Bioavailability of Elemental Iron Powders in Bread Assessed with an In vitro Digestion/Caco-2 Cell Culture Model

Chi Kong Yeung, Dennis D. Miller, Zhiqiang Cheng, and Raymond P. Glahn

ABSTRACT: Iron fortification of staple foods is arguably the most widely used strategy for increasing the iron intake of populations. Although FeSO₄ is a bioavailable form of iron, elemental iron powders are often used to fortify products with a long shelf-life, such as wheat flours, to avoid problems associated with the reactive nature of FeSO₄. Therefore, the objectives of this study were to compare the bioavailabilities of elemental iron powders manufactured with different production methods in wheat flour breads and to determine the effects of added ascorbic acid and baking, using an in vitro digestion/Caco-2 cell culture model. Two types of wheat flour (low-extraction and high-extraction) were fortified with 10 different commercial elemental iron powders and baked into breads. Iron bioavailabilities from the resulting breads, with and without added ascorbic acid, were evaluated using FeSO₄ as the control. Depending on the type of wheat flour, bioavailabilities of several powders were comparable to FeSO₄, but there was no consistent trend as to which production method produced the most bioavailable powder. In general, ascorbic acid enhanced, whereas the baking process reduced iron bioavailability from bread. Our results suggest that some elemental iron powders are potential alternatives to FeSO₄. Human studies are warranted before any of these powders are selected for national fortification programs.

Keywords: elemental iron, Caco-2, baking, wheat flour bread, ascorbic acid

Introduction

Iron deficiency is the most prevalent nutrient deficiency in the United States and in countries around the world. In the United States, iron deficiency is most common among children aged 1 to 2 years, adolescent girls, and women of childbearing age with prevalence for these groups ranging from 9% to 11% (Looker and others 1997). The situation is much more severe in many developing countries where up to 30% to 40% of young children and premenopausal women are iron-deficient (Yip 2001). Iron fortification of staple foods is arguably the most cost-effective and sustainable strategy for increasing the iron intake of populations at risk.

Cereal flours represent the most appropriate vehicle for iron fortification because they are widely consumed in many countries where iron deficiency is prevalent (Gillespie 1998). Although FeSO₄ is the preferred iron source for addition at the bakery, studies have indicated that undesirable odors and flavors occur in bakery products made from flours heavily fortified with FeSO₄ and stored for extended periods of time, presumably due to the reactive nature of FeSO₄ (Barrett and Ranum 1985; Bovell-Benjamin and others 1999). To avoid such problems, elemental iron powders have been used for fortification of products with a long shelf-life, such as home-use flours, breakfast cereals, and infant cereals. As the name implies, elemental iron powders consist of iron in the zero oxidation state (Fe⁰) in a fine particulate form. These powders are basically pure iron except for small amounts of contamination with other trace minerals and iron oxides. Three common types of commercial elemental iron powders are available (Patrick 1985). The 1st type is reduced iron, which is produced by reducing iron oxide with hydrogen or carbon monoxide gas and then milling to a fine powder. The 2nd type is electrolytic iron, which is produced by the electrolytic deposition of iron onto a cathode made of flexible sheets of stainless steel. The deposited iron is then removed by flexing the sheets and milled to a fine powder. The 3rd type is carbonyl iron, which is produced by heating scrap or reduced iron in the presence of CO under high pressure to form iron pentacarbonyl, Fe(CO)₅. The pentacarbonyl is then decomposed by heating to yield a very fine powder of high purity.

