (INTA) at the University of Chile between April and August 2011.

2.2. Sample size

The sample size was calculated to find a statistically significant difference when iron absorption differed by 5 percentage points, with a residual standard deviation of 3%. The sample size for an analysis of variance (ANOVA) was 9 subjects ($\alpha = 0.05$ and power = 80%). Thirteen subjects were chosen considering possible drop out during the protocol. A difference of five percentage points in geometric averages of iron absorption was considered biologically significant. In studies of iron absorption, a significant dispersion of values is observed due to the fact that the percentage of iron absorption is dependent on the body iron stores of the subject. For this reason, it is not surprising to find intragroup range values between -1 and + 1 SD. The present study was designed so that each subject was their own control. This removed the effect of the differences in iron stores between subjects.

2.3. Participants

Due to ethical considerations, only non-pregnant women were included in the study as a representative sample of an iron deficient demographic. Convenience sampling lead to the enrollment of 27 community-dwelling and apparently healthy women (35 to 49 years old). None of the women were pregnant, confirmed by a negative human chorionic gonadotropin (hCG) urine test, and all were using contraceptive methods. Women were recruited from southwest Santiago, an area of low socioeconomic status in Chile. Pregnant or lactating women and those who took micronutrient supplements within the past six months were excluded from the study.

2.4. Ethical considerations

After a comprehensive introduction regarding the study and its objective, each participant signed an informed consent form. The trial protocol was approved by the Ethics Committee of the Institute of Nutrition and Food Technologies of the University of Chile. The radiation dose was approved by the Chilean Nuclear Energy Commission (CChEN) and was established as 0.042 Rad; equivalent to 0.42 mSv. These calculations are similar to those reported in articles that use the same methodology.

2.5. Anthropometric measurements

Before the experiment, body mass index (BMI as kg/m^2) was obtained from weight and height measurements for each subject [11]. The anthropometric assessment was performed in order to exclude participants with obesity and to estimate blood volume in accordance with Nadler et al. [12].

2.6. Hematological analysis

Hemoglobin (Hb) and mean cell volume (MCV) (CELL-DYN 3200, ABBOTT Diagnostics, Abbott Park, IL); transferrin saturation (Sat) [13,14], zinc-protoporphyrin (ZPP) (ZP Hematofluorometer Model 206D; AVIV Biomedical Inc, Lakewood, NJ), and serum ferritin (SF) [15] were assessed to evaluate the iron nutrition status of the participants. Anemia was defined when Hb concentration was < 120 g/L; iron deficiency anemia (IDA) when participants had anemia and ≥2 abnormal biochemical parameters (MCV < 80 fL, ZPP > 70 μg/dL red blood cells, Sat < 15% or SF < 12 μg/L) [15]; iron deficiency without anemia (IDWA) was defined as Hb ≥120 g/L and ≥2 abnormal biochemical parameters; depleted iron storage status was defined just when serum ferritin was < 12 μg/L [15].

2.7. Iron radioisotope studies

High-specific activity non-heme iron radioisotopes (^{55}Fe and ^{59}Fe), as ferric chloride (Du Pont de Nemours & Co Inc., Wilmington, DE), were used as tracers. Radioisotopes were added to a solution which provided 5 mg of Fe as ferrous sulfate ($FeSO_4$). The iron solution was consumed after 8 h of overnight fasting, and no food or beverages other than water were consumed during the following 4 h. The total radioactivity ingested was counted in sextuplicate samples from aliquots of the labeled iron solution, as is standard protocol. Radioactivity measurements from the blood were obtained from duplicate venous samples following the method by Eakins and Brown [16]. Samples were counted sufficient times in order to ensure < 3% counting error. A liquid-scintillation counter (Beckman LS 5000 TD; Beckman Instruments Inc., Fullerton, CA) was used for the calculation of all radioisotope measurements. Percentage of absorption was calculated based on the blood volume, estimated from weight and height, and based on the assumption that 80% of the radioactive iron absorbed was incorporated into erythrocytes 14 days after ingestion [17].

