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Comparison of Iron Bioavailability from 15 Rice Genotypes: Studies Using an in Vitro Digestion/Caco-2 Cell Culture Model

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An in vitro digestion/Caco-2 model was used to compare iron bioavailability from 15 selected Fe-dense and normal genotypes of unpolished rice from the International Rice Research Institute. Iron uptake was determined using Caco-2 cell ferritin formation in response to exposure to a digest of the cooked rice. Iron bioavailabilities from all rice genotypes were ranked as a percent relative to a control variety (Nishiki). Iron concentration in the rice samples ranged from 14 to 39 μg/g. No correlation was observed between Fe uptake and grain-Fe concentration. Furthermore, phytic acid levels were not correlated with Fe bioavailability. Genotypes with low Fe bioavailability (Tong Lan Mo Mi, Zuchein, Heibao, and Xua Bue Nuo) were noticeably more brown to purple in color. The results suggest that certain unknown compounds related to rice grain color may be a major factor limiting Fe bioavailability from unpolished rice.

KEYWORDS: Iron; bioavailability; rice; in vitro digestion; Caco-2

INTRODUCTION

Iron deficiency is the most prevalent nutrient deficiency among humans on earth. Incredibly, over half of the world's population (≈3.5 billion people) is iron deficient (*1*, *2*). Among these, 1.48 billion women, children, and men suffer from the more severe form of iron deficiency—iron deficiency anemia. Over 90% of the affected people live in developing countries.

The causal factors responsible for the massive magnitude of iron deficiency globally are complex. These factors include inadequate dietary intakes of bioavailable iron, increased iron requirements during rapid growth periods (i.e., pregnancy and infancy), infectious diseases such as malaria, excessive blood loss from parasitic infections (e.g., hookworm) or urinary blood loss (e.g., schistosomiasis infections), and inordinate losses of blood from women during menstruation or child birth. Of these factors, inadequate intake of bioavailable iron is thought to be the primary cause for over half of the iron deficiency in the world.

Unexpectedly, the prevalence of iron deficiency has increased in some regions (South Asia and Sub-Saharan Africa) during the past 50 years expanding from ∼40% of the population in 1977 to >50% in 1987 (*3*, *4*). The reasons for the upward trend in iron deficiency are not known with any certainty, but the overall availability of dietary iron has declined in some regions. This decline in iron may be because the production of micronutrient-dense staple foods, such as pulses (beans, lentils, chickpeas, etc.), has decreased as a result of the expansion of "green revolution" cereal cropping systems in many world regions (e.g., South Asia) (*5*, *6*).

Because the major contributing factor to iron deficiency in the developing world is the low amount of bioavailable iron found in the major staple plant foods (rice, wheat, maize, beans, potatoes, and cassava), ways need to be found to increase the density of iron in these foods. Not only must the absolute amounts of iron be increased in the edible portions of these food crops, but also this iron must be in forms that are bioavailable to the person consuming them in typical meals.

Over the past few decades, the costs for performing animal and human trials on iron bioavailability have limited progress not only for iron nutrition but also for many other micronutrients (*7*). The development of an in vitro iron bioavailability model system that mimics the gastric and intestinal digestion of humans, coupled with culture of human intestinal epithelial cells (Caco-2) shows great promise in addressing iron bioavailability issues (*8*−*12*). Indeed, this model system was recently mentioned as a promising methodology at a conference sponsored by the federal Office of Dietary Supplements, the National Institutes of Health, and the American Society of Nutritional Sciences (*13*).

This system is a significant advancement over the use of in vitro digestion models alone, which measure only iron dialyzability and, therefore, are not a complete measure of iron

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bioavailability (*14*). 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 (*10*). Ferritin concentration is easily measured via radioimmunoassay, thus eliminating the need for radiolabeling of the food iron. This model system facilitates experiments that may not be feasible or practical to conduct in vivo. The objectives of the present study were to screen numerous varieties of unpolished rice and, if possible, to provide an explanation for observed differences in iron bioavailability. This information is needed by plant breeders when screening promising genotypes of rice having increased grain-iron density in order to advance lines in breeding programs (*15*).

MATERIALS AND METHODS

Chemicals, Enzymes, and Hormones. Unless otherwise stated, all chemicals, enzymes, and hormones were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passage 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.

Rice Samples. Sixteen grain-iron-dense genotypes of rice were provided by the International Rice Research Institute (IRRI), Los Baños, Philippines. Rice grains from selected genotypes were cultured in nutrient solutions (*16*). Grains were harvested at maturity. The hulls were removed, and subsamples of the brown rice were autoclaved for 15 min. The autoclaved rice was then homogenized in a Polytron homogenizer. The homogenate was frozen and then lyophilized to dryness.

