JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 2637–2642 **2637**

Comparison of Iron Uptake from Reduced Iron Powder and $FeSO_4$ Using the Caco-2 Cell Model: Effects of Ascorbic Acid, Phytic Acid, and pH

WAN-LING HE,[†,‡] YING FENG,[†,§] XIAO-LI LI,[‡] AND XIAO-E YANG*[,†]

Ministry of Education Key Laboratory of Polluted Environmental Remediation and Ecological Health, Zhejiang University, Hangzhou 310029, China, Institute of Animal Science and Technology, Henan Science and Technology University, Luoyang 471003, China, and Zhejiang Provincial Key Laboratory of Subtropic Soil and Plant Nutrition, Zhejiang University, Hangzhou 310029, China

The reduced iron powder has considerable potential for use as an iron fortificant because it does not change organoleptically during storage or food preparation for cereal flour, and its bioavailability is scarcely influenced by iron absorption inhibitors in foods. The objective of this article is to study the effects of ascorbic acid, phytic acid, and pH on iron uptake from reduced iron powder (43 μm) and $FeSO_4$, and to compare iron bioavailability of reduced iron powders among four selected granularity levels. The cell ferritin formation is used as a marker of iron uptake. Obviously, iron uptake of reduced iron powder is increased with decreasing of powder granularity and is much lower than $FeSO_4$ when the size is above 43 μm, but significantly higher at 40–60 nm. In the presence of ascorbic acid or phytic acid, Caco-2 cell iron absorption from reduced iron powder (43 μm) is significantly higher than that from $FeSO_4$. And iron uptake of Caco-2 cells is decreased with increasing of pH from 5.5 to 7.5. Moreover, the decrease trend is more obvious for reduced iron powder than for $FeSO_4$. Our results indicated that iron bioavailability of reduced iron powder by intestinal enterocytes is similar to that of iron salts, and reduced iron powder is more excellent than $FeSO_4$ as food fortificant, especially at ultramicroscopic granularity.

KEYWORDS: Caco-2 cell model; iron bioavailability; reduced iron powder; $FeSO_4$; ascorbic acid; phytic acid; pH

INTRODUCTION

Iron is an important essential micronutrient that is involved in oxygen transport and energy metabolism. Iron deficiency is a widespread major micronutrient malnutrition problem for human beings, and it is believed to affect 20–50% of the world's population (*1*). The problem is because of an insufficient amount and poor bioavailability of iron in the food (*2*). Strategies for eliminating iron deficiency include medication, food diversification, food fortification, and biological-aggrandizement. Food fortification is being recognized as a sustainable, relatively simple, and realistic way to reduce and prevent iron deficiency (*3, 4*). Many different forms of iron ranging from iron salts and iron chelates to reduced iron powder have been approved as iron sources for food fortification (*3, 5*). Previous studies indicated that the amount of dialyzable iron and bioavailability *in vitro* are changed with different sources of iron (*6–8*). In general, water soluble iron compounds, such as ferrous sulfate have an acceptable bioavailability but cause unacceptable organoleptic changes during the storage or preparation of grains. In addition, high iron bioavailability of some iron compounds, such as heme iron, ferrochel amino acid chelate, and NaFeEDTA are relatively expensive (*3, 4*). In contrast, the reduced iron powder does not cause organoleptic changes during storage or food preparation of cereal flours, but it is disputable on iron bioavailability (*6*). The effects of reduced iron granularity on iron availability have been demonstrated in other studies and indicate that iron availability enhanced with decreasing granularity (*9*) and that the average of iron relative bioavailability from fortified-wheat flour with reduced iron powder is 130% in human experiments (*10*).

The Caco-2 cell is a human adenocarcinoma cell line that has proven to be a useful model for studying iron absorption from foods and iron fortificants (*11, 12*). The cells differentiate into polarized monolayers with characteristics as a brush border membrane containing the enzymes present in normal absorptive epithelial enterocytes. Ferritin formation by the Caco-2 cells following exposure to an iron source has been used as a marker for iron uptake (*13, 14*).

The objectives of this study are to address the following questions: (1) Does reduced iron granularity have an influence

* To whom correspondence should be addressed. E-mail: xyang@zju.edu.cn.

[†] Ministry of Education Key Laboratory of Polluted Environmental Remediation and Ecological Health, Zhejiang University.

[‡] Henan Science and Technology University.

[§] Zhejiang Provincial Key Laboratory of Subtropic Soil and Plant Nutrition, Zhejiang University.

10.1021/jf0730946 CCC: $40.75 © 2008 American Chemical Society
Published on Web 04/01/2008

on iron bioavailability after simulated gastrointestinal conditions (*15–17*) in conjunction with *in vitro* intestinal absorption using the Caco-2 cells (*18–20*)? (2) Does pH have the same effects on iron uptake from reduced iron powder as from simple salts such as $FeSO_4$? (3) Do the promoter and inhibitor have the same effects on iron uptake from reduced iron powder as from $FeSO_4$? Therefore, we compared dialysis iron from reduced iron powder containing 125 μm, 74 μm, 43 μm, and 40–60 nm of granularity and studied the effects of ascorbic acid, phytic acid, and pH on *in vitro* iron bioavailability of reduce iron powder as well as $FeSO_4$.

