

Smaller iron particle size improves bioavailability of hydrogen-reduced iron–fortified bread

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Abstract

Bioavailability results, obtained in humans, of wheat flour fortified with elemental iron might not be reliable because these studies have been performed using tracer iron compounds that probably do not have the same physicochemical characteristics as the commercial elemental iron powders. The aim of the present study was to measure, in Caco-2 cells, the iron bioavailability of hydrogen-reduced (H-reduced) iron ($\leq 45\text{-}\mu\text{m}$ particle size) from 72%-extraction wheat flour bread. Bread was fortified with either 8- μm H-reduced iron (pro analysi compound used as control) or $\leq 45\text{-}\mu\text{m}$ H-reduced iron (industrialized food grade product). Solubility and dialyzability were determined from bread homogenate. Ferritin and intracellular iron content were assessed in Caco-2 cells incubated with either reduced iron or fortified bread homogenate. The 8- μm H-reduced iron had higher solubility and dialyzability than $\leq 45\text{-}\mu\text{m}$ H-reduced iron. Intracellular iron and ferritin concentrations in Caco-2 cells exposed to digest from bread fortified with 8- μm H-reduced iron were significantly higher than in bread fortified with $\leq 45\text{-}\mu\text{m}$ H-reduced iron ($P < .05$). When bread fortified with ferrous sulfate was used as a reference, the relative iron bioavailabilities of the fortified bread with 8- μm H-reduced iron and $\leq 45\text{-}\mu\text{m}$ H-reduced iron were 68.2% and 31.1%, respectively. In conclusion, there is an inverse relationship between H-reduced particle size and iron bioavailability. If H-reduced (food grade, $\leq 45\text{-}\mu\text{m}$ particle size) iron is used in wheat flour fortification, it should be added at 3 times the level of ferrous sulfate to provide the same absolute amount of absorbed iron.

Keywords:

Hydrogen-reduced iron; Iron fortification; Iron bioavailability; Caco-2 cells

1. Introduction

Iron deficiency continues to be a public health problem in most countries of the world. Food fortification is the most practical and best long-term strategy to prevent iron deficiency in the population [1]. Wheat flour bread is an appealing vehicle for iron fortification because of its relative low cost and widespread consumption.

An adequate absorption of the iron fortificant compound is one of the main technical factors that need to be considered

in the design of national fortification programs. Iron fortificants that are soluble in water or in weak acid are more readily available for absorption [2]. However, their reactivity is a disadvantage when considering stability in the foods used as vehicles of the fortification. Elemental iron powders are widely used in wheat flour fortification because of their low reactivity and high stability. Several forms of elemental iron powders are available depending on the method of preparation and the suppliers [3]. Four types of these compounds are currently being used: reduced iron by hydrogen (H-reduced) or carbon monoxide, atomized iron, electrolytic iron, and carbonyl iron. There are considerable variations between different types of elemental iron not only

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in particle size but also in dissolution rate, solubility, and reactive surface [3]. The physicochemical characteristics of the iron powder influence its bioavailability, with iron absorption being high when the particle size is small, uniform, and more soluble in diluted acid [3,4]. Electrolytic iron has the higher solubility and availability. However, H-reduced iron is the compound most commonly used for wheat flour fortification because of its low cost and low reactivity [3].

Few studies of the bioavailability of iron from wheat flour fortified with H-reduced iron have been performed in humans [4-7]. There is general concern about the extrapolation of the results of H-reduced iron bioavailability obtained from controlled studies to the actual absorption of iron from wheat flour fortified with industrially produced H-reduced iron. The use of cultured intestinal epithelial cells (Caco-2 line) provides an excellent model to study intestinal iron absorption, metabolism, and regulation [8,9]. Caco-2 cells, when cultured in bicameral inserts, behave as typical enterocytes, exhibiting both apical and basolateral iron uptake [10,11]. An *in vitro* Caco-2 technique has been used to predict iron bioavailability from foods and meals [12].