In Vitro Digestion. The preparation of digestion solutions (pepsin, pancreatin, and bile extract) and in vitro digestion was performed as previously published (*10*). Exactly 0.5 g of rice was used for each sample digestion. The term "intestinal digest" used in subsequent paragraphs refers to the mixture following the pepsin digestion period, with pancreatic enzymes and bile extract, at pH 7.0.

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, no. 41500; GIBCO, Inc.) at pH 7. This MEM was chosen as it contained no added iron and, upon formulation with the following ingredients, was always found to contain <8 μg of iron/L. The MEM was supplemented with 10 mmol/L piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES), 1% antibiotic−antimycotic solution (Sigma no. A-9909), hydrocortisone (4 mg/L), insulin (5 mg/L), selenium (5 μg/L), triiodothyronine (34 μg/L), and epidermal growth factor (20 μg/L). A fresh 1.0 mL aliquot of MEM covered the cells during the experiment. A sterilized insert ring fitted with a dialysis membrane was then inserted into the well, thus creating the two-chamber system. Then, a 2.5 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/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 time 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 as 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 h after the start of the intestinal digestion period, the cell monolayers were harvested. To harvest the cells, the medium 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, and 10 mmol of 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 min and then scraped from the plate surface and harvested along with the 2 mL volume of water in each well. The samples were immediately frozen and stored at −20 °C.

Experimental Design. Experiments were conducted using six-well plates on separate days in paired fashion with a control sample. The control sample was a rice grain variety known as Nishiki. Duplicate measurements of ferritin formation and dialyzable Fe (i.e., bottom chamber Fe) were made from each digest and averaged to represent a replication. Although this model has been shown to detect differences in bioavailable iron in the low nanogram range (5−10 ng), pilot studies indicated that it was necessary to add ascorbic acid to the samples in order to increase the amount of dialyzable Fe in the lower chamber significantly above background. This enabled greater and more consistent Caco-2 cell ferritin formation. Without the addition of ascorbic acid, only large differences in bioavailability could be detected. The molar ratio of added ascorbic acid to Fe was 10:1.

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 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. Analysis of the iron in solutions and digested biological samples was determined by inductively coupled argon plasma emission spectrometry (ICAP model 61E trace analyzer, Thermo Jarrell Ash Corp., Franklin, MA). Phytate analysis was done by HPLC (*17*). Total polyphenol content of the samples was measured using a vanillin, redox, and precipitation assay (*18*).

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 (*19*). Prior to analysis and when appropriate, data were log transformed to achieve equal variance. 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

The data for Caco-2 cell ferritin formation, relative to the Nishiki control, are summarized in **Figures 1** and **2**. Ferritin values are reported as a percent of that of the Nishiki genotype, which served as the reference control.

In the absence of ascorbic acid, no significant differences were observed relative to the control (**Figure 1**). Ferritin values for the control averaged 9.1 ± 0.9 ng of ferritin/mg of cell protein (mean ± SEM). It should be noted that overall pre-experimental baseline values for the Caco-2 cells ranged from 3 to 9 ng of ferritin/mg of cell protein. Typically, <20% variation in baseline ferritin was observed between wells of a given multiwell plate.

In the presence of ascorbic acid, the Heibao, Tong Lan Mo Mi, Xua Bue Nuo, and Zuchein genotypes had relatively low

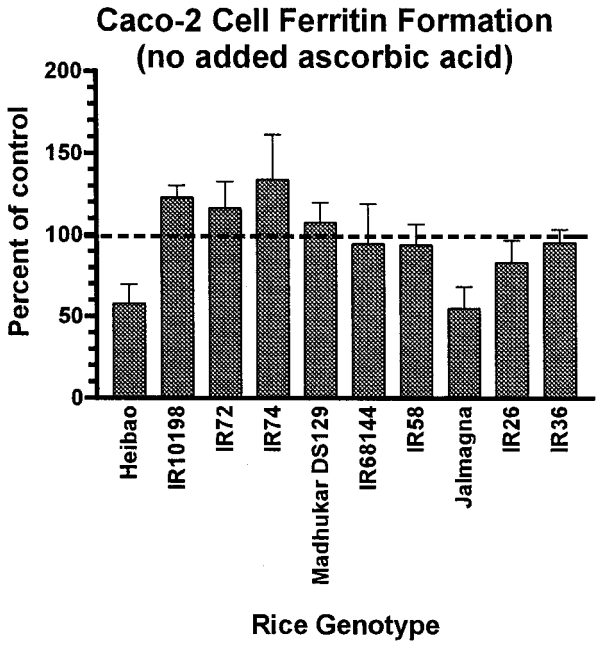

Figure 1. Iron bioavailability of unpolished rice relative to that of the control genotype (Nishiki). No ascorbic acid was added to the digest. Ferritin values for the control rice were 9.1 ± 0.9 ng of ferritin/mg of cell protein. Values are mean ± SEM, $n = 4$. No significant differences were observed.