MATERIALS AND METHODS

Chemical, Enzymes, and Hormones. Unless otherwise stated, all chemicals, enzymes, and hormones were purchased from sigma chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Reduced iron powders (125, 74, and 43 μm) were purchased from Shanghai Jinshan Smelt Factory (Shanghai, China). Nanoiron powder (40–60 nm) was purchased from Chengdu Ascend Technology Co., Ltd. (Chengdu, China).

Cell Culture. Caco-2 cells were obtained from the Institute of Biochemistry and Cell Biology, SIBS, CAS, Shanghai, China and were used between passage 20–43. The cells were grown in 25 cm^2 tissue culture flasks with 5 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 4 mL/L antibiotic mixture, 25 mg/L amphotericin B, and 100 μm nonessential amino acids. Medium was replaced every 2–3 days and cells grew to approximately 80% confluency before subculturing. Cells used in the uptake and transport of iron experiments were seeded on transwell (Corning, New York, USA) inserts at a density of 2.5 $\times$ A 10^5 cells per insert (4.71 cm^2) and grown for 21–22 days in supplemented DMEM. Medium (1.5 mL apical, 2.5 mL basolateral) was changed every other day for 14 days and then daily for another 7 days prior to use for uptake and transport studies on day 21. Formation of a monolayer was monitored by measuring the transepithelial electrical resistance (TEER) with a Millicell electrical resistance system (Millipose, Bedford, MA). Cell layers were used only after TEER had increased to greater than 250 Ohms$\cdot cm^2$, a level indicating the formation of an intact monolayer (*21–26*). All cells were maintained in an atmosphere of 5% CO_2/95% air at 37 °C.

Harvesting of Caco-2 Cell Monolayers for Ferritin Analysis. The cell monolayers were harvested 24 h after the start of the intestinal digestion period. To harvest the cells, the media covering the cells were removed, and the cells were washed twice with a 2 mL volume of a rinse solution containing 140 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol Piperazome-1,4-bis(2-ethanesulfonic acid) (PIPES) (Sigma, #CB-054) at pH 7. After the monolayers were rinsed, 2 mL of deionized water was placed on each monolayer. The plates were then placed on a rack with the bottom of each plate in contact with the water of a benchtop sonicator, which was kept in a cold room at 4 °C. The cells were sonicated for 15 min and then scraped from the plate surface and harvested, along with the 2 mL volume of water in each well, and stored at −20 °C.

Intestinal Digestion Simulation Treatments. Porcine pepsin (Sigma #P-7000, 800–2500 units/mg protein), pancreatin (Sigma #P-1750, activity) 4 $\times$ AU. S. P. specifications), and bile extract (Sigma #B-8631, glycine and taurine conjugates of hyodeoxycholic and other bile salts) were purchased from Sigma Chemicals. The preparation of gastric and enteric (pepsin, pancreatin, and bile extract) digestion solutions were performed as previously published (*8*).

Experimental Design. *In Vitro Digestion.* Four granularity levels, 125 μm, 74 μm, 43 μm, and 40–60 nm, were chosen to determine solubility and dialysis of reduced iron using the *in vitro* iron dialyzability assay method (*15*). To simulate gastric and enteric digestion, different iron forms were treated as previous described (*11*). Given the solubility of all sizes, iron powders are the same as $FeSO_4$, a certain amount of different size reduced iron powders as well as $FeSO_4$ were weighted (as iron content of different reduced iron powders and $FeSO_4$ are not the same, the amount of weighted powders are calculated by pure Fe, each 0.9 mg Fe) and dissolved in 5 mL of pepsin solution at pH 2.0 (pepsin content = 25 mg/mL) for 60 min on the rocking shaker (55 oscillation/min), respectively. The pH of these solutions were adjusted to 6.0 with 1 mol/L $NaHCO_3$, then added to 25 mL of pancreatin/bile extract (pancreatin 2 mg/mL, bile extract 10 mg/mL) and adjusted the pH to 7.0 with 1 mol/L $NaHCO_3$, and incubated for 120 min on the rocking shaker (55 oscillation/min). The digestion solutions were made to 100 mL volume with a pH 7.0 mixture solution including 120 mmol/L NaCl and 5 mmol/L KCl. The digested solutions were centrifuged (5 min, 4000 rpm, 4 °C), and the supernatants were used in the following dialysis experiment and Caco-2 cell absorption experiment.

