In Vitro Digestion/Caco-2 Cell Culture Model to Determine Optimal Ascorbic Acid to Fe Ratio in Rice Cereal

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ABSTRACT
The objectives were to determine if adding ascorbic acid (AA) to rice cereal enhanced iron availability, and if so, the best ratio of AA to Fe to maximize Fe availability. Also, we sought to determine whether mixing rice cereal with apple juice fortified with AA, compared to mixing it with water, would increase Fe availability. An in vitro digestion/cell culture model was used. Iron availability was increased at an AA:Fe molar ratio of 0.8:1 and maximal at 1.6:1. Mixing apple juice with rice cereal to approximate an AA:Fe ratio of 1.2:1 did not enhance Fe availability. Results indicate that factors in the apple juice offset any enhancement effects of AA on Fe availability.

Key Words: cereal, ascorbic acid, iron, rice, Caco-2

INTRODUCTION
We utilized the Caco-2 cell line in conjunction with in vitro digestion to develop a model whereby foods undergo simulated peptic digestion followed by intestinal digestion in the presence of Caco-2 cell monolayers (Fig. 1; Glahn et al., 1996, 1998a,b). This model measures iron solubility in addition to uptake via a Caco-2 cell monolayer. This model was a notable advancement over use of in vitro digestion alone, which measures iron solubility and therefore is not a complete measure of Fe availability (Glahn et al., 1998a; Miller and Berner, 1989). Published results have demonstrated that formation of ferritin, the intracellular Fe storage protein, occurs in response to Fe uptake and provides a measure of cell Fe uptake (Glahn et al., 1998b). Ferritin formation can be readily measured via radioimmunoassay, thus eliminating any need for radio labeling of the food Fe. 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.

Ascorbic acid (AA) can markedly enhance food iron availability. The magnitude of the effect can be influenced by the ratio of AA to ionized iron (Hallberg et al., 1986). When whole meals are being consumed, the total ratio of AA:Fe must be considered in addition to the degree of gastric and duodenal modification of iron and ascorbic acid.

For iron-fortified infant cereals, most of the iron is in the form of electrolytic iron powder, a form of elemental iron (Fe0). Hypothetically, Fe0 must be oxidized to Fe2+ or Fe3+ before it can be absorbed. The acid in the stomach reacts with elemental iron to form Fe2+. AA enhances the bioavailability of the ionized iron generated in the stomach by preventing Fe2+ from oxidizing to Fe3+. Fe2+ is more soluble at the pH of the upper small intestine and more bioavailable than Fe3+. When concentrations of AA are low, there may not be sufficient reducing power to prevent such oxidation. Thus, it is important to evaluate the effects of varying ratios of AA to iron on the availability of iron.

Our objectives were to determine whether adding AA to infant cereal enhances iron availability, and if so, the optimal molar ratio of AA to Fe to maximize Fe availability. We also determined whether mixing of infant cereal with apple juice fortified with AA, would increase Fe availability.

MATERIALS & METHODS

Cereal and apple juice
Samples of commercial infant rice cereal with and without AA added during processing, and apple juice fortified with AA were provided by Gerber Products Company (Fremont, Mich., U.S.A.). The measured Fe content of the infant rice cereal was 542±5 µg Fe/g cereal (mean±SEM; n = 6). Prepared rice cereal with AA contained at least 59 mg AA per 100-g dry rice cereal (data from product label). The apple juice was from sealed 118-mL jars of 100% freshly pressed apple juice. Each jar of apple juice was fortified with AA to contain at least 40 mg AA (from product label). For each replication of an experiment involving juice, a new jar was opened, and a fresh sample used.

Cell culture
Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Caco-2 cells were grown and maintained in monolayer culture as described by Glahn et al. (1996). Prior to use in the experiment, the cells were detached from the monolayer by incubation in trypsin (TrypLE Express; Invitrogen, Carlsbad, CA) for 2–3 min at 37°C. The detached cells were pelleted, counted, and resuspended in culture media at a density of 3×106 cells/mL.