bioavailable Fe levels, exhibiting ferritin levels ~10−20% of the control (**Figure 2B**). Dialyzable Fe as measured by the amount of Fe in the bottom chamber at the end of the intestinal digestion period were not correlated with ferritin values (**Figure 2A**). Control ferritin values in these experiments averaged 36.8 ± 3.8 ng of ferritin/mg of cell protein (mean ± SEM), indicating that the added ascorbic acid promoted Fe bioavailability relative to experiments without added ascorbic acid (**Figure 1**). The Tong Lan Mo Mi genotype contained the highest Fe concentration of all genotypes tested but showed the lowest bioavailable Fe level. The Jalmagna and Madhukar (HB6717) genotypes were ranked higher in bioavailable Fe compared to Tong Lan Mo Mi but were significantly lower than the control (54 and 62%, respectively). The remaining varieties, IR10198, IR72, IR74, Madhukar (DS129), IR68144, IR26, IR36, and Azucena, were all similar in Fe availability relative to the control. IR58 and Azucena were significantly higher in bioavailable Fe than Jalmagna but not different from Madhukar (HB6717).

Measurements of inositol phosphates [both inositol pentaphosphate (IP5) and inositol hexaphosphate (IP6)] in each rice genotype are summarized in **Table 1**. Iron bioavailability was not correlated with IP5, IP6, or IP5 + IP6.

Total polyphenol content was not correlated with Fe bioavailability (data not shown). Repeated measurements of the rice samples produced highly variable results and decreased sharply over time, indicating that the polyphenol content changed rapidly with storage of the samples. As we are not confident that the measured values represent the polyphenol levels at the time of the Fe bioavailability measurement, the results have been omitted here. Interestingly, genotypes with low Fe bioavailability tended to be more brown to purple in color (**Figure 3**).

DISCUSSION

A broad range of Fe bioavailability was observed among the 16 rice genotypes tested. Neither Fe concentration nor total IP5 + IP6 levels were correlated with Fe bioavailability. Low Fe