Despite their superior stability, elemental iron powders have been perceived to be less bioavailable than FeSO₄ with relative biological values (RBVs) reportedly ranging from about 13% to 90% in humans and about 8% to 76% in rats (Richardson 1990). Studies commissioned by the International Nutritional Anemia Consultative Group Task Force reported a RBV of 75% for electrolytic-type iron in humans fed a farina-based meal, using a double-isotope, extrinsic tag technique, and RBVs of 47% to 69% in rats using the same technique (Forbes and others 1989). Results from a human study also indicated that absorption of carbonyl-type iron in humans varied from 5% to 20% relative to the intrinsic iron present in wheat flour rolls in different breakfast meals (Hallberg and others 1986). Nonetheless, 2 other human studies testing the bioavailability of hydrogen-reduced elemental iron suggested otherwise. In one study, hydrogen-reduced elemental iron baked into bread rolls was equally available as FeSO₄, whereas absorption of sodium iron pyrophosphate was only 1/10 and ferric orthophosphate was 1/3 that of FeSO₄ (Cook and others 1973). In another study, Roe and Fairweather-Tait (1999) reported that percentage absorption from hydrogen-reduced iron (added to flour and baked into bread rolls) and a reference dose of ferrous ascorbate was 64.8% and 49.7%, respectively.

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The objectives of this study were to compare the iron bioavailabilities of breads made from low-extraction wheat (LEW) and high-extraction wheat (HEW) flours fortified with 10 different commercial elemental iron powders and to determine the effects of added ascorbic acid and baking, using an in vitro digestion/Caco-2 cell culture model.

Materials and Methods

Chemicals

All chemicals and digestive enzymes were obtained from Sigma Chemicals (St. Louis, Mo., U.S.A.) or Fisher Scientific (Fairlawrn, N.I., U.S.A.) unless stated otherwise. Ingredients for cell culture media were obtained from GIBCO, Life Technologies (Rockville, Md., U.S.A.). Water used in the preparation of reagents was double deionized. Glassware were soaked in 3 mol/L HCl for no less than 4 h and rinsed with deionized water before all experiments.

Elemental iron powders and flours

Ten elemental iron powders, assembled from various manufacturers, were provided by SUSTAIN (Sharing Science and Technology to Aid in the Improvement of Nutrition, Washington, D.C., U.S.A.). Two types of flours (LEW and HEW) were provided by ADM Milling Co. (Buffalo, N.Y., U.S.A.). The production methods of the iron powders are shown in Table 1. Each powder was added to the flours before bread making. Iron fortification of the LEW and HEW flours was designed to achieve total iron levels of 60 μg/g and 100 μg/g, respectively. In addition to elemental iron, breads made from unfortified flours and flours fortified with FeSO₄ were prepared for comparison.

Preparation of bread

For bread making, the iron-fortified flour (360 g), salt (9.6 g), shortening (24 g), sugar (12.5 g), yeast (5.7 g; SAF Perfect Rise, At- lanta, Ga., U.S.A.), and double deionized water (225 mL) were placed in the baking chamber of an automatic bread-maker (Regal Kitchen Pro collection no K6725, Kewaskum, Wis., U.S.A.) and baked using the “basic” setting. All bread samples were allowed to equilibrate to room temperature and were freeze-dried afterward. Samples were crushed after freeze-drying, and aliquots withdrawn for measuring iron content. All freeze-dried samples were stored in airtight plastic bags at –20 °C. On the day of the experiment, 1-g aliquots of freeze-dried and rinsed with deionized water before all experiments.

Iron bioavailability of each bread sample was assessed with and without ascorbic acid. The ascorbic acid was added at the start of the in vitro digestion process. The final concentration of ascorbic acid used was 200 μmol/L digest, and ascorbic acid–to–iron molar ratios were about 3:1 and 2:1 for the LEW and HEW flour breads, respectively. From our previous study, this amount of ascorbic acid would significantly promote iron bioavailability (Glahn and others 1999).

The effect of baking on iron bioavailability from the LEW flour bread was also determined. The bread was fortified with an iron fortificant (FeSO₄, FeCl₃, or elemental iron—Ferronyl) either before or after baking, and the resulting bioavailability was assessed.

Statistical analysis

Measurements of iron and phytate contents of the samples were done in triplicate. Iron bioavailability measurements for all treatments were independently replicated 6 times. Data were analyzed by analysis of variance (ANOVA) or Student t-test. Significance was defined at P < 0.05. If appropriate, means were compared by Tukey procedures.

