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A comparison of iron availability from commercial iron preparations using an in vitro digestion/Caco-2 cell culture model

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The objectives of this study were to compare iron availability from commercial preparations of $FeSO_4$, ferrous gluconate, ferrous fumarate, and a polysaccharide-iron complex using an in vitro digestion/Caco-2 cell culture model. In addition, we sought to determine if calcium carbonate and calcium acetate (common phosphate binding agents) inhibited iron availability from an oral iron supplement when digested simultaneously. Caco-2 cell ferritin formation following exposure to simulated gastric and intestinal digests of the iron supplements was used as a measure of iron uptake and availability. Plates without cell monolayers were included in each replication of the experiment to measure the total amount of soluble iron that resulted from the in vitro digestion. Significantly more iron was taken up from the $FeSO_4$, ferrous gluconate, and ferrous fumarate than the polysaccharide-iron complex. Similar results comparing $FeSO_4$ and the polysaccharide-iron complex have been observed in humans. In addition, less iron was taken up from digests with calcium carbonate relative to calcium acetate even though similar amounts of soluble iron were observed in these experiments. The results indicate that when iron supplements and phosphate binders are consumed simultaneously, calcium acetate may be the preferred phosphate binder to maximize iron availability. (J. Nutr. Biochem. 11:62–68, 2000)

Keywords: iron; availability; calcium acetate; calcium carbonate; Caco-2

Introduction

We have utilized the Caco-2 cell line in conjunction with in vitro digestion techniques and have developed a model whereby foods undergo simulated peptic digestion followed by intestinal digestion in the presence of Caco-2 cell monolayers.[1,2] This model both measures iron solubility and provides a measure of uptake via a living component, the Caco-2 cell monolayer (*Figure 1*). This system is a significant advancement over use of in vitro digestion alone, which only measures iron solubility and therefore is not a complete measure of iron availability.[1,3] Recent studies with this model have demonstrated that Caco-2 formation of ferritin, the intracellular iron storage protein, occurs in response to iron uptake and can be used as a measure of cell iron uptake.[2] Ferritin formation is easily measured via radioimmunoassay, thus eliminating the need for radiolabeling of the food iron. This model system is a unique tool, capable of conducting experiments that may not be feasible or practical to conduct in vivo. When used as a prelude to human trials, this model may enable improved design and productivity of the more expensive human experiment.

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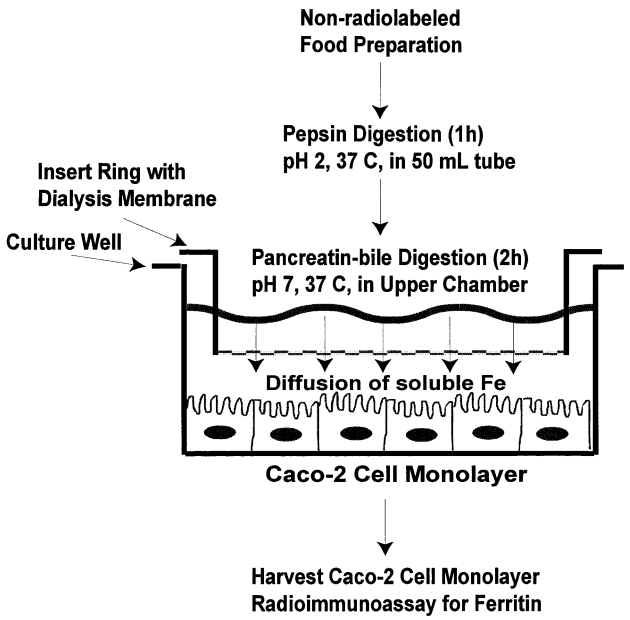

Figure 1 Diagram of in vitro digestion/Caco-2 cell culture model. This diagram represents a single well within a multiwell (six-well) culture plate.

In developed countries, iron fortification of foods has effectively alleviated iron deficiency; however, certain populations require iron supplementation as a result of a physiologic condition or disease state. Pregnant women and people with renal disease requiring dialysis are examples of those requiring supplementation. The effectiveness of iron supplementation is limited by iron availability and unpleasant side effects of high-dose (>200 mg) supplementation. In this regard, there is considerable incentive to develop iron supplements with improved availability.