A fresh 1.0 mL aliquot of supernatant was pipetted into 50 mL of polypropylene centrifuge tubes, and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, 50 mM final concentration) was added to the tubes to make it similar to the culture medium and facilitate cell viability, while deionized water was added to adjust the osmolarity to ~300 mOsmol/kg (Osmomat 030, Gonotec GmbH, Berlin, Germany) to obtain a final volume of 10 mL culture medium at pH 7.0 (adjusted with 1.0 mol/L $NaHCO_3$). Then all treatment solutions were transferred to a dialysis bag (molecular weight 8000 Da). The dialysis bag was fastened with thin string and placed in a 50 mL beaker with an appropriate volume of the above isotonic medium (without iron). Then the beaker was capped, placed horizontally, and incubated for 120 min on the rocking shaker (55 oscillations per minute) at 37 °C, and afterward, the media were harvested for analysis.

For the Caco-2 cell iron uptake experiment, the cell transport plates were used. A fresh 1.0 mL aliquot of previously prepared supernatant was mixed with 9 mL of minimum essential medium (MEM) to obtain the cell culture medium. The bottom chamber was filled with 2 mL of HBSS and the upper chamber filled with 1.5 mL of cell culture medium. The cell transport plates were then returned to the incubator for an additional 22 h, at 37 °C, after which time the cells were harvested for analysis.

Ascorbic Acid or Phytic Acid Ttreatments. To compare the iron bioavailability of reduced iron powder with ferrous sulfate and study the effects of ascorbic acid, phytic acid, and pH, both 43 μm reduced iron powder and $FeSO_4$ (the amounts are calculated on the basis of pure Fe, with each being 0.9 mg of Fe) were dissolved in 25 mL of 50% (v/v) HCl solution for 60 min on the rocking shaker (55 oscillation/min). A prepared ascorbic acid or phytic acid solution (Fe/ascorbic acid or phytic acid = 1/5 molar) was added to the pepsin solution, and the mixed solutions were freshly shaken for 30 min. Then 120 mmol/L NaCl and 5 mmol/L KCl was added to the mixed solution to 1 mL and the pH adjusted to 7.0 with 1 mol/L $NaHCO_3$ and incubated for 120 min on the rocking shaker (55 oscillations per minute) to obtain 100 mL of mixed solution.

One milliliter of mixed solution was mixed with MEM to obtain 10 mL of cell culture medium at pH 7.0 (pH was adjusted by 1 mol/L $NaHCO_3$). For Caco-2 cell culture, the prepared cell culture medium was transferred to the upper chamber, and cells were incubated for an additional 22 h before being harvested to measure protein and ferritin of cells.

pH Treatments. Three pH levels, pH 5.5, 6.5, and 7.6, were chosen to represent the gradual increase of pH in the intestinal lumen during digestion. Regular MEM was carefully titrated with either 0.1 mol/L HCl or 0.1 mol/L $NaHCO_3$ to obtain pH levels of 5.5, 6.5, and 7.6. Six milliliters medium was then supplemented with either 1 mL of 45 μm reduced iron or $FeSO_4$ acid solution (0.9 mg Fe were dissolved in 25 mL 18% HCl solution for 60 min on the rocking shaker), the pH adjusted to designed levels, and then made to a volume of 10 mL. For Caco-2 cell culture, the prepared cell culture medium was transferred to the upper chamber cells and incubated for an additional 22 h, then harvested to measure protein and ferritin of cells.

Analysis. The cells were harvested after 24 h in the intestinal digestion period. The Caco-2 cell protein was measured on samples that had been solubilized in 0.5 mol/L NaOH, using a semimicro adaptation of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). A one-stage, two-site immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-Iron II Ferritin Assay,

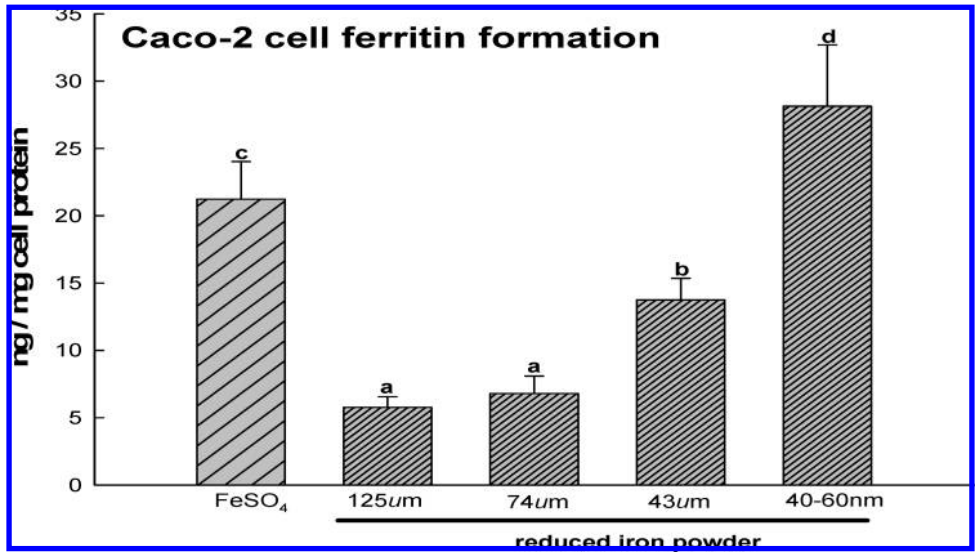

Figure 1. Measured variables of ferriitin formation of digests from reduced iron powder with different granularity and ferrous sulfate. Reduced iron powder with four granularity levels (125 μm, 74 μm, 43 μm, and 40–60 nm) was added to digests according to the iron concentration (16 μmol/L) of ferrous sulfate. Values represent the mean $\pm$ SEM ($n = 4$). Bar values with different letters are significantly different ($p < 0.05$).