Fig. 1—Diagram of in vitro digestion/Caco-2 cell culture model.
Collection (Rockville, Md., U.S.A.) at passage 17, and used in experiments at passage 25-33. Cells were seeded at a density of 50,000 cells/cm² in collagen-treated 6-well plates (Costar Corp., Cambridge, Mass., U.S.A.). The cells were grown in Dulbecco’s Modified Eagle Medium (GIBCO, Grand Island, N.Y., U.S.A.) with 10% v/v fetal calf serum (GIBCO), 25 mmol/L L HEPES, and 1% antibiotic antifungal solution (GIBCO). The cells were maintained at 37 °C in an incubator with a 5% CO₂, 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 d post seeding. Under these conditions, the amount of cell protein measured in each well was highly consistent from well to well within each culture plate.

**In vitro digestion**

Porcine pepsin (Sigma #P-7000, 800-2500 units/mg protein, St. Louis, Mo., U.S.A.), pancreatin (Sigma # P-1750, activity = 4 × U.S.P. specifications) and bile extract (Sigma # B-8631, glycline and taurine conjugates of hyodeoxycholic and other bile salts) were used to conduct the in vitro digestion. Further preparation of pepsin, pancreatin, and bile extract was performed to disperse the enzymes in solutions at physiological pH to remove any contamination Fe. 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, Calif., U.S.A.) and shaken gently on a platform tablopt shaker for 30 min. 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 eluted pepsin solution was 8 mL.

For intestinal digestion, 0.05 g of pancreatin and 0.3 g of bile extract were dissolved in 25 mL of 0.1 mol/L NaHCO₃. Chelex-100 (12.5 g) was added and the resulting mixture was shaken for 30 min on a tabletop shaker. The mixture was poured into a 1.6-cm dia filtration column to filter out the Chelex. An additional 10 mL of 0.1 mol/L NaHCO₃ was added to the column, and the filtrate collected into the pancreatic/bile solution. The final total volume of pancreatic/bile solution was 27 mL. Treatment of the pepsin and pancreatin-bile solutions by the described methods 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, Miss., U.S.A.) in an incubator at 37 °C with a 5% CO₂, 95% air atmosphere maintained at constant humidity. The intestinal digestion was carried out in the upper chamber of a two-chamber system in 6-well plates, with the cell monolayer attached to the bottom surface of the lower chamber (Fig. 1). The upper chamber was formed by fitting the bottom of an appropriate-sized Transwell® insert ring (gift from Costar Corp., Cambridge, Mass., U.S.A.) with a 15,000 molecular weight cut-off dialysis membrane (Spectra/Por 2.1, Spectrum Medical Industries Inc., Gardena, Calif., U.S.A.). 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, N.Y., U.S.A.). 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, 0.2 g of cereal was placed in a 50 mL screw cap culture tube with 10 mL of 140 mmol/L NaCl, 5 mmol/L KCl, and the mixture adjusted to pH 2.0 with 5.0 mol/L HCl. Then, 0.5 mL of pepsin solution was added. The tube was capped, placed horizontally, and incubated for 60 min on the rocking shaker and rocked at speed #7 (55 oscillations/min). For intestinal digestion, the pH of the sample (also referred to as the “digest”) was raised to pH 6 by adding 1 mol/L NaHCO₃ dropwise. Then 2.5 mL of the pancreatin-bile extract mixture was added. The mixture was adjusted to pH 7 with 1 M NaOH, and the volume was brought to 15 mL with 120 mmol/L NaCl, 5 mmol/L KCl.

**Preparation of 6-well culture plates with cell monolayers**

Immediately before intestinal digestion, 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 Inc., Grand Island, N.Y., U.S.A.) at pH 7. This MEM was chosen because it contained no added Fe, and upon formulation with the added ingredients, was always found to contain less than 8 µg Fe/L. The MEM was supplemented with 10 mmol/L PIPES (piperazine-N,N’-bis-[2-ethanesulfonic acid]), 1% antibo-tic-antimyctic solution (Sigma #A-9909), hydrocortisone (4 mg/L), insulin (5 µg/mL), selenium (5 µg/L), triiodothyronine (34 µg/L), and epidermal growth factor (20 µg/L), all from Sigma Co. 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 intestinal digest was pipetted into the upper chamber. The plate was covered and incubated on the rocking shaker at 6 oscillations/min for 120 min.