Current oral iron supplementation regimens are unable to provide iron stores adequate to reverse anemia in dialysis patients. Factors contributing to iron deficiency in renal failure are impaired iron absorption (due to altered diet, interference from other medications, and possibly undefined factors specific to the uremic state), loss of blood (through the dialysis procedure, phlebotomy, and increased bleeding through anticoagulation), and relative iron resistance (erythrogenesis is not maintained and anemia is not reversed in dialysis patients until iron stores are greater than normal). Because of these factors it is unusual to maintain adequate iron stores in dialysis patients without intermittent intravenous iron supplementation or red blood cell transfusions. At best, oral iron supplementation merely delays the need for intravenous iron therapy. In fact, recently published and widely adopted guidelines advocate for the routine use of intravenous rather than oral iron supplementation.[4] Following these guidelines, it is clear that iron stores adequate to correct renal failure associated anemia can be maintained with intravenous iron supplementation. Cost is also an issue because intravenous iron administration is many times more expensive than oral iron. If the factors responsible for inadequate oral iron absorption could be identified and resolved, adequate iron stores and hematocrit could be maintained in dialysis patients without the risks and expense of intravenous iron. The technique described in this article provides a method for addressing some of the factors responsible for inadequate enteric iron absorption.

The objectives of the present study were to compare iron availability of four common iron supplements. The forms of iron were $FeSO_4$, ferrous gluconate, ferrous fumarate, and a polysaccharide-iron complex. In addition, because people with renal failure must limit phosphorous intake and absorption, we sought to determine if simultaneous consumption (i.e., digestion) of phosphate binding agents (calcium carbonate and calcium acetate) affects iron availability from $FeSO_4$.

Material and methods

Chemicals, enzymes, and hormones

Unless otherwise stated, all chemicals, enzymes, and hormones were purchased from Sigma Chemical Co. (St. Louis, MO USA). The commercial iron preparations used in this study were: $FeSO_4$ (65 mg iron per tablet, Geneva Pharmaceuticals, Broomfield, CO USA); ferrous gluconate (Fergon, 36 mg iron per tablet, Bayer Corporation, Myerstown, PA USA); ferrous fumarate (Nephro-Fer, 115 mg iron per tablet, R & D Laboratories, Inc., Marina del Rey, CA USA); and polysaccharide-iron complex (Niferex, 150 mg iron per capsule, Central Pharmaceuticals, Inc., Seymour, IN USA). For some experiments, preparations of calcium carbonate (Tums, SmithKline Beecham, Pittsburgh, PA USA) and calcium acetate (PhosLo, Braintree Laboratories, Braintree, MA USA) were used to investigate the effects of these products on iron availability.

Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD USA) at passage 17, and used in experiments at passage 25-33. Cells were seeded at a density of 50,000 cells/cm^2 in collagen-treated six-well plates (Costar Corp., Cambridge, MA USA). The cells were grown in Dulbecco's Modified Eagle Medium (GIBCO, Grand Island, NY USA) with 10% v/v fetal calf serum (GIBCO), 25 mmol/L HEPES, and 1% antibiotic antimycotic solution (GIBCO). The cells were maintained at 37°C in an incubator with a 5% carbon dioxide (CO_2)/95% air atmosphere at constant humidity, and the medium was changed every 2 days. The cells were used in the iron uptake experiments at 13 days postseeding. Under these conditions, the amount of cell protein measured in each well was found to be highly consistent from well to well within each culture plate.

Preparation of iron supplements for in vitro digestion

Because the iron supplements used in this study were in tablet or capsule form, it was necessary to solubilize the tablet or capsule contents in 250 mL of 0.2 mol/L HCl immediately prior to start of the peptic digestion. In addition, because the supplements contained different amounts of iron per tablet, the volume used from each preparation was adjusted to achieve 42 μg iron from each form of iron per digest. The total volume of each digest was 15 mL at the time when 1.5 mL aliquots of each were placed in the upper chamber of the digestion plate; thus, the iron concentration in each digest was 50 μmol/L. For each replication of the experiment, a new tablet or capsule was prepared.