2.8. Study protocol

The study was designed to determine the effect of seven different calcium salts on the absorption of 5 mg of non-heme iron as $FeSO_4$ radio labeled with ^{55}Fe and ^{59}Fe. The dose of elemental calcium was 800 mg in each experiment, which is the recommended amount for pregnant women. Five mg of iron was used because this is a typical amount found in common meals and it has been reported that this amount of iron is not effected by 800 mg of calcium chloride [18]. The 27 women were randomly divided into one of two groups. Experimental Group-1 (n = 13) received calcium gluconate (JT Baker, N Cat 299-28-5, Netherlands), calcium citrate (Tate y Lyle, N Cat GH5D281G3D, USA), and calcium carbonate (Mallinckrodt Chemicals, N Cat 471-34-1, USA). Experimental Group-2 (n = 14) received calcium lactate (Merck, N Cat 248-953-3, Germany), calcium sulfate (JT Baker, N Cat 778-18-9, Netherlands), and calcium phosphate (Sigma, N Cat 7758-87-4, Germany). In addition, both groups 1 and 2 received calcium chloride (CaCl Merck, N Cat 233-140-8, Germany) as the control salt. The digestion of calcium chloride does not release conjugated compounds at the intestinal brush border and is assumed not to interfere with iron absorption in the amount used. For both groups 1 and 2, the calcium salts were given in a random order. On day 1, 2, 14 and 15, calcium salts were given with the iron solution. On days 1 and 14, 50 mL of a solution with 5 mg of $FeSO_4$ labeled with 74 kBq/mL of ^{55}Fe was given and on days 2 and 15, the solution was given labeled with 25.9 kBq/mL of ^{59}Fe. Venous blood samples were obtained on days 14 and 28 to measure erythrocyte radioactivity.

2.9. Statistical analysis

Results are expressed as geometric means ± 1 SD range because serum ferritin concentration and iron bioavailability had skewed distributions. These values were first transformed into their logarithms before calculating means and SD and performing statistical analysis. The results were then turned into anti-logarithms to recover the original units and were expressed as geometric means ± 1 SD range. Fe absorption differences between the calcium salts were determined by repeated measures ANOVA (RM-ANOVA) and Dunnett's post hoc test

Table 1
Demographic characteristics of the participants.

Variables[1]	
Age (y)	39.5 ± 4.2
Weight (kg)	62.4 ± 7.7
Height (m)	1.57 ± 0.03
BMI (kg/m2)	25.2 ± 2.9
Hb (g/L)	142 ± 11
MCV (fL)	87 ± 4
Serum iron (μg/dL)	93 ± 32
TIBC (μg/dL)	384 ± 83
ZPP (μg/dL red blood cells)	65.7 ± 14.7
Sat (%)	25.4 ± 14.7
SF[2] (μg/dL)	14 (5 – 34)

BMI: body mass index; Hb: Hemoglobin; MCV: mean cell volume; TIBC: total iron-binding capacity; ZPP: zinc-protoporphyrin; Sat: saturation of transferrin; SF: serum ferritin.
[1] Data presented as mean ± standard deviation (SD).
[2] Data presented as geometric mean and ± 1 SD range.

($p < 0.05$). Data were analyzed using PRISMA 5.0 software (GraphPadPrismTM, Version 5.0, and GraphPad Software Incorporated).

3. Results

The mean ± SD for age, weight, height, and BMI was 39.5 ± 4.2 years old, 62.4 ± 7.7 kg, 1.57 ± 0.03 m, and 25.2 ± 2.9 kg/m^2, respectively. The mean ± SD of hemoglobin, MCV, ZPP, and Sat of the participating subjects was 142 ± 11 g/L, 87 ± 4 fL, 65.7 ± 14.7 μg/dL red blood cells, and 25.4 ± 14.7%, respectively. The geometric mean ± 1 SD range for SF was 14 (5–34) μg/dL (Table 1). Two of the women who participated in the study had IDA, seven women had IDWA, 10 women had DIS, and eight women had normal iron nutrition status (Fig. 1).

Geometric means ± 1 SD range of non-heme iron bioavailability for calcium chloride (as control salt), calcium gluconate, calcium citrate, and calcium carbonate in experimental group-1 were 17.8 (9.6–32.8) %, 13.5 (5.9–30.8) %, 11.9 (5.9–24.2) %, and 16.5 (7.9–34.4) %, respectively (RM–ANOVA, *F = 3.79, p = 0.018,* and Dunnett's post hoc test: chloride vs. citrate, $p < 0.05$) (Table 2 and Fig. 2). Geometric means ± 1 SD range of non-heme iron bioavailability for calcium chloride (as control salt), calcium lactate, calcium sulfate, and calcium phosphate in experimental group-2 were 10.2 (4.9–21.1) %, 12.3 (6.8–22.4) %, 8.0 (1.5–42.8) %, and 9.0 (4.3–18.9) %, respectively. (RM–ANOVA, *F = 0.69, p = 0.56)* (Table 3 and Fig. 2).