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 h, after which the cells were harvested for analysis.

**Harvesting of Caco-2 cell monolayers for ferritin analysis**

Exactly 24 h after the start of the intestinal digestion period, the cell monolayers were harvested. The media covering the cells was removed, and the cells were washed once with a 2 mL volume of a “rinse” solution containing 140 mmol/L NaCl, 5 mmol/L KCl, 10 mmol PIPES, at pH 7. The “rinse” solution was then aspirated, and a 2 mL volume of a freshly prepared “removal” solution was placed on the cell monolayer for 10 min. The “removal” solution, designed to remove non-specifically bound Fe, consisted of the above rinse solution with an additional 5 mmol/L sodium hydro-sulfite and 1 mmol/L bathophenanthroline disulfonic acid (BPD). We had found that lowering the BPDS level in the removal solution to 1 mmol/L was equally effective (unpublished results) as the 5 mmol/L BPD solution used previously (Glahn et al., 1995).

The removal solution was then aspirated, and the cell monolayer washed with a 2 mL volume of rinse solution. The rinse solution was then aspirated, and 2 mL of deionized water was placed on each monolayer. The plates were then placed on a rack such that the bottom of each plate was in contact with the water of a bench top sonicator that was kept in a cold room at 4 °C. The cells were sonicated for 15 min, 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**

Experiments involving cell cultures were replicated four to five times for each experimental protocol. Each treatment was performed in duplicate for each replication. The averages of duplicates were used as data points for each replication. The position of each experimental treatment in the multiwell plate was different for each replication. Replicates of each experiment were conducted on separate days.

In order to determine the optimal AA to Fe molar ratio of the infant rice cereal, we used a 100 mM AA solution, prepared immediately prior to use, and rice cereal without added AA. Aliquots of the AA stock solution were added to 10 mL of 140 mmol/L NaCl, 5 mmol/L KCl containing 0.2 g of the rice cereal. The ratios of AA to Fe were 0.8:1, 1.2:1, 1.6:1, 2.0:1, and 2.4:1. In order to determine the effect of mixing AA fortified apple juice on Fe availability, we used 6 different digests, all containing the same amount of Fe (Table 1).
Determination of optimal AA to Fe ratio

Analyses

Caco-2 cell protein was measured on samples that had been solubilized in 0.5 mol/L NaOH, using a semi-micro adaptation of a protein assay kit (Bio-Rad Laboratories, Hercules, Calif., U.S.A.). A one stage, 2-site immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-IRON II Ferritin Assay, RAMCO Laboratories, Houston, Texas, U.S.A.). A 10-μL sample of 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 solutions, foods, digests, and Caco-2 cell monolayers were conducted using an inductively coupled plasma emission spectrometer (ICAP Model 61E Trace Analyzer, Thermo Jarrell Ash Corporation, Franklin, Mass., U.S.A.). Measurement of AA in the apple juice was determined via titration using 2,6-dichloroindophenol as a reducing agent according to the methods of Haddad (1977).

Statistics

Statistical analysis of the data was performed using the software package GraphPad Prism™ (GraphPad Software, San Diego, Calif., U.S.A.). Statistical analyses were conducted according to the methods of Motulsky (1995). Prior to analysis, data were log transformed to achieve equal variance. Since each replication of an experiment in our study was a paired comparison, a repeated measures ANOVA was performed with the Tukey’s post test to compare the various means of each series of experiments. Means were expressed as means ± SEM of quadruplicate samples. "p ≤ 0.05."