In vitro digestion

Porcine pepsin (Sigma #P-7000, 800–2,500 units/mg protein), pancreatin (Sigma # P-1750, activity = 4 × U.S.P. specifications), and bile extract (Sigma # B-8631, glycine and taurine conjugates of hyodeoxycholic and other bile salts) were used. Further preparation of the pepsin, pancreatin, and bile extract was performed as follows. Shortly before use, 0.2 g pepsin was dissolved in 5 mL 0.1 mol/L HCl, added to 2.5 g of Chelex-100 (Catalogue #142-2842, Bio-Rad Laboratories, Hercules, CA USA), and shaken on a rotating titer plate tabletop shaker (Lab Line Instruments, Melrose Park, IL USA) for 30 minutes. The pepsin solution with Chelex was then poured into a 1.6 cm diameter filtration column to filter out the Chelex from the pepsin solution. An additional 5 mL of 0.1 mol/L HCl was added to the column and the filtrate collected into the pepsin solution. The final total volume of the eluted pepsin solution was 8 mL.

For intestinal digestion, 0.05 g pancreatin and 0.3 g bile extract were dissolved in 25 mL of 0.1 mol/L $NaHCO_3$. Chelex-100 (12.5 g) was added and the resulting mixture was shaken for 30 minutes on the rotating titer plate tabletop shaker. The mixture was then poured into a 1.6 cm diameter filtration column to filter out the Chelex. An additional 10 mL of 0.1 mol/L $NaHCO_3$ was added to the column and the filtrate collected in the pancreatin/bile solution. The final total volume of the pancreatin/bile solution was 27 mL. Treatment of the pepsin and pancreatin-bile solutions via the methods described above did not affect the activity of the enzymes.

Peptic and intestinal digestions were carried out on a rocking platform shaker (Reliable Scientific, Inc., Hernando, MS USA) in an incubator at 37°C with a 5% CO_2/95% air atmosphere maintained at constant humidity. The intestinal digestion was carried out in the upper chamber of a two-chamber system in six-well plates, with the cell monolayer attached to the bottom surface of the lower chamber (*Figure 1*). The upper chamber was formed by fitting the bottom of an appropriately sized Transwell® insert ring (Costar Corp., Cambridge, MA USA) with a 15,000 molecular weight cut-off dialysis membrane (Spectra/Por 2.1, Spectrum Medical Industries, Inc., Gardena, CA USA). The membranes were soaked in deionized water prior to use. The dialysis membrane was held in place with a silicone ring (item no. 2-215-S604, Web Seal, Inc., Rochester, NY USA). After fastening the dialysis membrane to the insert ring, the entire unit was sterilized in 70% ethanol and then kept in sterile water until use.

To start the peptic digestion, a sample of each solubilized iron supplement was placed in a 50 mL screw cap culture tube containing 10 mL of 140 mmol/L NaCl, 5 mmol/L KCl at pH 2.0 (adjusted with 1.0 mol/L HCl). Then, 0.5 mL of the pepsin solution was added. The tube was capped, placed horizontally, and incubated for 60 minutes on the rocking shaker and rocked at speed no. 7 (55 oscillations/min). For the intestinal digestion step, the pH of the sample (also referred to as the "digest") was raised to pH 6 by adding 1 mol/L $NaHCO_3$ dropwise. Then 2.5 mL of the pancreatin-bile extract mixture was added. The pH was adjusted to pH 7 with NaOH, and the volume was brought to 15 mL with 120 mmol/L NaCl, 5 mmol/L KCl.

Preparation of the six-well culture plates with cell monolayers

Immediately before the intestinal digestion period, the growth medium was removed from each culture well and the cell layer was washed twice with 37°C minimum essential media (MEM, #41500; GIBCO) at pH 7. This MEM was chosen because it contained no added iron, and upon formulation with the following ingredients, was always found to contain less than 8 μg iron/L. The MEM was supplemented with 10 mmol/L PIPES (piperazine-N,N′-bis-[2-ethanesulfonic acid]), 1% antibiotic-antimycotic solution (Sigma #A-9909), hydrocortisone (4 mg/L), insulin (5 mg/L), selenium (5 μg/L), triiodothyronine (34 μg/L), and epidermal growth factor (20 ug/L). A fresh 1.0 mL aliquot of MEM covered the cells during the experiment. A sterilized insert ring, fitted with dialysis membrane, was then inserted into the well, thus creating the two-chamber system. Then, a 1.5-mL aliquot of the intestinal digest was pipetted into the upper chamber. The plate was covered and incubated on the rocking shaker at 6 oscillations per minute for 120 minutes.

When the intestinal digestion was terminated, the insert ring and digest were removed. The solution in the bottom chamber was allowed to remain on the cell monolayer and an additional 1 mL of MEM was added to each well. The cell culture plate was then returned to the incubator for an additional 22 hours, after which the cells were harvested for analysis.

