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Red Grape Juice Inhibits Iron Availability: Application of an in Vitro Digestion/Caco-2 Cell Model

FRANCESCA BOATO,† GARY M. WORTLEY,† RUI HAI LIU,† AND RAYMOND P. GLAHN*,‡

Institute of Food Science, Cornell University, Ithaca, New York 14853, and U.S. Plant, Soil and Nutrition Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Tower Road, Ithaca, New York 14853

Adequate bioavailable Fe intake is essential for optimal growth and intellectual development of infants and children. Fruit juices are nutritious and popular drinks for infants and children and are known to contain Fe uptake inhibitors (e.g., polyphenolic compounds) and a dominant promoter, ascorbic acid. Ascorbic acid is naturally present in fruit juices and is added during processing to almost all juices found in supermarkets. With these facts taken into account, an in vitro digestion/Caco-2 cell culture model was developed to compare the effects of apple, pear, white grape, red grape, prune, grapefruit, and orange juices on iron bioavailability. In two series of experiments, juices from a local supermarket were combined with $FeCl_3$ or commercial infant cereal fortified with elemental iron and subject to simulated gastric and intestinal digestion. Caco-2 cell ferritin formation in response to exposure to the digests served as the measure of Fe uptake. The pear, apple, grapefruit, orange, and white grape juice significantly increased Fe bioavailability from $FeCl_3$. For the infant cereal studies, the apple, orange, pear, and white grape juices increased the Fe bioavailability of the infant cereal. In contrast, the red grape juice and prune juice had profound inhibitory effects on iron bioavailability. These inhibitory effects were likely due to high levels of polyphenolic compounds that bind and thereby prevent absorption of soluble Fe. These inhibitory compounds appeared to counteract the promotional effects of ascorbic acid as they were in considerable molar excess relative to ascorbic acid and Fe in the digest. From a nutritional standpoint, the results suggest that individuals in need of optimal Fe absorption should avoid red grape and prune juice or at least vary the types of juices consumed. Alternatively, individuals seeking to limit Fe uptake (e.g., hemochromatitics and astronauts) may be able to utilize red grape or prune juice as effective inhibitors of Fe uptake. Consumers should be aware that the compounds that inhibit Fe availability are also linked to anticancer benefits; thus, a dietary balance of the above juices may be optimal.

KEYWORDS: Iron; bioavailability; juice; infant cereal; Caco-2; phenolics

INTRODUCTION

The intake and bioavailability of dietary iron is a key concern for infants and young children. Infant cereals, infant formula, and juices are examples of products in which iron is a critical nutrient. As such, manufacturers must assess the bioavailability of iron as it is influenced by proteins, organic acids (e.g., citric acid, ascorbic acid, and phytic acid), other minerals (e.g., zinc and calcium), and polyphenolic compounds (e.g., tannic acid and chlorogenic acid).

Given the high costs and limitations of human and animal feeding trials, initial development and screening of iron bioavailability of foods is best accomplished by in vitro methods. In the past, simple measurements of iron solubility or dialyzability have been used (*1*); however, the preferred approach involves the combination of in vitro digestion coupled with a living component such as human intestinal epithelial cells. The goal of these methods is to determine if the iron is "bioaccessible", in other words, to determine if the iron can cross the brush border surface of the intestinal epithelial cell and thus be available for absorption should the individual require it.

The in vitro digestion/Caco-2 cell culture model developed by Glahn et al. (*2*, *3*) shows promise as a rapid and cost-effective tool to predict iron uptake by humans. It combines simulation of human digestion with iron uptake by Caco-2 cells, a human intestinal cell line. This model has been specifically developed for measurement of iron availability from foods using Caco-2 cell ferritin formation as the marker of iron uptake. This approach negates the need for radioisotopic labeling of the food

* Author to whom correspondence should be addressed [telephone (607) 255-2452; fax (607) 255-1132; e-mail rpg3@cornell.edu].
† Institute of Food Science.
‡ U.S. Plant, Soil and Nutrition Laboratory.

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iron, thus making it more useful for a broad range of users. In general, the system is able to address experimental objectives not feasible or affordable to study in vivo.