The aim of the present study was to measure, in Caco-2 cells, the iron bioavailability of a commercial H-reduced iron ($\leq 45\text{-}\mu\text{m}$ particle size) from 72%-extraction wheat flour bread.

2. Methods and materials

2.1. Fortification of wheat flour with reduced iron

Wheat flour (72% extraction) was fortified (30 mg/kg) with the following:

1. H-reduced iron (pro analysi) with particle size of $8\ \mu\text{m}$ (8- μm H-reduced) (Merck, Darmstadt, Germany)—this compound has more than 96% of metallic iron; this compound was used as a positive control because of its potential high absorption due to the small particle size;
2. H-reduced (food grade, industrially produced) iron with particle size above 20 and below $45\ \mu\text{m}$ ($\leq 45\text{-}\mu\text{m}$ H-reduced) (Roche Vitamins Ltd, Basel, Switzerland)—this iron comes in a premix, which also contains zinc (21.8 g/100 g), vitamin A (0.55 g/100 g), folic acid (0.5 g/100 g), pyrophosphates (33.5 g/100 g), vitamin B₁ (0.5 g/100 g), and vitamin B₂ (0.3 g/100 g); this is one of the most commonly elemental iron powder premix used in food fortification; or
3. FeSO₄ · 7H₂O, above 99.9% purity (Merck Darmstadt, Germany), as a reference iron compound. Ferrous sulfate was combined with ascorbic acid (AA) (Merck Darmstadt, Germany) in molar ratio of Fe/AA of 1:2.

H-reduced iron was solubilized in 0.1 mol/L HCl. The iron concentration in flour was determined from an acid-wet digestion extract [13] by colorimetric assay using bathophenanthroline reagent (Sigma Chemical, Saint Louis, Mo) at 540 nm. Bread buns (100 g each) were prepared from the fortified wheat flour. The mean iron concentration in the unfortified wheat flour was 1.27 ± 0.25 mg/100 g of flour. The mean iron concentration in the breads fortified with 8- μm H-reduced, $\leq 45\text{-}\mu\text{m}$ H-reduced, and ferrous sulfate were 4.22 ± 0.14 , 3.94 ± 0.88 , and 4.27 ± 0.15 mg/100 g of flour, respectively (analysis of variance [ANOVA], $P = \text{NS}$).

2.2. *In vitro* peptic digestion

8- μm H-reduced iron, $\leq 45\text{-}\mu\text{m}$ H-reduced iron, ferrous sulfate, and bread made from wheat flour fortified with 8- μm H-reduced iron, $\leq 45\text{-}\mu\text{m}$ H-reduced iron, and ferrous sulfate were digested by *in vitro* peptic digestion [14]. A homogenate was prepared with ion-free water. To determine the solubility of reduced iron in the extract, 2 mL of pepsin solution (0.2 g pepsin per 5 mL 0.1 mol/L HCl; Sigma Chemical, Saint Louis, Mo) was added to 80 g of the homogenate (pH 2.0) and incubated for 2 hours at 37 °C in a shaker. The extract was then aliquoted (20 g each), and the iron content in the extract was determined by atomic absorption spectroscopy (Perkin Elmer, model 2280, Norwalk, Conn). To determine the H-reduced iron and ferrous sulfate dialyzabilities, a dialysis bag (cutoff point 8000 D, Spectrum Medical Industries, Inc, Los Angeles, Calif) with 20 mL of Pipes buffer (pH 7.3), was introduced in the extract and incubated for 30 minutes in a shaker. The pH was raised to 6 with 1 mol/L NaHCO₃, and 5 mL of bile/pancreatin solution (Sigma Chemical) (0.05 g pancreatin; 0.3 g bile extract [glycine and taurine conjugates and other bile salts] in 25 mL 0.1 NaHCO₃) was added. After incubation, both the homogenate and the Pipes buffer (Sigma-Aldrich Corp, St. Louis, Mo) were weighed. The total iron content was determined in the Pipes buffer by atomic absorption spectroscopy. The dialyzability was determined by