To accurately determine the amount of iron that diffused into the bottom chamber during the intestinal digestion period, plates without cells were used and treated identically to those with cells for each replication of the experiment. At the end of the intestinal digestion period, the entire volume of solution in the bottom chamber was collected for measurement of total iron.

Harvesting of Caco-2 cell monolayers for ferritin analysis

Exactly 24 hours after the start of the intestinal digestion period, the cell monolayers were harvested. To harvest the cells, the media covering the cells was removed and the cells washed once with a 2 mL volume of a "rinse" solution containing 140 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol PIPES, at pH 7. After rinsing, 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 minutes 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.

Experimental design

Two series of experiments are presented in this article. The first series of experiments was designed to compare the iron availability from the various forms of iron; therefore, simulated digestion of each sample preparation containing identical amounts of iron (42 μg) were conducted. A "blank" digest (i.e., no added iron) and a digest containing $FeSO_4$ ($FeSO_4 \cdot 7H_2O$, laboratory grade, Mallinckrodt, Inc., Paris, KY USA) with ascorbic acid (vitamin C) at a concentration of 1 mmol/L were included as a baseline and positive control, respectively. The second series of experiments was designed to determine the effects of the phosphate binding compounds calcium carbonate and calcium acetate on iron availability. For these studies, a $FeSO_4$ tablet (same as in first series of experiments) was solubilized in 250 mL of 0.1 M HCL with and without the addition of two tablets of calcium carbonate or calcium acetate. A 1:2 ratio of $FeSO_4$ tablets to calcium carbonate or calcium acetate tablets was used because it is a common prescription consumed by patients receiving dialysis therapy. Samples of each preparation were then subjected to the digestion/uptake procedure. As in the first series of experiments, the digests were designed to contain 42 μg iron. For both series of experiments, measurements of the digest iron concentration were conducted to confirm similar iron concentrations. Digests containing only calcium carbonate and calcium acetate also were prepared, as was a blank digest and $FeSO_4$ plus vitamin C digest.

Analyses

All glassware used in the sample preparation and analyses was acid-washed. Caco-2 cell protein was measured on samples that had been solubilized in 0.5 mol/L NaOH, using a semi-micro adaptation of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories). A one-stage, two-site immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-Iron II Ferritin Assay, RAMCO Laboratories, Houston, TX USA). A 10 μL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL of water, was used for each ferritin measurement. Pilot studies had determined that centrifugation of the Caco-2 cell sample prior to sampling was not necessary for accurate ferritin measurement. Analysis of the iron content of the experimental solutions and digests were conducted using an inductively coupled plasma emission spectrometer (ICAP Model 61E Trace Analyzer, Thermo Jarrell Ash Corporation, Franklin, MA USA).

Statistics

Statistical analysis of the data was performed using the software package GraphPad Prism™ (GraphPad Software, San Diego, CA USA). Statistical analyses were conducted according to the methods of Motulsky.[5] Prior to analysis, data were log transformed to achieve equal variance. As each replication of an experiment in our study was a paired comparison, a repeated measures analysis of variance was performed with the Tukey's post-test to compare the various means of each series of experiments. Means were considered significantly different if *P*-values were less than or equal to 0.05. Variance within treatment groups are expressed as standard error of the mean (SEM).

Results

A comparison of iron availability from the various supplements is summarized in *Figure 2*. Relative to the blank digest, the bottom chamber of the $FeSO_4$, ferrous gluconate, and ferrous fumarate digests contained approximately four to six times as much iron at the end of the intestinal digestion period (0.41 ± 0.08 μg iron, 0.59 ± 0.08 μg iron, 0.38 ± 0.04 μg iron, and 0.10 ± 0.02 μg iron; mean ± SEM, $n = 4$, for the $FeSO_4$, ferrous gluconate, ferrous fumarate, and blank digests, respectively). No significant increase in bottom chamber iron was observed for the polysaccharide-iron complex digest. The digest of $FeSO_4$ plus ascorbic acid contained the greatest amount of iron in the bottom chamber, which was significantly greater than that of the $FeSO_4$ and ferrous fumarate digests, but not significantly different from the ferrous gluconate digest.