Results and Discussion

Iron and phytate contents

The total iron contents of breads with and without fortification are shown in Table 1. All values are expressed on a dry weight basis. The intrinsic iron and phytate contents of the flours are shown in Table 2. For the LEW flour, there was no measurable phytate in the form of inositol pentaphosphates (IP5) or inositol hexaphosphates (IP6), both of which are the forms of phytate known to significantly inhibit iron bioavailability (Sandberg and others 1999; Skogland and others 1999). In contrast, a significant amount of intrinsic phytate was present in the HEW flour, approximately a 5-fold molar excess relative to the intrinsic iron of the flour. The additional ingredients used in bread making produced a negligible change in iron content (comparing iron levels of the flours and unfortified breads).

Iron bioavailability of bread

Figure 1 shows the ferritin/protein levels from Caco-2 cells exposed to breads made with the LEW and HEW flours, with and without fortification and added ascorbic acid. The ferritin/protein level of untreated Caco-2 cells typically ranges from 6 to 10 ng/mg, whereas cells exposed to the digests of unfortified breads made with the LEW and HEW flours yielded ferritin/protein levels of 20 and 35 ng/mg, respectively. These results indicated that some of the intrinsic iron from the flours was released after in vitro digestion and taken up by the cells, and that the Caco-2 system was responding positively. A factorial (2 × 2 × 2) ANOVA revealed that the effects of fortification (P < 0.0005), type of wheat flour (P < 0.0005), and added ascorbic acid (P < 0.0005) on ferritin formation in Caco-2 cells were all significant. There was also a significant interaction between fortification and the type of wheat flour (P = 0.007). Fortification iron in the form of FeSO₄ significantly increased the ferritin/protein...
level of Caco-2 cells exposed to both types of breads, but the magnitude of increase was greater in breads made with the LEW flour, even though the HEW flour bread had a higher total iron content (Table 1). The HEW flour contained an approximately 5:1 phytate-to-iron molar ratio, whereas the phytate content of the LEW flour was much lower (Table 2). Although the iron content of the HEW flour was increased approximately 3-fold as a result of fortification and yeast fermentation during bread making helped reduce phytate level in bread (Hurrell and others 2002), the inhibitory effect of phytate would still be present if the phytate-to-iron molar ratio remained higher than 1:1. Human studies have suggested that the molar ratio of phytate to iron in wheat flour bread and liquid soy protein-isolate formula should be reduced to below 1:1 and preferably below 0.4:1 to produce a substantial improvement in iron bioavailability (Hallberg and others 1989; Hurrell and others 1992). This could explain why the effect of fortification was greater on ferritin formation in Caco-2 cells exposed to the LEW flour bread, compared with the HEW flour bread.

Addition of ascorbic acid at a level of 200 μmol/L before in vitro digestion significantly enhanced ferritin formation in Caco-2 cells (Figure 1). There has been mounting evidence, as reviewed by Monsen (1988) and Hurrell (2002), that ascorbic acid enhances iron absorption from low-bioavailability diets in humans. For example, ascorbic acid has been shown to increase iron absorption from white bread with added phytate in human subjects (Siegenberg and others 1991). Nonetheless, ascorbic acid has an enhancing effect only if ingested together with meals and would play no role if ingested at other times (Fleming and others 1998; Backstrand and others 2002). It should be noted that ascorbic acid is often added to wheat flour for improving the baking properties of wheat flour dough. In the United States, the amount of ascorbic acid allowable in wheat flour is specified by the Code of Federal Regulations (Title 21, Section 137.105), which states that “It (wheat flour) may contain ascorbic acid in a quantity not to exceed 200 parts per million as a dough conditioner.” Addition of as little as 15 ppm of ascorbic acid to wheat flour increases both dough strength and loaf volume, compared with flour with no added ascorbic acid (Melville and Shattock 1938; Sandstedt and Hites 1945; Elkassabany and Hoseney 1980). The loss of ascorbic acid, nevertheless, is continuous and substantial during mixing, proofing, and baking of the dough, as well as subsequent

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**Table 1—Total iron contents (μg per g of flour used) in breads made from flours fortified with different elemental iron powders**