Fe dialyzability (%)¹²

$$= \frac{[\text{Pipes Fe } (\mu\text{g/g}) \times \text{final Pipes (g)}] + [\text{Pipes Fe } (\mu\text{g/g}) \times \text{final homogenate big (g)}]}{\text{Initial Fe homogenate (Ag/g)} \times \text{initial homogenate (g)} \times 100} \quad (1)$$

where (a) Pipes Fe ($\mu\text{g/g}$) is the iron concentration in Pipes buffer at the end of the dialysis, (b) final Pipes (g) is the Pipes buffer weighed at the end of the dialysis, (c) final homogenate (g) is the homogenate weighed at the end of the dialysis, (d) initial homogenate Fe ($\mu\text{g/g}$) is the homogenate iron concentration before the dialysis, and (e) initial homogenate (g) is the homogenate weighed before the dialysis.

The product of the dialyzability was centrifuged at 10000 g for 15 minutes at 4 °C; the pH was then adjusted to 6.0, and the final solution was filtered (0.20 μm),

aliquoted and stored at $-20\text{ }^{\circ}\text{C}$. The iron concentration was determined as described above. This extract was used as a source of reduced iron from homemade bread digest.

2.3. Studies in Caco-2 cells

Caco-2 cells were obtained from American Type Culture Collection (Rockville, Md) and were cultured in Dulbecco's modified Eagle medium (DME, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hybritech, Sigma Chemical, St Louis, Mo), 10 IU/mL penicillin/streptomycin, and 25 $\mu\text{g}/\text{mL}$ fungizone. Caco-2 cells were used in a range of 20 to 25 passages, and the cultured media were changed every 3 days. Cells were incubated at $37\text{ }^{\circ}\text{C}$ in a 5% CO_2 atmosphere, grown in T-25 plastic tissue culture bottles. Relative survival of cells exposed to different experimental conditions was evaluated by using trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction assays [15]. The concentration of metals present in the incubation medium (Dulbecco's modified Eagle medium, 10% FBS) were determined by total reflection x-ray fluorescence spectrometry (TXRF) [16]. The metal content in this solution was 0.24 $\mu\text{mol}/\text{L}$ for Cu, 4.69 $\mu\text{mol}/\text{L}$ for Fe, and 3.60 $\mu\text{mol}/\text{L}$ for Zn. All buffers solutions were filtered through Chelex 100 (Sigma Chemical) to minimize the concentrations of contaminating heavy metals.

To obtain equilibrium loading of a monolayer of Caco-2 cells with H-reduced iron or ferrous sulfate, the cells were seeded at 1×10^5 cells per 25-cm² flask and incubated for 1 week in DME media [10], 10% low Fe FBS [8], supplemented with (a) variable amounts of H-reduced iron (0.5, 5, and 20 $\mu\text{mol}/\text{L}$) obtained from the digestion of bread fortified with both H-reduced iron compounds or (b) 50 μL of digest of bread fortified with 8- μm H-reduced iron, $\leq 45\text{-}\mu\text{m}$ H-reduced iron, and ferrous sulfate. The medium was changed every 2 or 3 days. After the cells reached confluence, with a density of 2.0 to 2.5×10^6 cell per 25-cm² flask, they were rinsed twice with phosphate-buffered saline (PBS) and then trypsinized. Cell viability assessed by trypan blue exclusion was typically 90% to 95%. Cells were then reseeded at a density of 1×10^5 cells per flask and cultured as above. To perform the uptake experiments, after 2 passages, preequilibrated cells were seeded onto 4.7 cm² polycarbonate cell culture inserts with 0.3- μm pore size membrane (Transwell, Costar, Cambridge, Mass) with the same culture media supplemented with H-reduced iron or ferrous sulfate, as above [8,10]. Culture media (700 μL) were placed inside the insert (apical cell side) and 1.5 mL in the chamber (basolateral cell side). The media were changed every 3 days. A volume of 14 μL per insert was calculated considering an area of 4.7-cm² insert and a cell height of 30 μm [17]. The formation of a monolayer was monitored by measuring the transepithelial electrical resistance (TEER) using a Millicell electrical resistance system (Millipore, Bedford, Mass). Stabilization of a TEER (250-300 $\Omega \cdot \text{cm}^2$) took between 13 and 15 days.