Caco-2 cell ferritin formation was highest for the $FeSO_4$ plus ascorbic acid digest, with a value two to three times that of the $FeSO_4$, ferrous gluconate, and ferrous fumarate digests (374 ± 31 ng ferritin/mg cell protein, 102 ± 17 ng ferritin/mg cell protein, 135 ± 29 ng ferritin/mg cell protein, and 117 ± 17 ng ferritin/mg cell protein, for the $FeSO_4$ plus ascorbic acid, $FeSO_4$, ferrous gluconate, and ferrous fumarate digests, respectively). No significant difference in ferritin formation was observed between the $FeSO_4$, ferrous gluconate, and ferrous fumarate digests; however, ferritin formation from digests of these iron supplements was approximately three to four times greater than that of the polysaccharide-iron complex digest (32 ± 3 ng ferritin/mg cell protein). Relative to the blank digest (11 ± 2 ng ferritin/mg cell protein), ferritin formation from the polysaccharide-iron complex digest was significantly higher.

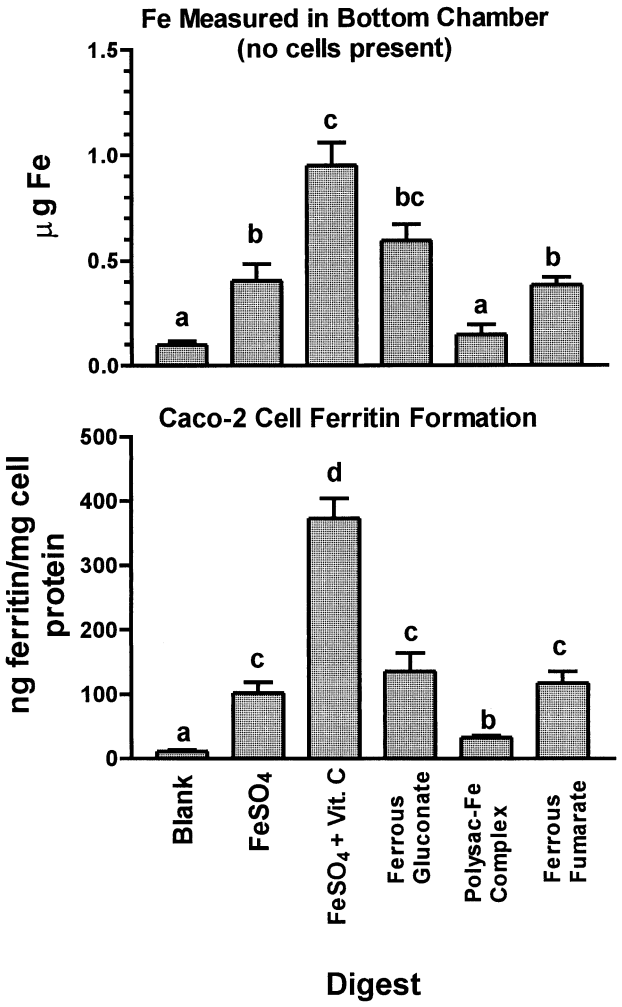

Figure 2 Comparison of iron availabilty from digests of $FeSO_4$, $FeSO_4$ plus ascorbic acid (Vit. C), ferrous gluconate, ferrous fumarate, and a polysaccharide-iron complex (Polysac-Fe). Digest iron concentrations (42 μg iron per 15 mL) were identical for all forms of iron. For each form of iron, 1.5 mL of the intestinal digest (4.2 μg iron) was placed in the upper chamber. The lower panel represents Caco-2 cell ferritin formation 24 hours after the start of the intestinal digestion period. The upper panel represents the amount of iron in the bottom chamber (no cells present) at the end of the 2-hour intestinal digestion period. Values (mean ± SEM; $n = 4$) with no letters in common are significantly different ($P < 0.05$).

The effects of combining calcium carbonate or calcium acetate with $FeSO_4$ are summarized in *Figure 3*. No significant difference was observed in the amount of bottom chamber iron for the digests of $FeSO_4$, $FeSO_4$ plus calcium carbonate, and $FeSO_4$ plus calcium acetate (0.66 ± 0.20 μg iron, 0.50 ± 0.09 μg iron, and 0.62 ± 0.11 μg iron, respectively). As in the first series of experiments, the amount of bottom chamber iron was greatest for the $FeSO_4$ plus ascorbic acid digest (1.51 ± 0.10 μg iron), which served as a positive control. As additional controls, digests of calcium carbonate and calcium acetate without iron were conducted. Bottom chamber iron for the calcium carbonate (0.06 ± 0.01 μg iron) and calcium acetate (0.12 ± 0.05 μg