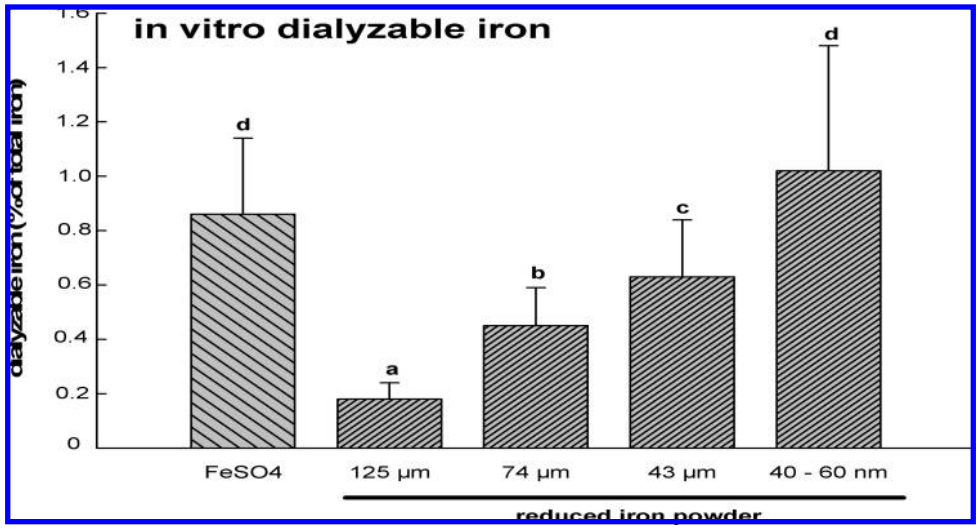

Figure 2. Percentage of *in vitro* dialyzable iron from reduced iron powders at different granularity levels and FeSO4. The percentage of dialyzable iron = amount of dialyzable iron (dialyzable iron of 1 mL supernatant $\times$ 100 mL)/0.9 mg (amount of added iron to digestion solution). The values represent the mean $\pm$ SEM, $n = 4$. Bar values with different letters are significantly different ($p < 0.05$).

RAMCO Laboratories, Houston, TX). A 10 uL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL of water was used for each ferritin measurement. Total iron content in digest solutions and in the bottom chamber was determined by inductively coupled argon plasma-Mass spectrometer (ICP-MS Agilent 7500a America).

Statistics. Statistical analysis of the data was performed using the software package DPS6.0. Statistical analysis was conducted according to the methods of Motulsky (*27*). Prior to analysis and when appropriate, data were log transformed to achieve equal variance. The figures were created using the software Sigmaplot10.0. ANOVA was performed with Tukey's post test to compare the various means of each series of experiments. Means were considered to be significantly different if p values were ≤ 0.05.

RESULTS

Ferritin Formation of Reduced Iron Powder at Selected Granularity Levels. Ferritin concentrations in Caco-2 cells treated with reduced iron powder at four granularity levels, 125 μm, 74 μm, 43 μm, and 40–60 nm, are shown in **Figure 1**. Iron uptake of reduced iron powder was increased with decreasing of granularity, which was in agreement with a previous study (*9*). Solubility of reduced iron powder was gradually increased with decreasing the powder size, which resulted in a gradual increase in the total amount of dialyzable iron (**Figure 2**). Compared to ferrous sulfate, iron bioavailability of reduced iron powder at 125 μm, 74 μm, and 43 μm granularity levels were significantly low in the intestinal digestion period, only 27.1%, 32%, and 64.8%, respectively. But iron uptake of 40–60 nm iron powder was comparable to $FeSO_4$, and its relative iron bioavailability was 132.5%, which indicated that the smaller the reduced iron powder size, the higher the bioavailability.

Effect of Ascorbic Acid and Phytic Acid on Ferritin Formation. Iron bioavailability was affected by many promoters and inhibitors in the daily diet. In general, ferritin formation of reduced iron powder was about 46.7% lower than that of $FeSO_4$ (**Figure 3**). When ascorbic acid was added to the Caco-2 cell culture system, cell ferritin formation was significantly improved, about 3.8-fold and 1.8-fold to iron powder and $FeSO_4$, respectively (**Figure 3**). However, in the presence of phytic acid,