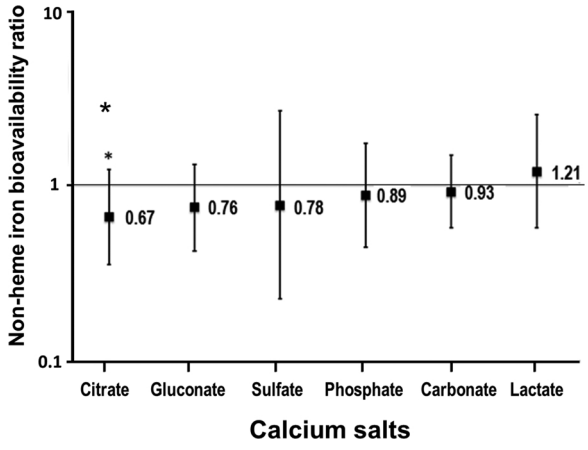

Fig. 1. Data presented as percentages (%) with confidence intervals of 95% (CI95%).
IDA: iron deficiency anemia; IDWA: iron deficiency without anemia; DIS: depleted iron storage.

Table 2
Individual nonheme iron bioavailability of Experimental Group-1.

Participants[1]	^{55}Fe-Calcium Gluconate	^{59}Fe-Calcium chloride	^{55}Fe-Calcium Citrate	^{59}Fe-Calcium Carbonate
1	4.4	10.6	8.4	11.2
2	6.0	8.7	7.9	9.0
3	36.8	20.9	29.7	38.4
4	45.9	32.0	27.7	44.7
5	18.0	23.2	23.3	17.3
6	9.6	7.3	8.5	7.3
7	24.0	25.9	10.9	20.5
8	46.4	61.3	42.2	61.7
9	16.8	11.3	10.4	25.0
10	10.6	34.0	4.8	15.5
11	5.1	13.7	4.3	5.9
12	6.5	12.7	9.0	8.3
13	9.8	16.3	10.3	13.1
Nonheme iron bioavailability (%)	**13.5 (5.9–30.8)**	**17.8 (9.6–32.8)**	**11.9 (5.9–24.2)**	**16.5 (7.9–34.4)**

[1] Data presented as geometric mean and ± 1 SD range. Repeated measurements –ANOVA, F = 3.79, p = 0.018, and Dunnett's post hoc test: chloride vs. citrate, $p < 0.05$.

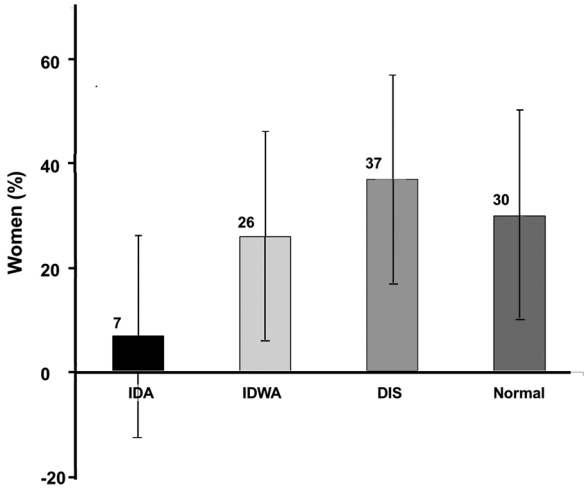

Fig. 2. Data presented as geometrical means of absorption ratios ± 1 SD range (Ratio = iron bioavailability with calcium salts/iron bioavailability with calcium chloride as control).
* p value (< 0.05) after repeated measures Analysis of Variance (RM-ANOVA) and Dunnett's post hoc test.