RESULTS & DISCUSSION

Determination of optimal AA to Fe ratio

For each digest, the amount of Fe was constant as the same amount of cereal (0.2 g) had been added to each. As the average concentration of Fe in the rice cereal with or without added AA was 542 μg Fe/g cereal, the total Fe in each digest was 108 μg. Different levels of AA were added immediately prior to beginning the digestion to provide molar AA:Fe ratios of 0:1, 0.8:1, 1.2:1, 1.6:1, 2.0:1, and 2.4:1. Fe availability was maximized at an AA:Fe ratio of 1.6:1 (Fig. 2). Note that the AA was added immediately prior to the start of digestion; thus, there was no opportunity for oxidation of AA due to subsequent processing or storage. Oxidation of AA during processing and storage should be determined and taken into account during formulation to achieve optimal Fe availability.

AA added to rice cereal during manufacturing enhanced Fe availability (Fig. 3). Based on the nutrition label information, each 15-g serving contained at least 9 mg of AA; thus, the minimal AA:Fe ratio in this cereal should be 0.35:1. Food processors routinely fortify with amounts greater than nutrition label claims in order to ensure compliance. This is especially true for AA, which is known to oxidize during processing and storage (deMan, 1989). Measurement of the AA concentration was not done on the AA-fortified cereal samples. Based on the Caco-2 cell ferritin formation from digests containing a 1.2:1 ratio of AA:Fe and the commercially AA-fortified cereal (Fig. 3), there appears to be considerable potential for improved Fe availability. Increased Fe availability could likely be achieved by reducing the loss of AA during processing and storage, possibly by microencapsulation of the AA (Knezetic et al., 1998).

Mixing apple juice with rice cereal

Mixing the rice cereal with apple juice did not enhance Fe availability (Fig. 3). Measurement of total AA (i.e., AA plus dehydroascorbic acid) in a sample of apple juice averaged 0.520 ± 0.011 mg/mL. Based on that data, the AA:Fe ratio in the digest containing juice should have been 1.2:1. Using freshly prepared AA, an AA:Fe ratio of 1:2:1 was found to increase Fe uptake (Fig. 2 and 3). Analysis of the juice to quantify the amount of oxidized AA was done by eliminating the reducing agent from the titration protocol (Haddad, 1977). Without the reducing agent, the AA content of the juice was 0.471 ± 0.002 mg/mL, indicating that only 9% of the AA was in the oxidized form. Values for both measurements were expressed as means ± SEM of quadruplicate samples from a freshly opened container.

These results suggest that other

Table 1—Contents of digests used to determine the effects of ascorbic acid or apple juice on Fe availability from infant rice cereal

<table>
<thead>
<tr>
<th>Digest</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>No Fe or AA, No cereal.</td>
</tr>
<tr>
<td>Rice</td>
<td>Rice cereal. No AA added by manufacturer or prior to experiment.</td>
</tr>
<tr>
<td>Rice (+AA)</td>
<td>Rice cereal with AA added by manufacturer.</td>
</tr>
<tr>
<td>Rice (+Juice)</td>
<td>Rice cereal mixed with commercially prepared Apple juice immediately prior to experiment (0.2 g of cereal plus 0.8 mL of apple juice).</td>
</tr>
<tr>
<td>1.5 AA: 1 FeSO4</td>
<td>FeSO4 mixed with AA at molar ratio of 1.5:1. No cereal present in this digest. This was a positive control.</td>
</tr>
</tbody>
</table>
CONCLUSIONS

ADDITION OF AA TO INFANT RICE CEREAL ENHANCED IRON availability. To obtain maximal iron availability from cereal with approximately 542 µg Fe/g cereal, this in vitro model would recommend a molar ratio of at least 1.6:1 (AA:Fe). Ratios greater than 1.6:1 would have no increased benefit on iron availability; however, oxidation of AA during processing and storage would likely occur. Thus, a higher ratio of AA:Fe may be warranted for maximal Fe availability. Mixing apple juice with rice cereal at a “desirable consistency” did not enhance iron availability. The amount of AA in the juice added to the cereal should have enhanced Fe availability in the absence of counteracting factors. Additional testing of apple juice is needed to determine if other factors, such as presence of polyphenols, would inhibit the Fe uptake enhancing effects of AA.

REFERENCES

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