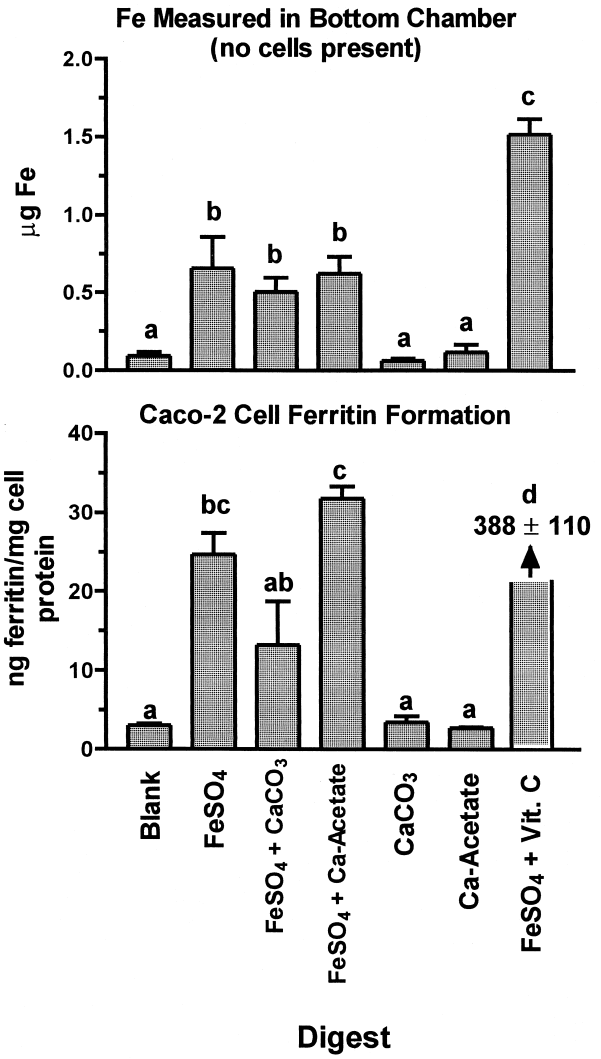

Figure 3 Comparison of iron availability from digests of $FeSO_4$, $FeSO_4$ plus $CaCO_3$, $FeSO_4$ plus Ca-acetate, $CaCO_3$, Ca-acetate, and $FeSO_4$ plus ascorbic acid (Vit. C). Digest iron concentrations (42 μg iron per 15 mL) were identical for all forms of iron. For each form of iron, 1.5 mL of the intestinal digest (4.2 μg iron) was placed in the upper chamber. The lower panel represents Caco-2 cell ferritin formation 24 hours after the start of the intestinal digestion period. The upper panel represents the amount of iron in the bottom chamber (no cells present) at the end of the 2-hour intestinal digestion period. Values (mean ± SEM; $n = 3$) with no letters in common are significantly different ($P < 0.05$).

iron) were not different from the blank digest (0.09 ± 0.02 μg iron).

Caco-2 cell ferritin formation for the calcium carbonate and calcium acetate digests were not different from the blank digest (3.4 ± 0.8 ng ferritin/mg cell protein, 2.8 ± 0.1 ng ferritin/mg cell protein, and 3.1 ± 0.2 ng ferritin/mg cell protein, respectively). Ferritin formation for the $FeSO_4$ plus calcium carbonate digest was highly variable (13.2 ± 5.5 ng ferritin/mg cell protein) and thus not significantly different from the blank, $FeSO_4$, calcium carbonate, and calcium acetate digests, but was significantly less than the $FeSO_4$ plus calcium acetate digest (31.8 ± 1.5 ng ferritin/mg cell protein). The ferritin formation of the $FeSO_4$ digest (24.7 ± 2.7 ng ferritin/mg cell protein) was not different from that of the $FeSO_4$ plus calcium carbonate or $FeSO_4$ plus calcium acetate digests. As expected, the $FeSO_4$ plus ascorbic acid digest (388 ± 110 ng ferritin/mg cell protein) was significantly greater than all of the above digests.