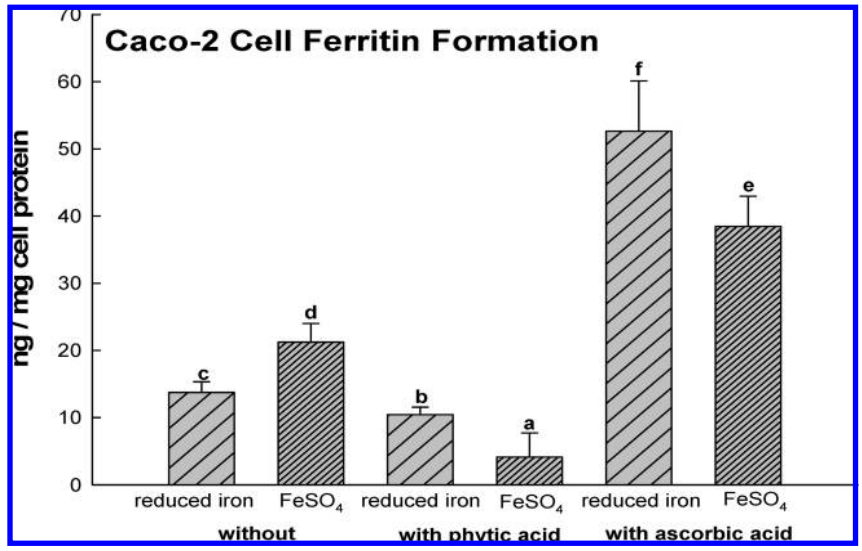

Figure 3. Caco-2 cell ferritin formation in response to different chemicals. Measured variables for digests containing reduced iron and $FeSO_4$ (16 μmol Fe/L digests) in CK and phytic acid (phytic acid, Fe = 5:1) or ascorbic acid (ascorbic acid, Fe = 5:1), respectively. The values represent mean ± SEM, $n = 4$. Bar values with different letters are significantly different ($p < 0.05$).

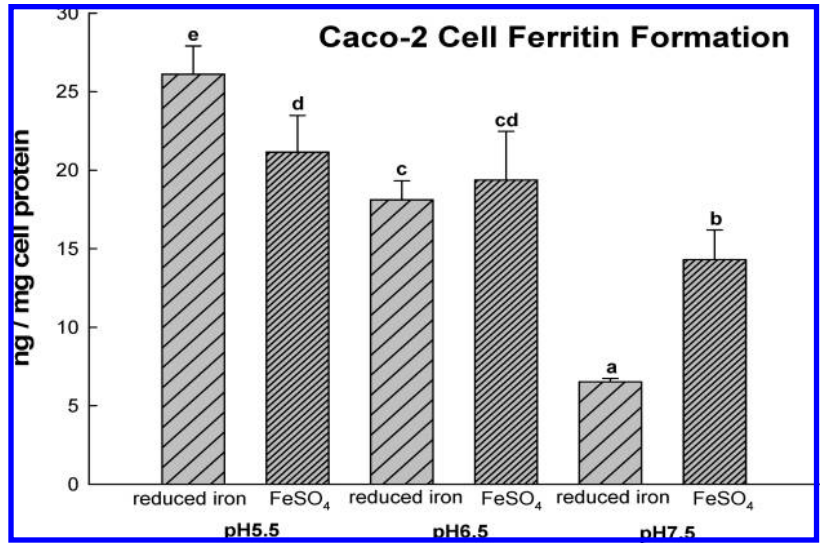

Figure 4. Ferritin formation at three pH levels of 5.5, 6.5, and 7.5. The final concentration was 16 μmol/L for all treatments. The values represent the mean ± SEM ($n = 4$). Bar values with different letters are significantly different ($p < 0.05$).

ferritin formation from the two iron sources was significantly inhibited, decreasing by 24% for iron powder and by 80.6% for $FeSO_4$ (**Figure 3**).

Ferritin Formation at Selected pH Levels. The ferritin formation response on pH in the medium was tested at three pH levels: 5.5, 6.5, and 7.5. Regardless of the iron sources, the patterns of ferritin formation were qualitatively comparable, decreased with increasing pH (**Figure 4**). At pH 5.5, ferritin formation in cells treated with reduced iron powder was significantly higher than that of $FeSO_4$ treatment. However, at pH 7.5, iron bioavailability of $FeSO_4$ in the intestinal digestion period appeared to be 2.2-fold that of iron powder (**Figure 4**).

DISCUSSION

Elemental iron powders are widely used as fortificants for their low reactivity and high stability. Studies of iron bioavailability from reduced iron powders have been preformed in Caco-2 cells (*28–30*), pigs (*30*), and humans (*29*). However, most researchers have been concerned about the extrapolation of the results of reduced iron bioavailability obtained from controlled studies to the actual absorption of fortified wheat or corn with commercial fortificants (*28*). Arredondo et al. (*28*) compared the iron bioavailability of 8 μm H-reduce iron and 45 μm H-reduce iron by Caco-2 cells and concluded that an inverse relationship existed between H-reduced particle size and iron bioavailability in Caco-2 cells. In agreement with their work, our results showed that iron bioavailability of reduced iron powder was enhanced with decreasing granularity and that iron absorption of 40–60 nm reduced iron powder was significantly higher than 45 μm and above the size. The solubility of iron powder gradually increased with decreasing granularity, resulted in easier desolution in digestion simulation and much higher bioavailability.