As with any in vitro model, validation of the system relative to human trials is critical. This model system has demonstrated qualitatively similar results with human studies under a multitude of conditions. For example: iron uptake from digests of beef, chicken, and fish was 300−400% of the iron uptake from a digest containing casein (*2*). In other studies, investigators demonstrated the benefits of a human milk-based versus a bovine milk-based infant formula on iron availability (*4*). The enhancing effects of meat and ascorbic acid on iron availability have also been demonstrated in this system (*3*, *5*, *6*). Also, in a comparison of several commercial iron supplements, the in vitro iron availabilities from a polysaccharide−Fe complex and an $FeSO_4$ preparation were qualitatively identical to a closely matched human study (*6*, *7*). Taken together, the above studies impart confidence in the use of this system as the literature abounds with similar effects on iron availability to humans.

The applications for this model system are numerous. It can be used to improve the bioavailable Fe from commercial food products such as ready-to-eat breakfast cereals, infant cereals, and infant formula. It can be used as a screening tool for determining relative iron availability from varieties of staple food crops such as rice, wheat, corn, and beans, thereby identifying those with improved or poor nutritional quality. In addition to evaluating individual foods, the system can be used to determine Fe availability from meals or specific food and drink, the combinations of which are endless. It represents a means to systematically study or screen numerous factors, compounds, or conditions and thereby refine and improve the design of the more expensive and definitive human trial. Indeed, investigators using this model have found that with the support of one to two full-time technical staff, several hundred samples can easily be analyzed in a calendar year (R.P.G., personal observations). For all of these applications, it is imperative that appropriate amounts of food and iron be considered when the experiments are designed so as not to overload the system. In general, the standard conditions developed for this system are very sensitive to small changes in iron availability and capable of handling a broad range of foods (*2*−*6*).

The objective of the present study was to perform an in vitro assessment of the effects of different juices on iron bioavailability, especially iron of infant cereal as it is common for infants to consume fortified infant cereal mixed with various fruit juices.

MATERIALS AND METHODS

Chemicals, Enzymes, and Hormones. Unless otherwise stated, all chemicals, enzymes, and hormones were purchased from Sigma Chemical Co. (St. Louis, MO). The source of $FeCl_3$ was a 1040 μg of Fe/mL solution in 1% HCl (Sigma I-9011).

Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passages 25−33. Cells were seeded at a density of 50000 cells/cm^2 in collagen-treated six-well plates (Costar Corp., Cambridge, MA). The cells were grown in Dulbecco's Modified Eagle Medium (GIBCO, Grand Island, NY) 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% 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. On day 12 postseeding, the growth medium was removed from the cell culture plates, the plates were washed once with 2 mL of MEM (Minimum Essential Medium, GIBCO) at pH 7, and then an additional 2 mL of MEM was added; the cells were returned to the incubator until use the following day. Immediately prior to use in the experiment, the cells were again washed with MEM and exactly 1.0 mL of MEM was placed on each monolayer. As per the in vitro digestion model and protocol (see below), a sterilized insert ring with attached dialysis membrane was then placed in each well. This MEM was chosen because it contained no added Fe and, upon formulation with the added ingredients, was always found to contain <8 mg Fe/L. The MEM was supplemented with 10 mM PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid], 1% antibiotic−antimycotic solution (Sigma), hydrocortisone (4 mg/L), insulin (5 mg/L), selenium (5 mg/L), triiodothyronine (4 mg/L), and epidermal growth factor (20 mg/L), all from Sigma Co.

In Vitro Digestion. The in vitro digestion protocol was performed as described previously (*3*). Briefly, peptic and intestinal digestions were prepared for each food sample. Aliqouts (1.5 mL) of the intestinal digest were placed in the upper chamber of each well. As per our previously published diagrams, the "upper chamber" was created by attaching a dialysis membrane (15000 molecular weight cutoff) to an insert ring. The Caco-2 cells are cultured below this membrane on the plastic surface of the culture plate for each well. Iron from samples placed in the upper chamber can dialyze through the membrane and be accessible for uptake by the Caco-2 cells. Ferritin formation by the Caco-2 cells, a marker for cell Fe uptake, is used as the indicator of Fe bioavailability. Measurement of Caco-2 ferritin formation eliminates the need for radiolabeling of the food Fe and negates the controversy associated therewith. Ferritin formation is easily measured via RIA using commercially prepared assay kits (Ramco Laboratories, Houston, TX).