Discussion

The time and costs associated with measuring iron absorption in vivo have limited the number and scope of investigations of iron availability from foods and iron supplements. Thus, there has been great demand for an adequate in vitro model that is representative of the conditions found in the intestinal lumen and capable of estimating iron availability. Iron availability from foods, supplements, and interactions can be investigated initially in vitro, resulting in enhanced productivity from the more expensive in vivo trials that remain the standard for nutritional studies. Many of the important questions that are not feasible to address in vivo can be explored in an in vitro model.

The in vitro digestion/Caco-2 cell culture model developed by Glahn and co-workers[1,6] has been accepted as a valuable tool for studies of iron availability. In this model system, factors known to enhance iron availability, such as meat and ascorbic acid, have been documented. In addition, factors that inhibit iron uptake, such as inositol phosphates and iron chelates, have also been reproduced.[2,7,8] Thus, there is confidence that results observed in this model system will be reflected in human studies. However, it is important to keep in mind that this model system only measures iron uptake and is designed to be used as a screening tool. Effects that occur in vivo at levels other than uptake, such as the intracellular handling or transcellular transfer of iron, would not be detectable in this model system. Human studies measure the sum of the various components of the entire absorption process; therefore, continued application of this model system in conjunction with human trials is necessary to define the degree to which this system can be useful.

In the present study, the results indicate that the iron in the $FeSO_4$, ferrous gluconate, and ferrous fumarate were equal in availability (*Figure 2*). As expected, addition of ascorbic acid enhanced the iron availability of $FeSO_4$. The polysaccharide-iron complex was significantly less available than all of the other forms of iron. These results agree with a human study that documented higher iron uptake in healthy human subjects from $FeSO_4$ relative to the specific polysaccharide-iron complex used in this study.[9] Interestingly, in this human study, absorption of iron was poor from both compounds for patients on continuous ambulatory peritoneal dialysis. Thus, from an availability standpoint, the results using the in vitro model may not reflect iron availability under certain disease states, unless it is possible to alter the in vitro model to match the physiology of the condition in question.

The exact chemical nature of the polysaccharide-iron complex has been examined. Mossbauer spectroscopy and X-ray powder diffraction indicate that the iron-rich core of this compound is similar to the mineral akagenéite.[10] Akagenéite is a polymorphous mineral with feroxyhyte, goethite, and lepridocite that is commonly found in mines

and meteorites and considered to be formed in flight or by alteration.[11] Iron in this compound exists as FeOH and $FeCl_3$. Given what is currently known of factors that enhance iron availability, there do not appear to be any characteristics present in the above complex that suggest that it should be more available than the other forms used in this study.

Most patients on dialysis therapy require iron supplementation and many report gastrointestinal discomfort associated with oral iron therapy.[12,13] Numerous studies report improved tolerance of particular iron supplements, yet a common opinion among clinicians is that it is the amount of iron ingested and not the form of iron that is primarily responsible for adverse side effects.[13–15] Thus, there remains considerable demand for an iron supplement that is highly available and that would result in better maintenance of iron status while decreasing gastrointestinal discomfort as a result of less ingested iron. $FeSO_4$ in the presence of excess ascorbic acid represents an attractive form of iron to achieve this goal; however, stability of the ascorbic acid is likely to be a problem.[16,17] Inclusion of ascorbic acid within an iron supplement may not be necessary, because an ascorbic acid supplement could easily be ingested simultaneously with the iron supplement. Additional research should be done to determine if supplementation with ascorbic acid improves iron absorption in patients with renal insufficiency. Alternatively, heme iron may be an attractive alternative to nonheme iron supplements because this form of iron may produce fewer side effects.[14]

Many studies document the inhibitory effects of calcium on iron absorption in humans.[18,19] Although several of the studies are conflicting, the balance of evidence clearly indicates that calcium present at levels common in meals and from ingestion of supplements inhibits the absorption of heme and nonheme iron.[18]

In the present study, uptake of iron from the digest containing calcium carbonate was significantly less than from the digest with calcium acetate (*Figure 3*). During solubilization of the $FeSO_4$ tablets and the subsequent addition of the calcium tablets, we observed that the calcium carbonate neutralized the acid of the digest whereas the calcium acetate did not affect the pH of the pepsin digest; thus, the digest with calcium carbonate was more likely to be in the ferric versus ferrous state and less available. These results suggest that for patients who wish to maximize iron uptake and must ingest a phosphate binder with their iron supplement, calcium acetate would be preferred.