A lot of researchers have demonstrated that ascorbic acid is a promoter of iron bioavailability (*12, 32–34*). In the presence of ascorbic acid, ferritin formation of $FeSO_4$ was about 27% lower than that of reduced iron. We speculated that the high bioavailability of reduced iron powder is possibly due to the interaction of ascorbic acid with reduced iron. Fe^{2+}, for the reduced iron was simultaneously linked by a weak ligand of

ascorbic acid, and to increase the solubility of iron by stabilizing it from oxidation and precipitation at near neutral pH (*35, 36*). However, because $FeSO_4$ compounds decompose faster, at least some of the Fe^{2+} may be oxidized to Fe^{3+} before forming a complex with ascorbic acid.

Phytic acid is an exoteric inhibitor of iron absorption, which has been shown to inhibit iron uptake in humans (*4, 8, 37*). Phytic acid mostly binds Fe (II) but not others; therefore, it is possible that a majority of Fe^{2+} from $FeSO_4$ was chelated by phytic acid and only a few Fe^{2+} for uptake. Though phytic acid did decrease the uptake of iron from reduced iron, the ferritin formation from cells treated with reduced iron powder was significantly higher than that of $FeSO_4$. This finding supports the hypothesis that reduced iron was possibly absorbed by the enterocyte as free ions, because phytic acid is unlikely to affect reduced iron uptake if it is acquired as free ions via paracellular routes. Therefore, during the 22 h incubation in the presence of inhibitors such as phytic acid, the amount of inhibited iron from reduced iron was lower than that from $FeSO_4$ (**Figure 3**).

Iron uptake of cells from digested media with reduced iron powder was higher than those with $FeSO_4$ in similar pH conditions (about pH 5–6) of the duodenum, the most important part for iron uptake. The first step of dietary iron absorption is iron transport from the intestinal lumen into the enterocytes, which involves a membrane protein called divalent metal transporter (DMT-1) (*38–41*). DMT-1 appears to be specific for divalent cations. Because the active divalent metal transport process mediated by DMT-1 has been shown to be proton-coupled, it is no surprise that DMT-1-mediated metal transport is more effective as pH decreases (*38*), and iron uptake at pH 5.5 was the highest among all three pH levels tested (**Figure 4**). As pH increases, the efficiency of DMT-1 decreases, so does the solubility of reduced iron powder and $FeSO_4$, hence iron uptake decreased. Because $FeSO_4$ has a high solubility at pH 2.0−7.0, iron uptake of $FeSO_4$ is mainly influenced by DMT-1, and iron bioavailability of reduced iron powder might be influenced by primary solubility as well as the efficiency of DMT-1.

Generally, iron salts of food fortification have always formed a complex with phytic acid, and because the reduced iron does not link with phytic acid, a lot of Fe^{2+} is possibly released from iron-fortified food with reduced iron powder in the intestinal lumen. Therefore, we conclude that reduced iron powder is better than $FeSO_4$ as food fortification, especially at granularity that is fine enough.

ABBREVIATIONS USED

SIBS, Shanghai Institutes for Biological Sciences; CAS, Chinese Academy of Sciences; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; TEER, transepithelial electrical resistance; MEM, minimum essential medium; PIPES, piperazome-1,4-bis(2-ethanesulfonic acid); HEPES, [4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid; HBSS, Hank's buffered salt solution; ICP-MS, inductively coupled argon plasma-mass spectrometer; DMT-1, divalent metal transporter; ANOVA, analysis of variance.

ACKNOWLEDGMENT

We sincerely thank Professor Donald Oberlea from Texas Tech University of USA for his critical comments and English corrections on the manuscript.

LITERATURE CITED

(1) Beard, J.; Stoltzfus, R. J. Iron-deficiency anemia: Reexamining the nature and magnitude of the public health problem. *J. Nutr.* **2001**, *131*, 563S–703S.

(2) Forbes, A. L.; Arnaud, M. J.; Chichester, C. O.; Cook, J. D.; Harrison, B. N.; Hurrell, R. F.; Kahn, S. G.; Morris, E. R.; Tanner, J. T.; Whittaker, P. Comparison of in vitro, animal, and clinical determinations of iron bioavailability: International Nutritional Anemia Consultative Group Task Force report on iron bioavailability. *Am. J. Clin. Nutr.* **1989**, *49*, 225–238.

(3) Hurrell, R. F. Preventing iron deficiency through food fortification. *Nutr. ReV.* **1997**, *55*, 210–222.

(4) Hurrell, R. F. Fortification: Overcoming technical and practical barriers. *J. Nutr.* **2002**, *132*, 806S–812S.

(5) Whittaker, P.; Ali, S. F.; Imam, S. Z.; Dunkel, V. C. Acute toxicity of carbonyl iron and sodium iron EDTA compared with ferrous sulfate in young rats. *Regul. Toxicol. Pharmacol.* **2002**, *36*, 280–286.