4. Discussion

Iron and calcium deficiencies are common nutritional problems worldwide [19]. Micronutrient supplements containing both iron and calcium salts are often used as a treatment strategy against these micronutrient deficiencies. There is evidence that shows that calcium is a significant inhibitor of iron absorption [7–10,20–23]. These previously published studies were performed using the same methodology-they assessed micronutrient supplementation combined with fortified foods or with test meals [23]. The problem with using this methodology to measure iron absorption is that the ingestion of a meal may be adding compounds which act as either inhibitors or enhancers of iron absorption [21]. Contrary to these findings, when calcium was given separate from a meal in studies performed on iron deficient populations, no effect of calcium on iron absorption was observed [24,25]. Based on the results of a previously published study on iron absorption protocols

Table 3
Individual nonheme iron bioavailability of Experimental Group-2.

Participants[1]	^{55}Fe-Calcium Lactate	^{59}Fe-Calcium chloride	^{55}Fe-Calcium Sulfate	^{59}Fe-Calcium Phosphate
1	11.1	11.3	1.7	5.5
2	7.9	6.3	23.8	22.9
3	5.8	8.7	3.9	7.6
4	9.2	4.2	3.3	3.3
5	4.3	14.2	32.3	5.4
6	15.8	15.5	23.3	7.7
7	30.4	15.8	24.4	11.6
8	6.9	6.6	8.4	7.3
9	13.3	2.8	0.1	9.9
10	19.6	50.0	53.5	60.0
11	32.8	17.2	34.1	13.7
12	19.3	18.2	9.4	10.7
13	14.8	5.1	1.8	5.0
14	11.5	9.2	16.0	4.9
Nonheme iron bioavailability (%)	**12.3 (6.8–22.4)**	**10.2 (4.9–21.1)**	**8.0 (1.5–42.8)**	**9.0 (4.3–18.9)**

[1] Data presented as geometric mean and ± 1 SD range. Repeated measurements –ANOVA, F = 0.69, p = 0.56.

in fasting subjects, the present study controls for the potential confounding factors of different calcium salts and doses.

The results from the present study show that calcium citrate exerted an inhibitory effect on non-heme iron bioavailability in fasted women of childbearing age. No statistically significant effects of calcium gluconate, calcium carbonate, calcium sulfate, calcium lactate, or calcium phosphate on non-heme iron bioavailability were observed. There are a few possible mechanisms which may explain the reported decrease in iron absorption in the presence of high amounts of calcium. First, the presence of calcium decreases the affinity of the divalent metal-ion transporter-1 (DMT1) for iron. Through the expression of human DMT1 in a cell model using Xenopus oocytes, Shawki and Mackenzie examined DMT1 activity using radiotracer assays and a voltage clamp. The study concluded that Ca^{2+} is an inhibitor, but not a transported substrate, of DMT1 [26]. In addition to this, Thompson et al. reported a dose dependent negative response of calcium on DMT1 apical membrane expression and suggested that this could explain the reduction in non-heme iron absorption [27]. Additionally, Hallberg et al. suggested that calcium can negatively affect ferroportin activity [8].

Cook et al. have shown that both calcium citrate and calcium phosphate affect iron absorption, when taken without food, in a study using 600 mg elemental calcium and 18 mg Fe. They also found that calcium carbonate, calcium citrate, and calcium phosphate all have an inhibitory effect on iron absorption when taken with test meals [21]. Cook et al. suggested that calcium phosphate exerts its inhibitory effect on iron through a reaction between iron and the free phosphate anions. Other researchers have supported this assertion through studies that suggest that both iron and phosphate may react to form an insoluble salt at the intestinal brush border [22]. The mechanism by which calcium citrate affects iron absorption remains unknown.

In the present study, all participants received the same amount of calcium (800 mg elemental calcium). Therefore, using calcium chloride as a control, any effect on iron absorption in the presence of the varying calcium salts can be isolated to their conjugated elements such as citrate, phosphate, carbonate, sulfate, gluconate or lactate.

The bioavailability of iron was negatively correlated with SF levels (data not shown). This finding is in concordance with the literature that states that total body iron is the most important regulatory factor of iron absorption in the gut. The proposed mechanism is mediated by decreased levels of hepcidin, an important hormonal modulator of the expression of both the iron transporter protein DMT1 (in the enterocyte apical membrane) and of IREG1 (the basolateral transfer-protein of iron to the circulation) [28,29].