<table>
<thead>
<tr>
<th>Iron powder/name</th>
<th>Production method</th>
<th>Low-extraction wheat (μg/g)</th>
<th>High-extraction wheat (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No added Fe</td>
<td>—</td>
<td>9.0</td>
<td>30.8</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>—</td>
<td>58.7</td>
<td>104.3</td>
</tr>
<tr>
<td>Atomel 95SSP</td>
<td>Reduced</td>
<td>61.6</td>
<td>104.8</td>
</tr>
<tr>
<td>Atomel 75</td>
<td>Reduced</td>
<td>61.4</td>
<td>107.6</td>
</tr>
<tr>
<td>RSI-Hi-Sol</td>
<td>Reduced</td>
<td>58.9</td>
<td>106.8</td>
</tr>
<tr>
<td>AC-325</td>
<td>H-reduced</td>
<td>61.8</td>
<td>104.2</td>
</tr>
<tr>
<td>RSI-325</td>
<td>CO-reduced</td>
<td>60.2</td>
<td>104.1</td>
</tr>
<tr>
<td>MH300.29</td>
<td>CO-reduced</td>
<td>58.5</td>
<td>104.3</td>
</tr>
<tr>
<td>Ferronyl</td>
<td>Carboxyl</td>
<td>59.1</td>
<td>107.2</td>
</tr>
<tr>
<td>OF</td>
<td>Carboxyl</td>
<td>58.7</td>
<td>103.7</td>
</tr>
<tr>
<td>A-131</td>
<td>Electrolytic</td>
<td>61.6</td>
<td>104.3</td>
</tr>
<tr>
<td>Electrolytic</td>
<td>Electrolytic</td>
<td>58.7</td>
<td>107.6</td>
</tr>
</tbody>
</table>

*aValues are average of triplicate measurements with variability between measurements of less than 5%.

**Table 2—Intrinsic iron and phytate contents of the wheat flours**

<table>
<thead>
<tr>
<th>Flour</th>
<th>Intrinsic iron (μg/g)</th>
<th>Intrinsic phytate (μmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-extraction wheat</td>
<td>7.14</td>
<td>0.128</td>
</tr>
<tr>
<td>High-extraction wheat</td>
<td>28.9</td>
<td>0.517</td>
</tr>
</tbody>
</table>

*aValues are average of triplicate measurements with variability between measurements of less than 5%.

**Figure 1—Effect of FeSO₄ and ascorbic acid on iron bioavailabilities from breads made with low-extraction and high-extraction wheat flours.** Ferritin/protein level of unfortified Caco-2 cells typically ranges from 6 to 10 ng/mg. +aa = addition of ascorbic acid at the start of in vitro digestion; HEW = high-extraction wheat flour bread; LEW = low-extraction wheat flour bread. Bar values (mean + SEM, n = 6) with no letters in common are significantly different (P < 0.05).

**Figure 2—Relative iron bioavailabilities of elemental iron powders from bread made with the low-extraction wheat flour without added ascorbic acid.** Bar values (mean + SEM, n = 6) with no letters in common are significantly different (P < 0.05). Asterisk indicates significant difference (P < 0.05) versus FeSO₄.
storage of the bread (Lu and Seib 1998). The resulting bread generally is not considered a source of ascorbic acid. In the present study though, ascorbic acid was added to the bread samples at the start of in vitro digestion (that is, after baking and freeze-drying), and the enhancing effect of ascorbic acid was prominent.

Figures 2 through 5 show the results of iron bioavailability measurements from breads fortified with elemental iron powders, both in the presence and absence of ascorbic acid. All elemental iron powders were compared with FeSO₄, which was added to the flours in the same way the elemental iron powders were added. Results were expressed as percentages relative to breads fortified with FeSO₄. Therefore, breads fortified with FeSO₄ had a relative ferritin formation of 100% by definition in Figures 2 through 5.