Experimental Design. *Study 1*. Experiments were designed to determine the effects of various fruit juices on iron absorption. The juices used were as follows: apple (Gerber Products Co., Fremont, MI); grapefruit (Wegman's Food Markets, Inc., Rochester, NY); orange (The Minute Maid Co., Houston, TX); pear (Gerber Products Co.); prune (Wegman's Food Markets, Inc.); red grape (Welch's, Concord, MA); and white grape juice (Welch's). All samples were purchased at a local supermarket in volumes of 0.945 or 1.89 L. All claimed to be 100% juice with added ascorbic acid. For each in vitro digestion, 25 μg of Fe (added as $FeCl_3$ solubilized in 1% HCl) and 0.73 mL of juice were added to 10 mL of 140 mM NaCl/5 mM KCl. Then, the in vitro digestion procedure including pH adjustment and addition of digestive enzymes was performed as per our standard protocol (*3*). A digest without juice containing the 25 μg of $FeCl_3$ plus 0.73 mL of deionized water, 10 mL of 140 mM NaCl and 5 mM KCl, plus the digestive enzymes, served as a reference control. All titrations and testing of the digests were done simultaneously on the same day, with separate independent replications ($n = 5$) for each sample.

Study 2. Experiments were designed to determine the effect of apple juice, orange juice, pear juice, red grape juice, and white grape juice on Fe availability from infant cereal. These juices were chosen as they are commonly fed to infants via mixing with infant cereal. The apple, pear, white grape, and orange juice samples were from individual vacuum-sealed serving sizes (118 mL) designed for infants (Gerber Products Co.) and were purchased at a local supermarket. The red grape sample was from a 1.89 L container (Welch's). As per study 1, the standard in vitro digestion conditions were used and all replicates were performed simultaneously on the same day in an independent manner. The source of iron was a commercial single-grain infant rice cereal (Gerber Products Co.) containing a total of 540 μg of Fe/g of rice cereal. It was estimated that >98% of the total Fe in the cereal was added as electrolytic Fe. According to the label claims, no ascorbic acid was added to the rice cereal. The ratio of Fe/juice used for each sample digestion was based on the serving size listed on each container (i.e., one serving of cereal per serving of juice). On the basis of this information, 0.2 g of cereal and 1.57 mL of juice was used for each digest. The control digest received cereal plus deionized water in place of juice.

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 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

Table 1. Measured Amounts of Phenolics, Quantified as Gallic Acid Equivalents, and Ascorbic Acid in Juice Samples of Studies 1 and 2

	apple	pear	white grape	red grape	prune	grapefruit	orange
			Study 1 Measurements				
μg of gallic acid/mL of juice	609	819	593	1405	1805	435	613
mg of ascorbic acid/mL of juice	0.66	0.84	0.38	0.36	0.24	0.16	0.31
molar ratio of ascorbic acid to Fe	6.12	7.77	3.52	3.27	2.19	1.46	2.87
molar ratio of gallic acid to Fe	5.84	7.85	5.69	13.47	17.30	4.17	5.88
molar ratio of gallic acid to ascorbic acid	0.95	1.00	1.61	4.17	7.69	2.86	2.04
			Study 2 Measurements				
mg of ascorbic acid/mL of juice	0.456	0.547	0.453	0.649			0.603
molar ratio of ascorbic acid to Fe	2.15	2.59	2.14	3.07			2.85