Cells grown for 14 days were used in the experiments. The TEER of the inserts was measured at the beginning and at the end of each experiment to ensure integrity of the cell barrier, discarding all inserts with TEER lower than 240 $\Omega \cdot \text{cm}^2$ at the end of the experiment.

Cellular extracts from Caco-2 cells were prepared [18]. In brief, the cells were washed with Dulbecco's PBS and then incubated with Tris-saline-EDTA buffer (40 mmol/L Tris-HCl, 100 mmol/L NaCl [pH 7.5], and 1 mmol/L EDTA) for 10 minutes at $37\text{ }^{\circ}\text{C}$. The cells were centrifuged at 1000 rpm for 10 minutes. The cellular pellet was washed with Tris-saline buffer without EDTA. The pellet was diluted with 25 $\mu\text{L}/1 \times 10^6$ cells in lysis buffer (0.5%), NP-40, 10 mmol/L Hepes (pH 7.5), 3 mmol/L MgCl_2 , 40 mmol/L KCl, 1 mmol/L PMSF, 10 $\mu\text{g}/\text{mL}$ leupeptin, 0.5 $\mu\text{g}/\text{mL}$ aprotinin, 0.7 $\mu\text{g}/\text{mL}$ pepstatin A, 5% glycerol, and 1 mmol/L dithiothreitol). The pellet was then incubated for 15 minutes at $4\text{ }^{\circ}\text{C}$ and centrifuged for 5 minutes at 10000 g. The supernatant was diluted at 25 $\mu\text{L}/1 \times 10^6$ cells in lysis buffer without NP-40, aliquoted and stored at $-70\text{ }^{\circ}\text{C}$. This cellular extract was used to determine protein content [19], iron concentration, and ferritin. The intracellular iron content was determined by TXRF [16]. The cells were centrifuged and resuspended in 1 mL of PBS and repelleted. The cellular pellet was resuspended in 0.5 mL of lysis buffer (0.1 N NaOH in PBS), and 20 μL was kept for protein determination. The remaining sample was mixed with 0.5 mL of subboiling ultrapure nitric acid and digested at room temperature for 48 hours. Total intracellular iron analysis was determined by TXRF using a Seifert EXTRA-II spectrometer (Rich Seifert & Co, Ahrensburg, Germany) and the digested sample standardized with 1 $\mu\text{g}/\text{mL}$ of selenium (Merck ICP selenium standard solution, Darmstadt, Germany). The concentration of metals present in the cell-free PBS-sodium hydroxide/nitric acid solution was also measured, resulting in values of 0.11, 1.5, and 0.41 $\mu\text{mol}/\text{L}$ for Cu, Zn, and Fe, respectively.

Intracellular ferritin was determined using a sandwich enzyme-linked immunosorbent assay, as described by Arredondo et al [20]. In brief, cellular extracts were incubated with polyclonal rabbit antihuman ferritin, and peroxidase-labeled rabbit antihuman ferritin antibodies were from DAKO (Carpinteria, Calif).

2.4. Statistical analysis

In all of the experiments, measurements were made in triplicate wells, and the experiments were repeated at least 2 times with independent digested samples or 4 times with reduced iron solutions. Experiments were considered valid if the coefficient of variation of 3 replicates was less than 20%. Regression analysis of iron uptake data was computed using the ENZFITTER program (Elsevier Biosoft, Amsterdam, Netherlands). One-way ANOVA was used to test differences in mean values, and post hoc *t* tests (Scheffé) were used for correction of multiple comparisons. Differences were considered significant at

Table 1
Solubility and dialyzability of H-reduced iron alone and in fortified bread