One report in the literature indicates a dose effect relationship between the amount of calcium chloride ingested and the degree of inhibition of iron absorption.[20] In that study, 3.8 mg of radiolabeled iron was fed to human subjects in wheat rolls with or without various amounts of calcium. The researchers observed maximal inhibition of iron absorption (50–60%) when greater than 300 mg calcium was consumed with the radiolabeled iron meal. The same amount of calcium also inhibited heme iron absorption, suggesting that the effect of calcium is at least partially related to the mucosal transfer of iron and is not merely an effect on mucosal iron uptake. Interestingly, the authors also observed that as little as 40 mg of calcium added to the wheat roll dough reduced phytate degradation during fermentation and baking, resulting in higher phytate levels that corresponded to inhibition of iron absorption. This study is evidence that calcium can affect iron absorption at both uptake and transfer stages of the absorption process, and that the amount of calcium determines not only the degree but the mechanism of inhibition of iron absorption. In our current model system, we are not able to measure transfer of iron across the mucosal cell. Our results only reflect the effects of calcium on mucosal iron uptake.

In the present study, the ratio of calcium to iron in each digest was 15.4:1 and 5.1:1 for the calcium carbonate and calcium acetate digests, respectively. These ratios are within a range that has been shown to inhibit iron absorption in humans.[20] These amounts were chosen based on a commonly prescribed regimen for iron supplementation and intake of the phosphate binders. For example, most dialysis patients are on a multitude of medicines that are to be taken four times per day. In practical terms, this usually means immediately before each meal and at bedtime. Thus, a $FeSO_4$ tablet containing 65 mg iron is commonly ingested twice daily with two to three tablets of calcium carbonate (500 mg calcium/tablet) or calcium acetate (332 mg calcium/tablet). Given the above conditions, our results suggest that calcium does not interfere with iron uptake, but that the anion of the calcium salt may affect iron availability. For example, the acid-neutralizing capacity of the calcium carbonate alone could account for the difference in iron availability observed in *Figure 3*. The above statements should be noted in the context of human studies, where calcium is suspected to inhibit iron absorption at the intracellular transfer step.[18] Thus, one must be cautious in considering the calcium to iron ratio and aware of the total amount of calcium in the meal or supplement when discussing iron availability in the presence of dietary calcium.

Other studies have been conducted that investigated the effects of different calcium salts on iron absorption. In a study of 61 healthy human subjects, the effects of calcium carbonate, calcium citrate, and calcium phosphate on the absorption of nonheme iron were examined.[19] When taken without food, calcium carbonate did not inhibit the absorption of ferrous sulfate with doses of 300 mg calcium and 37 mg iron or 600 mg calcium and 18 mg iron. Calcium citrate and calcium phosphate reduced iron absorption by 49% and 62%, respectively, at levels of 600 mg calcium and 18 mg iron. All of the calcium supplements inhibited absorption of the iron supplement when ingested with food, and in addition were found to inhibit iron absorption of the nonheme iron present in the food. The authors concluded that no adverse effects of iron absorption will occur if calcium carbonate is used and is taken between meals. In addition, they concluded that if taken regularly with meals, calcium supplements may make it more difficult to meet daily iron requirements. In dialysis patients, calcium carbonate or calcium acetate must be ingested with the meal if they are going to function as a phosphate binder. Unfortunately, this represents another inhibitory effect on iron absorption in this patient population.

Although the inhibitory effects of calcium on human iron absorption appear to be well established, considerable variation in the effects of calcium exist. Results have varied from no effect to significant inhibition of up to 62%.[18,19]

Complicating factors across studies include different ratios of calcium to iron, different forms of calcium, and conditions such as whether the calcium is ingested with or without food. In reviewing the literature we did not find any studies that attempted to separate out the effects of the calcium salt anion on iron uptake. For example, citrate is an excellent iron chelate and evidence exists that under certain conditions, increased levels of iron chelates such as citrate inhibit mucosal iron uptake.[1] Alternatively, the acid neutralizing effects of calcium carbonate may play a role in reducing iron solubility.

Given the nature of iron bioavailability studies and the endless combination of food conditions that exist, it is likely that it will be some time before we have a thorough understanding of the effects of calcium on iron absorption. Moreover, underlying disease states such as renal failure further complicate the nutritional considerations of iron deficiency and calcium intake, because the patient with end-stage renal disease may have an altered iron absorption process relative to normal or merely iron-deficient subjects.

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