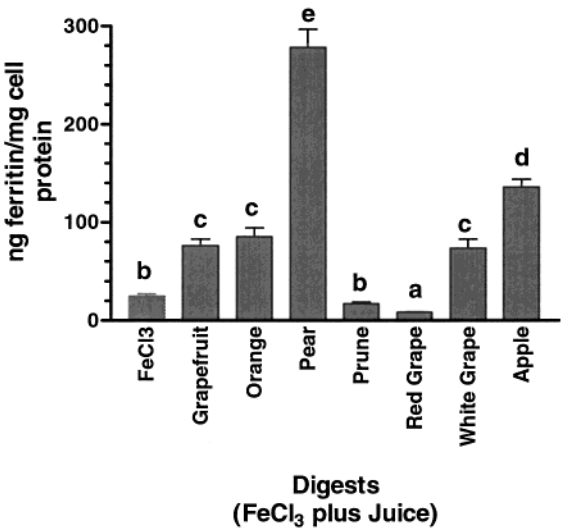

Figure 1. Caco-2 cell uptake of $FeCl_3$ (as measured by cell ferritin formation) added to each juice sample and subject to the in vitro digestion process (study 1). Equal amounts of iron (25 μg) were combined with juice samples (0.73 mL) purchased directly from the local supermarket and subjected to the standard conditions of the in vitro digestion/cell uptake process. Values represent mean ± SEM, $n = 5$. Bar values with no letters in common are significantly different ($p < 0.05$).

measure Caco-2 cell ferritin content (FER−Iron II ferritin assay, RAMCO Laboratories, Houston, TX). 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. Analyses 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 Corp., Franklin, MA). Measurement of the ascorbic acid in the juices was determined via titration using 2,6-dicloro-indophenol as a reducing agent according to the methods of Haddad (*8*).

Total phenolic content was determined by using a method described previously, and the content in phenolics was reported as micrograms of gallic acid equivalents per milliliter of juices (*9*).

Statistics. Statistical analysis of the data was performed using the software package GraphPad Prism (GraphPad Software, San Diego, CA). Statistical analyses were conducted according to the methods of Motulsky (*10*). Prior to analysis, data were log transformed to achieve equal variance. Tukey's post test was used to compare the means of each series of experiments. Means were considered to be significantly different if p values were ≤ 0.05.

RESULTS AND DISCUSSION

The effects of various fruit juices on nonheme iron availability in the form of $FeCl_3$ (study 1) are summarized in **Figure 1**. The pear juice sample produced the greatest increase in ferritin (1018%), followed by apple juice (455%), orange juice (243%), grapefruit juice (207%), and then white grape juice (196%). The prune juice and red grape juice decreased Caco-2 cell ferritin formation by 31 and 67%, respectively. As shown in **Figure 1**, there are three statistically different levels of enhancement of Caco-2 ferritin formation. The pear juice was the highest followed by apple juice, with grapefruit, orange, and white grape juices being relatively equal in effect. There was no net effect on ferritin formation (relative to control) in the presence of prune juice, and the red grape juice actually decreased the ferritin levels relative to the control. These results indicate that the red grape juice and prune juice decrease the availability of the Fe from $FeCl_3$.

As shown in **Table 1**, the red grape and prune juices were considerably higher in phenolics than the other juices. These results represent measurement of total phenolics in the juices and thus are to be considered a mixture of various phenolic compounds. The high levels of phenolics relative to the other juices are the most likely cause of the low or inhibited Fe uptake in the prune and red grape juice digests. Human studies have shown that the content of iron-binding galloyl groups is associated with inhibition of iron absorption, presumably by binding the iron and thus preventing uptake by the iron transporter (*11*).

South and Miller (*12*) documented the iron binding of tannic acid in the presence of different iron ligands with various mixing sequences of the tannic acid, ligands (ascorbic acid, EDTA, nitrilotriacetic acid, and citric acid), and iron. In their studies, the Fe/ligand ratio was 1:1 or perhaps slightly higher than 1:1 in regard to tannic acid. At these molar ratios, ascorbic acid was able to counteract binding of the iron by tannic acid if it was mixed with iron before the tannic acid. A simultaneous mixing of the ascorbic acid and tannic acid with Fe resulted in more Fe binding by tannic acid, but some preventive effect was observed. Mixing of the tannic acid with Fe prior to the addition of the ascorbic acid resulted in immediate, complete, and apparently nonreversible binding of the Fe by tannic acid. Commercial preparations of tannic acid are generally considered to be a 10 unit polymer of gallic acid with an approximate molecular weight of 1700 Da; thus, in molar galloyl equivalents, the ratio of galloyl equivalents to ascorbic acid (or Fe) in the South and Miller study could be approximated to 10:1. In our study, the galloyl equivalents value was always in considerable molar excess relative to iron and equimolar or greater relative to ascorbic acid (**Table 1**), ranging from 4 to 17 times that of ascorbic acid. Also, the ascorbic acid and phenolic compounds of the juice would have mixed prior to the experiment and thus interacted simultaneously with the Fe at the start of the digestion process, a sequence similar to that shown to result in binding of Fe by tannic acid in the South and Miller study. It is important to note that the observations by South and Miller (*12*) were measured over a period of only 5 min. In our study, conditions