Compounds	n	Solubility ^a (%)	Dialyzability ^a (%)
8- μ m H-reduced iron	3	60.4 \pm 6.1	11.2 \pm 1.4
8- μ m H-reduced iron (bread)	3	45.6 \pm 2.2	7.5 \pm 2.1
\leq 45- μ m H-reduced iron	3	37.6 \pm 11.7	6.7 \pm 4.9
\leq 45- μ m H-reduced iron (bread)	3	29.4 \pm 10.6	3.4 \pm 3.6
FeSO ₄ : AA (1:2)	3	90.4 \pm 8.7	45.7 \pm 1.7
FeSO ₄ : AA (1:2) (bread)	3	85.2 \pm 2.8	20.6 \pm 1.5

^a Mean \pm SD.

$P < .05$. Statistical analyses were performed using the program Statistica for Windows (release 4.5, StatSoft Inc, Tulsa, Okla).

3. Results

The percentages of iron solubility and dialyzability of the different samples are shown in Table 1. A lower solubility and dialyzability was observed of both H-reduced iron compounds, compared with the ferrous sulfate that served as a reference. 8- μ m H-reduced iron had a higher solubility than \leq 45- μ m H-reduced iron. Similar results were obtained when the solubility and dialyzability of these 3 iron compounds was measured in bread. However, lower iron solubility and dialyzability percentages were obtained in bread.

To further our investigation of the absorption of reduced iron, we repeated the experiment with epithelial Caco-2 cells. A positive correlation ($R^2 = 0.85$) between intracellular ferritin concentration and intracellular iron concentration was observed when Caco-2 cells were incubated with both reduced iron compounds at different extracellular iron concentrations (Fig. 1).

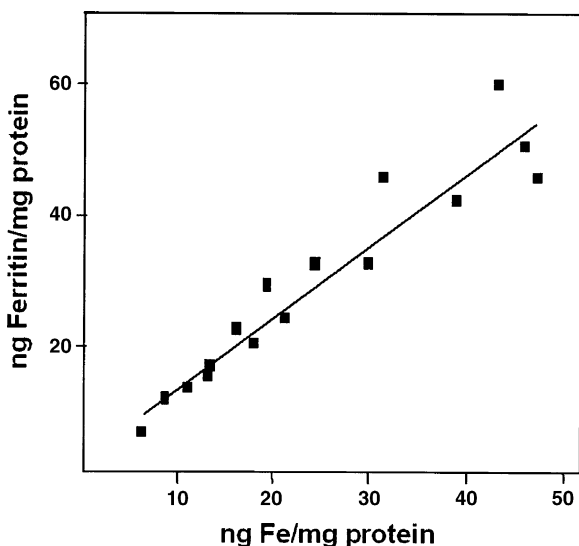


Fig. 1. A positive correlation ($R^2 = 0.85$) was found between intracellular iron and ferritin concentrations in extracts of Caco-2 cells grown in different hydrogen-reduced iron concentrations (mean of 3 independent experiments).

Table 2

Intracellular iron and ferritin concentrations in Caco-2 cells incubated with fortified bread digests (50 μ L)

Fortificant compounds	n	Iron ^a (ng Fe/mg protein)	Ferritin ^a (ng ferritin/mg protein)
8- μ m H-reduced iron	3	266 \pm 41	26.1 \pm 3.8
\leq 45- μ m H-reduced iron	3	108 \pm 28	17.2 \pm 3.1
FeSO ₄	3	388 \pm 35	37.5 \pm 5.8
ANOVA (P)	3	<.001 ^b	.005 ^c

8- μ m H-reduced vs \leq 45- μ m H-reduced ($P < .05$).

^a Mean \pm SD, Scheffé post hoc test.

^b Ferrous sulfate vs both H-reduced Fe compounds ($P < .001$).

^c Ferrous sulfate vs both H-reduced Fe compounds ($P < .01$).