In the present study, 7% of the participants had IDA (mean Hb 142 ± 11 g/L). A previous report from our laboratory found a prevalence of IDA of 8% (mean Hb 134 ± 13 g/L) between 2001 and 2010 amongst women of childbearing age who participated in different iron absorption protocols [30]. According to the Chilean National Health Survey in 2003, in which data on anemia was collected, the prevalence of anemia in Chilean women ≥17 years old was 5.1%, (mean Hb 137 ± 4 g/L), and was greatest (7.8%) in women of low socioeconomic status [31]. The results of the present study are similar to those of the 2003 National Health Survey. The women enrolled in the present study from the low-income southwest metropolitan region of Santiago, Chile, displayed similar levels of IDA as previously found. Anemia in Chilean women of childbearing age remains a public health problem, especially for those living in low-income areas.

Calcium phosphate has been shown to negatively affect iron absorption through the donation of a phosphorus anion which may form an insoluble precipitate with iron [21]. In the present study, however, calcium phosphate did not exert a statistically significantly effect on iron absorption. This may potentially be explained by the different forms of calcium salts used in our study compared to those used by Cook et al. The present study used dry calcium phosphate, while Cook et al. used pills which contain excipients that may increase the inhibitory effect of calcium on iron absorption or which may be inhibitory themselves. A limitation of the present study is that only women of childbearing age were included in the study sample. Different findings may be reported in studies including both sexes and different age groups. Differences in iron status between the sexes may be exacerbated by the fact that females lose iron through menstruation. The present study was conducted in a small sample of women of childbearing age from low-income communities. Therefore, the findings may not be representative of all Chilean women of childbearing age.

According to the Institute of Medicine [32], the most common forms of supplemental calcium in North America are calcium carbonate and calcium citrate. While calcium citrate produces less digestive symptoms and its absorption is less dependent on stomach acid than calcium carbonate, the present results emphasize the importance of promoting the consumption of calcium citrate supplements between meals. The consumption of calcium citrate supplements before, rather than during, meals will avoid negatively influencing the absorption of dietary iron In Chile, calcium carbonate is the most widely used calcium salt due to its low cost. Calcium phosphate is the second most commonly used calcium salt and is used to supplement both calcium and phosphorus during pregnancy [33]. Calcium citrate, commonly used in Europe, has high calcium bioavailability even in patients with achlorhydria, although it also has a high cost [34]. Currently in Chile, the only readily available calcium salts are calcium carbonate and calcium phosphate. According to the results of the present study, there is no inhibitory effect of either of these calcium salts on non-heme iron bioavailability when consumed on an empty stomach. In a previous study, we also reported no effect of one month of calcium carbonate supplementation (600 mg elemental calcium/d) on both heme and non-heme iron bioavailability in women of childbearing age [35]. In the present study, the calcium dose used was 800 mg for three reasons. First, it is the recommended amount of calcium to supplement to pregnant women [36]. This amount also comprises 80% of the daily recommendation of calcium (1000 mg/d) and 800 mg of elemental calcium provided from calcium salts has been reported as the maximum amount of elemental calcium that does not affect the bioavailability of 5 mg of non-heme iron as $FeSO_4$ [18]. This allows the present study to ascribe the inhibitory effects from the calcium salts to their conjugated elements, and not to the presence of elemental calcium.

The present study showed an acute effect of calcium salt consumption on iron bioavailability. Inferences on the chronic effects of various calcium salt supplementation cannot be made due to the short duration of the study. The strength of the present study design was that

it controlled for the potential confounding variables of fed status and iron nutritional status.

This study was performed on women selected from a specific community and from a relatively small age range. Because of this, the participants do not represent all women found in Chile. Despite the limited study setting, the results highlight the implications of combined calcium and iron supplementation in populations at high risk for both iron and calcium deficiency. The results have public policy implications regarding the design and implementation of micronutrient supplementation programs to these vulnerable populations (infants, pregnant and lactating women and the elderly).

In conclusion, 800 mg of elemental calcium supplemented as calcium citrate had a significant inhibitory effect on non-heme iron bioavailability when compared to 800 mg of calcium delivered as calcium chloride control in fasted women of childbearing age. No significant reductions in non-heme iron bioavailability were found from supplementation with the five other calcium salts used.

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Conflict of interest

The authors declare no conflicts of interest.

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