(6) Willem, K.; Danielle, O. D. K.; Leo, A. In vitro iron availability from iron-fortified whole-grain wheat flour. *J. Agric. Food Chem.* **2004**, *52*, 8132–8136.

(7) Yeung, C. K.; Glahn, R. P.; Miller, D. D. Inhibition of iron uptake from iron salts and chelates by divalent metal cations in intestinal epithelial cells. *J. Agric. Food Chem.* **2005**, *53*, 132–136.

(8) Glahn, R. P.; Wortley, G. M.; South, P. K.; Miller, D. D. Inhibition of iron uptake by phytic acid, tannic acid, and ZnCl2: Studies using an in vitro digestion/Caco-2 cell model. *J. Agric. Food Chem.* **2002**, *50*, 390–395.

(9) *ASM. Powder Metal Technologies and Applications (ASM Handbook)*; ASM International: Novelty, OH, 1996; pp 826−828.

(10) Roe, M. A.; Fairweather-tait, S. T. High bioavailability of reduced iron added to UK flour. *Lancet* **1999**, *353*, 1938–1939.

(11) Glahn, R. P.; Lee, O. A.; Yeung, A.; Goldman, M. I.; Miller, D. D. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. *J. Nutr.* **1998**, *128*, 1555–1561.

(12) Yun, S.; Habicht, J. P.; Miller, D. D.; Glahn, R. P. An in vitro digestion/Caco-2 cell culture system accurately predicts the effects of ascorbic acid and polyphenolic compounds on iron bioavailability in humans. *J. Nutr.* **2004**, *134*, 2717–2721.

(13) Etcheverry, P.; Wallingford, J. C.; Miller, D. D.; Glahn, R. P. Calcium, zinc, and iron bioavailabilities from a commercial human milk fortifier: A comparison study. *J. Dairy Sci.* **2004**, *87*, 3629–3637.

(14) Glahn, R. P.; Wien, E. M.; Van Campen, D. R.; Miller, D. D. Caco-2 cell iron uptake from meat and casein digests parallels in vivo studies: Use of a novel in vitro method for rapid estimation of iron bioavailability. *J. Nutr.* **1996**, *126*, 332–339.

(15) Luten, J.; Crews, H.; Flynn, A.; van Dael, P.; Kastenmayer, P.; Hurrell, R. F.; Deelstra, H.; Shen, L.-H.; Fairweather Tait, S. J.; Hickson, K.; Farre, R.; Schlemmer, U.; Frohlich, W. Interlaboratorytrial on the determination of the *in* V*itro* iron dialysability from food. *J. Sci. Food Agric.* **1996**, *72*, 415–424.

(16) Miller, D. D.; Scricker, B. R.; Rasmussen, R. R.; Van Campen, D. A. An *in* V*itro* method for estimation of iron availability from meals. *Am. J. Clin. Nutr.* **1981**, *34*, 2248–2256.

(17) Miller, D. D.; Berner, L. A. Is solubility *in Vitro* a reliable predictor of iron bioavailability. *Biol. Trace Elem. Res.* **1989**, *19*, 11–24.

(18) Au, A. P.; Reddy, M. B. Caco-2 cells can be used to assess human iron bioavailability from a semipurified meal. *J. Nutr.* **2000**, *130*, 1329–1334.

(19) Garcia, M. N.; Flowers, C.; Cook, J. D. The Caco-2 cell culture system can be used as a model to study food iron availability. *J. Nutr.* **1996**, *126*, 251–258.

(20) Tallkvist, J.; Bowlus, C. L.; Lonnerdal, B. Functional and molecular responses of human intestinal Caco-2 cells to iron treatment. *Am. J. Clin. Nutr.* **2000**, *72*, 770–775.

(21) Alvarez-Hernadez, X.; Nichols, G. M.; Glass, J. Caco-2 cell line: A system for studying intestinal iron transport across epithelial cell monolayers. *Biochim. Biophys. Acta* **1991**, *1070*, 205–208.