When Caco-2 cells were incubated with 50 μ L of a digest from bread fortified with 8- μ m H-reduced iron, \leq 45- μ m H-reduced iron, or ferrous sulfate, both the mean intracellular iron concentration and mean intracellular ferritin concentration with both H-reduced iron compounds were significantly lower than with ferrous sulfate ($P < .001$ and $< .01$, respectively) (Table 2). Intracellular iron concentration and intracellular ferritin concentration were higher with 8- μ m H-reduced iron than \leq 45- μ m H-reduced iron ($P < .05$) (Table 2).

The relative iron bioavailability of bread fortified with 8- μ m H-reduced and \leq 45- μ m H-reduced iron to the standard of ferrous sulfate (intracellular iron test/standard $\times 100$) was 68.2% \pm 3.2% and 31.1% \pm 1.8%, respectively.

4. Discussion

The measurement of the bioavailability of reduced iron in humans is based on the concept of a *non-hem pool in the meal*, where the elemental iron will exchange with the other non-hem iron in the pool. The tracer used to measure bioavailability needs to have the same physicochemical characteristics of the iron fortificant.

The results of H-reduced bioavailability from wheat flour, performed in humans, might not be reliable because these studies have used labeled iron compounds that probably do not have the same physicochemical characteristics as those of commercial elemental iron powders. For instance, most of the studies performed have used very low particle size of H-reduced iron, which has better solubility and bioavailability [5,7]. On the other hand, the absorption of commercial H-reduced iron has been difficult to measure in humans because of the nonexistence of industrially produced H-reduced iron in a radio/stable isotopic form.

At present, there is not enough information about the iron bioavailability of wheat flour fortified with H-reduced iron produced industrially. Recent publications have provided information that the Caco-2 cell model is able to predict iron bioavailability in humans [21-23]. We determined, as a measure of iron bioavailability, the iron status of

Caco-2 cell grown with a digest of wheat flour, which had been fortified with 1 of 3 different iron compounds: an 8- μm H-reduced particle size pro analysi, which served as positive control; an $\leq 45\text{-}\mu\text{m}$ H-reduced particle size (industrial specification for flour fortification); and ferrous sulfate (reference compound).

Results from the present study show that the solubility and dialyzability from both H-reduced iron compounds were lower than that of ferrous sulfate and that H-reduced iron with lower particle size has higher solubility and dialyzability than the commercial premix containing H-reduced iron with a particle size 45 μm or less. The relationship between particle size and percentage of solubility was within the range reported by Björn-Rasmussen et al [4].

Iron bioavailability, relative to the standard (ferrous sulfate), for bread fortified with 8- μm H-reduced iron and $\leq 45\text{-}\mu\text{m}$ H-reduced iron was 68.2% and 31.1%, respectively. Walter et al [7] found a relative bioavailability of approximately 65% in humans who had consumed white wheat bread fortified with H-reduced ^{58}Fe (particle size average of 15 μm), which is comparable with the bioavailability we obtained in Caco-2 cells with an H-reduced iron with a particle size of 8 μm . On the other hand, Cook et al [5] found a higher relative bioavailability (95%) using 8- to 10- μm H-reduced iron. Björn-Rasmussen et al [4] studied the relative bioavailability of H-reduced iron where 91% of the iron had a particular size below 7 μm . A high relative bioavailability (82%-90%) was found for the more soluble H-reduced samples. However, the relative bioavailability of coarser H-reduced iron powder was 66% and 13% for more or less soluble forms, respectively. As previously reported, we also found a positive correlation between iron and ferritin intracellular concentrations [18].

In conclusion, solubility and dialyzability studies predict H-reduced iron bioavailability in Caco-2 cells. The H-reduced iron compound with higher solubility and dialyzability had the higher iron bioavailability. There is an inverse relationship between H-reduced particle size and iron bioavailability in Caco-2 cells. This in vitro digestion/Caco-2 method could be potentially used to measure the bioavailability of wheat flour fortified with different particle sizes of H-reduced iron. If industrialized food grade $\leq 45\text{-}\mu\text{m}$ H-reduced particle size is used in wheat flour fortification, it should be added at 3 times the level of ferrous sulfate to provide the same absolute amount of absorbed iron.

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