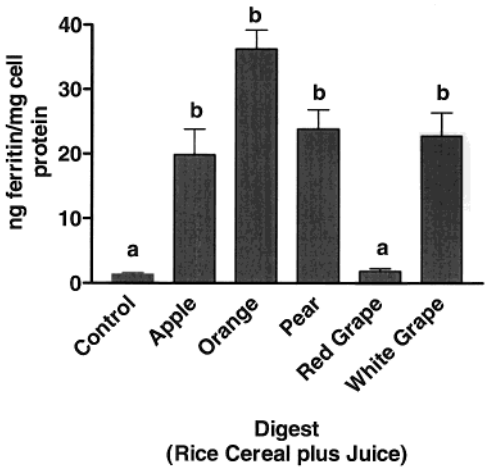

Figure 2. Effects of various juices on Caco-2 cell uptake of iron from digests of infant cereal (study 2). Equal amounts of infant cereal (0.2 g) were combined with juice samples (1.57 mL) purchased directly from the local supermarket and subjected to the standard conditions of the in vitro digestion/cell uptake process. Bar values with no letters in common are significantly different ($p < 0.05$). Values are means ± SEM, $n = 5$.

were different as longer periods of time (i.e., a 1 h peptic digestion period and a 2 h intestinal digestion period) were involved. It is possible that under these conditions, ascorbic acid may have even less counteracting effect on the galloyl groups in the juice.

The effects of various juices on iron availability from infant cereal are summarized in **Figure 2**. The control sample was simply the cereal without added juice. It is interesting to note that the mean cell ferritin value for the control (1.4 ng of ferritin/mg of cell protein) was below the range we routinely observe in our cultures prior to the start of the experiment, ~6−8 ng of ferritin/mg of cell protein. We have observed similar effects in the past when analyzing foods known to be high in phytic acid and/or tannins (*13*). The conclusion from these observations was that the ferritin formation method is sensitive to small changes in available Fe and that the inhibitors are in sufficient quantity to eliminate even trace contaminant amounts of Fe from the digest and the cell culture medium in the well during the experiment. These results should not be interpreted to mean that the human gastrointestinal tract would not absorb any Fe from the infant cereal under these conditions (i.e., cereal alone, no added juice), but they do indicate that the Fe bioavailability from the cereal is relatively low. In a previous study of commercial infant rice cereal using this model, an increase in Caco-2 cell iron uptake was observed from the rice sample with no added ascorbic acid or juice (*5*). This difference either reflects variability in the response of the Caco-2 model or is due to differences in formulation, such as the particle size of the elemental Fe or levels of inhibitors in the infant rice cereal. The levels of inhibitors (e.g., phytic acid and phenolics) could vary due to genotype of rice, agricultural practices, or processing.

The apple, orange, pear, and white grape juices all promoted Fe uptake from the infant cereal, whereas the red grape juice had no promotional effect (**Figure 2**). Therefore, if optimal Fe availability from an infant cereal is desired, these results indicate that juices other than red grape should be mixed with the cereal to promote iron uptake. It is interesting to note that in studies utilizing this model to investigate the iron bioavailability from rice, unpolished rice samples that were brown to purple in color were also very low in bioavailable iron (*13*). For those studies, the total phenolic content was related to iron bioavailability, suggesting that a specific phenol or class of phenol was responsible.

To summarize, juices such as apple, pear, orange, white grape, and grapefruit promoted iron uptake in this in vitro model. The dark-colored juices such as red grape and prune juices had a profound inhibitory effect on iron bioavailability from $FeCl_3$ or elemental Fe of infant cereal. Levels of phenolics were highest in the red grape and prune juices and appeared to overwhelm the promotional effect of ascorbic acid. As it is common practice for parents to offer juice or mix it with infant cereals, light-colored juices such as pear and apple appear to be best for optimal iron bioavailability.

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