(22) Alvarez-Hernadez, X.; Smith, M.; Glass, J. Regulation of intestinal iron uptake by transferrin in Caco-2 cells, an intestinal cell line. *Biochim. Biophys. Acta* **1994**, *1192*, 215–222.
(23) Nunez, M. T.; Alvarez-Hernandez, X.; Smith, M.; Tapia, V.; Glass, J. Role of redox systems on Fe^{3+} uptake by transformed human intestinal epithelial (Caco-2) cells. *Am. J. Physiol.* **1994**, *267*, C1582–1588.
(24) Nunez, M. T.; Nunez-Millacura, C.; Beltran, M.; Tapia, V.; Alvarez-Hernandez, X. Apotransferrin and holotransferrin undergo different endocytic cycles in intestinal epithelia (Caco-2. *J. Biol. Chem.* **1997**, *272*, 19425–19428.
(25) Hidalgo, I. J.; Kato, A.; Borchardt, R. T. Binding of epidermal growth factor by human colon carcinoma cell (Caco-2) monolayers. *Biochem. Biophys. Res. Commun.* **1989**, *160*, 317–324.
(26) Audus, K. L.; Bartel, R. L.; Hidalgo, I. J.; Borchardt, R. T. The use of cultured epithelial and endothelial cells for drug transport and metabolism studies. *Pharm. Res.* **1990**, *7*, 435–451.
(27) Motulsky, H. Comparing Three or More Means. In *Intuiti Ve Biostatistics*; Motulsky, H., Ed.; Oxford University Press: Oxford, NY, 1995; pp 255–262..
(28) Miguel, A.; Valentina, S.; Fernando, P.; Manuel, O. Smaller iron particle size improves bioavailability of hydrogen-reduced iron-fortified bread. *Nutr. Res.* **2006**, *26*, 235–239.
(29) Yeung, C. K.; Miller, D. D.; Cheng, Z. Q.; Glahn, R. P. Bioavailability of elemental iron powders in bread assessed with an in vitro digestion/Caco-2 cell culture model. *J. Food. Sci.* **2005**, *70*, S199–S203.
(30) Maekawa, A. A.; Glahn, R. P.; Lei, X. G.; Miller, D. D. Effect of bread baking on the bioavailability of hydrogen-reduced iron powder added to unenriched refined wheat flour. *J. Agric. Food Chem.* **2006**, *54*, 8362–8368.
(31) Tomas, W.; Fernando, P.; Erick, B.; Steven, A. A. The poor bioavailability of elemental iron in corn masa flour is not affected by disodium EDTA. *J. Nutr.* **2004**, *134*, 380–383.
(32) Hallberg, L.; Brune, M.; Rossander, L. The role of vitamin C in iron absorption. *Int. J. Vitam. Nutr. Res. Suppl.* **1989**, *30*, 103–108.
(33) Reddy, M. B.; Cook, J. D. Assessment of dietary determinants of nonheme-iron absorption in humans and rats. *Am. J. Clin. Nutr.* **1991**, *54*, 723–728.
(34) Zhu, L.; Raymond, P. G.; Yeung, C. K.; DENNIS, D. M. Iron uptake by Caco-2 cells from NaFeEDTA and FeSO4: Effects of ascorbic acid, pH, and a Fe (II) chelating agent. *J. Agric. Food Chem.* **2006**, *54*, 7924–7928.
(35) Hallberg, L.; Brune, M.; Rossander, L. Effect of ascorbic acid on iron absorption from different types of meals. Studies with ascorbic-acid-rich foods and synthetic ascorbic acid given in different amounts with different meals. *Hum. Nutr. Appl. Nutr.* **1986**, *40*, 97–113.
(36) Hopping, J. M.; Ruliffson, W. S. Effect of chelating agents on radioiron absorption and distribution in rats in vivo. *Am. J. Physiol.* **1963**, *205*, 885–889.
(37) Glahn, R. P.; Cheng, Z.; Welch, R. M.; Gregorio, G. B. Comparison of iron bioavailability from 15 rice genotypes: Studies using an in vitro digestion/Caco-2 cell culture model. *J. Agric. Food Chem.* **2002**, *50*, 3586–3591.
(38) Gunshin, H.; Mackenzie, B.; Berger, U. V.; Gunshin, Y.; Romero, M. F.; Boron, W. F.; Nussberger, S.; Gollan, J. L.; Hediger, M. A. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature.* **1997**, *388*, 482–488.
(39) Anderson, G. J.; Frazer, D. M. Recent advances in intestinal iron transport. *Curr. Gastroenterol. Rep.* **2005**, *7*, 365–372.
(40) Flanagan, P. R. Mechanisms and regulation of intestinal uptake and transfer of iron. *Acta Paediatr. Scand. Suppl.* **1989**, *361*, 21–30.
(41) Fleming, M. D.; Trenor, C. C., III; Su, M. A.; Foernzler, D.; Beier, D. R.; Dietrich, W. F.; Andrews, N. C. Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nat. Genet.* **1997**, *16*, 383–386.
(42) Oikeh, S. O.; Menkir, A.; Maziya-Dixon, B.; Welch, R.; Glahn, R. P. Assessment of concentrations of tron and zinc and bioavailable iron in grains of early-maturing tropical maize varieties. *J. Agric. Food Chem.* **2003**, *51*, 3688–3694.
(43) Lucca, P.; Harrell, R.; Potrykus, I. Fighting iron deficiency anemia with iron-rich rice. *J. Am. Coll. Nutr.* **2002**, *21*, 182S–190S.

Received for review October 22, 2007. Revised manuscript received January 5, 2008. Accepted January 25, 2008. This study was financially supported by Key International Cooperative Project from the Science and Technology Ministry of China (2006DFA31030), Harvestplus-China (#8022), Project from Zhejiang Province (#2006C32019), and Program for Changjiang Scholars and Innovative Research Team in University (#IRT0536).

JF0730946