For the LEW flour, without ascorbic acid, breads fortified with AC-325, Ferronyl, and OF induced higher ferritin formation in Caco-2 cells relative to FeSO₄, and all other elemental iron-fortified breads were not different from the FeSO₄ control (Figure 2). With the addition of ascorbic acid, 9 of the 10 elemental iron-fortified breads were not different from the FeSO₄ control, except for the bread fortified with MH300.29, which induced lower ferritin formation (Figure 3). The finding that 3 powders showed higher bioavailabilities than FeSO₄ in the absence of ascorbic acid is surprising because elemental iron has been perceived to be less bioavailable than FeSO₄. We do not have a definite explanation for this observation, but because the LEW flour contains very little phytate, these results suggest that in the absence of inhibitors, most of the elemental iron powders tested show comparable bioavailability to FeSO₄.

Unlike the LEW flour, iron bioavailabilities from breads made with the HEW flour and fortified with elemental iron powders, both in the presence and absence of ascorbic acid, were less than or at best equal to the FeSO₄ controls (Figures 4 and 5). FeSO₄ generally appears to be more bioavailable than elemental iron in high-phytate breads. In addition, despite the differences in their total iron contents, ferritin formation of unfortified breads were comparable to most breads fortified with elemental iron powders. AC-325 and RSI Hi-Sol breads were the only 2 that induced greater ferritin formation than the unfortified bread in the absence of ascorbic acid, whereas in the presence of ascorbic acid, all breads were not significantly different from the unfortified bread, suggesting that fortification in the form of elemental iron did not significantly increase the amount of bioavailable iron in the HEW flour breads.

Figure 6 shows the effect of baking on iron bioavailability from the LEW flour bread. Baking significantly reduced the bioavailabilities of FeSO₄ and Ferronyl, but showed no effect on FeCl₃. These results suggest that the baking process promotes oxidation of iron from FeSO₄ and Ferronyl to ferric iron, and this oxidation negatively affects iron bioavailability. Presumably, iron from FeCl₃ is already in ferric form so baking does not affect the bioavailability of FeCl₃.

There was no consistent trend on which production method produced the most or the least bioavailable elemental iron powder for wheat flour bread. MH300.29 induced less ferritin forma-
tion than FeSO₄ in the LEW flour bread with ascorbic acid, as well as in the HEW flour bread with and without ascorbic acid, suggesting that it may not be suitable for fortifying wheat flours. However, another CO-reduced iron powder, RSI-325, only showed inferior bioavailability in the HEW flour bread with ascorbic acid when compared with FeSO₄. Besides, some individual powders seem to have better bioavailability in certain flours (for example, Ferronyl in the LEW, but not HEW, flour), suggesting that the food matrix may also play a role in the bioavailability of elemental iron.

Electrolytic-type elemental iron has been touted as the only type of iron powders useful for food fortification (Hurrell 2002), but the results of our study do not appear to support any particular type of powders that is superior in terms of bioavailability in wheat flour bread. Swain and others (2003) recently reported RBVs of 21% to 64% when rats were fed a casein-based rat chow fortified with 6 of the 10 commercial elemental iron powders tested in the present study, with carbonyl-type iron (Ferronyl) being the most bioavailable. While all 6 powders tested in their study were significantly less bioavailable than FeSO₄, our results suggest that some elemental iron powders are potential alternatives to FeSO₄ when added to flours and baked into breads. Because the food matrices used in the 2 studies were quite different (casein-based rat chow versus wheat flour bread) and Swain and others did not subject their powders to any heat treatment, these results may not be directly comparable. Further research is clearly needed. Our results, nevertheless, are consistent with the data from 2 human studies that evaluated the bioavailability of hydrogen-reduced iron in wheat flour bread (Cook and others 1973; Roe and Fairweather-Tait 1999).

**Conclusions**

Fortification of low-extraction and high-extraction wheat flours with commercial elemental iron powders increased the total iron contents of the resulting breads. Iron bioavailabilities of several powders were comparable to FeSO₄. Our results showed no clear and consistent trend as to which production method produced the most bioavailable powder, but in general, addition of ascorbic acid enhanced iron bioavailability from bread, whereas the baking process reduced iron bioavailability from bread. If any of these powders are to be selected for fortification programs at a national level, human absorption trials are warranted.

**Acknowledgments**

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Hurrell RF, Reddy MB, Burr J, Cook JD. 2002. Phyto degradation determines the effects of industrial processing and home cooking on iron absorption from cere-


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