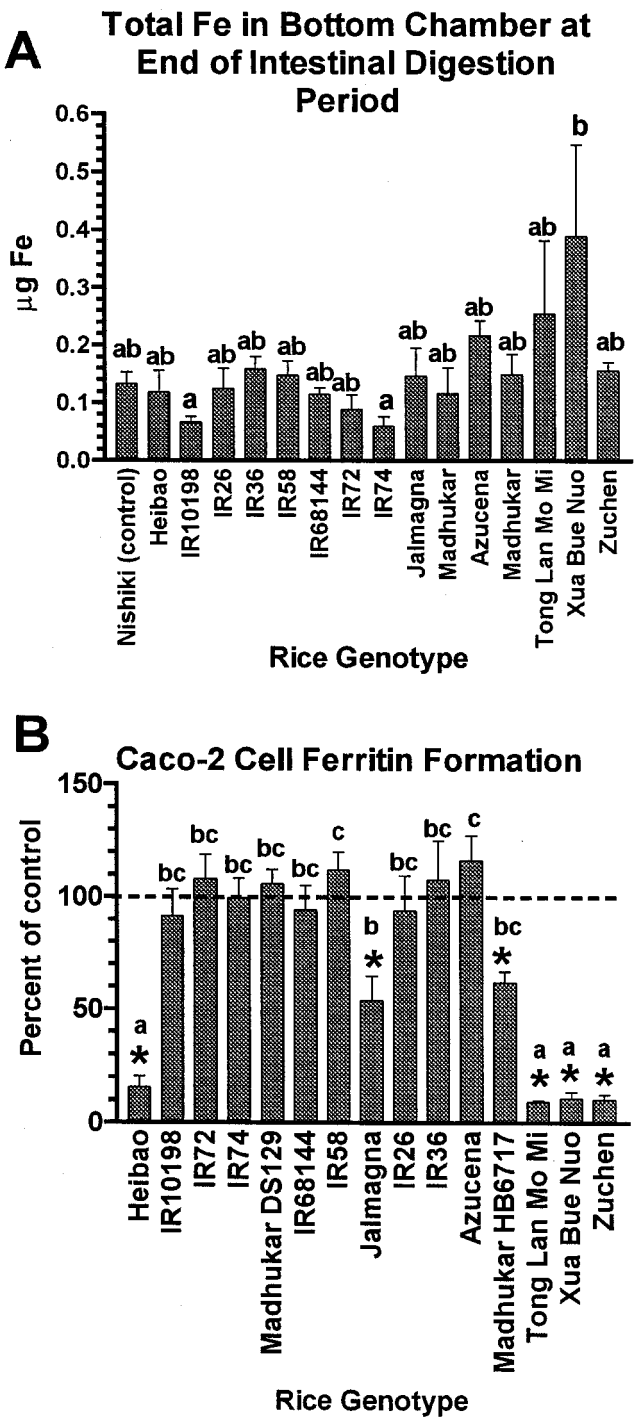

Figure 2. (A) Total Fe present in the bottom chamber at the end of the intestinal digestion period; (B) iron bioavailability of unpolished rice relative to the control genotype (Nishiki). Ascorbic acid was added to the samples at a 10:1 (ascorbic acid/Fe) molar ratio, prior to the start of the pepsin digestion. Values are mean ± SEM, $n = 5$. Asterisk indicates significant difference from control ($p < 0.05$). Bar values with no letters in common are significantly different ($p < 0.05$).

bioavailability was observed only in genotypes having grain noticeably more purple to brown in color (**Figure 3**). This suggests that polyphenolic compounds had a profound effect on Fe bioavailability in the Heibao, Jalmagna, Tong Lan Mo Mi, Xua Bue Nuo, and Zuchein genotypes. Anthocyanins, proanthocyanidins, and other tannins and tannin-like compounds are known to be potent inhibitors of iron absorption; however, quantification of the exact profile of compounds present in foods

Table 1. Concentration of Fe, Total IP5 + IP6, and (IP5 + IP6)/Fe Molar Ratios in 16 Genotypes of Brown Rice Grain[a]

rice genotype	Fe (μg/g)	total IP5 + IP6[b] (μmol/g)	(IP5 + IP6)/Fe (molar ratio)
Nishiki (control)	14.4	11.4	44.2
IR26	14.8	21.3	80.4
IR10198	17.3	21.1	68.1
Madhukar (DS129)	17.3	17.7	57.1
Madhukar (HB6717)	17.5	10.7	34.2
IR36	17.9	20.1	62.7
IR74	18.0	19.0	59.0
IR72	18.2	19.2	58.9
Heibao	19.6	18.8	53.4
IR58	19.9	23.1	64.8
IR68144	20.4	20.8	57.0
Jalmagna	20.5	19.5	53.1
Azucena	23.1	5.7	13.8
Zuchein	23.7	13.5	31.8
Xua Bue Nuo	24.5	15.0	34.2
Tong Lan Mo Mi	38.6	14.2	20.6

[a] Values are the average of duplicate measurements. Variability of duplicates was <5% for all measurements. [b] IP5 + IP6 = *myo*-inositol pentaphosphate + *myo*-inositol hexaphosphate.

is extremely difficult due to chemical transformation during processing and storage (*20*, *21*). Certainly additional research needs to be done to determine if measurement of specific polyphenols or a class of polyphenols can be used as a predictor of iron bioavailability to humans.

Both in vivo and in vitro studies have shown that the inhibitory effect of phytic acid on nonheme Fe uptake can be overcome by the presence of ascorbic acid in the meal (*11*, *22*−*25*). In the present study, we observed that it was necessary to add ascorbic acid in order to increase the Caco-2 cell ferritin formation significantly above baseline so that ranking of the genotypes could be accomplished (**Figures 1** and **2**). Our decision to add ascorbic acid was based on the following: Baseline ferritin levels in our Caco-2 cells prior to exposure to the digestion conditions range from 3 to 9 ng of ferritin/mg of cell protein, with <20% variability between wells of a multiwell (six-well) plate for a given day of replications. After exposure to digested rice samples without added ascorbic acid, ferritin values were relatively unchanged (4−11 ng of ferritin/mg of cell protein). Simultaneously, we observed that little or no Fe dialyzed into the bottom chamber under these conditions. Pilot studies indicated that a 10-fold molar excess of ascorbic acid to Fe would increase the Fe bioavailability but not maximize Fe uptake under these in vitro digestion conditions (*10*, *24*, *26*). The addition of ascorbic acid was presumed to counteract the effects of the inhibitors of Fe availability and allow some available Fe to form. The 10:1 ratio of ascorbic acid to Fe used in these in vitro conditions was calculated to be similar to consuming a small glass of orange juice (6−8 oz) with a reasonable serving of rice (30−50 g). Although many populations consume large quantities of rice per meal, it is common knowledge that other foods containing ascorbic acid such as fruits are consumed in the same meal. Thus, we are confident that inclusion of this amount of ascorbic acid represents a reasonable condition for comparison of the rice samples.

Although the addition of ascorbic acid increased the amount of dialyzable or soluble Fe, these values were not correlated with Fe availability (**Figure 2A**). This is not surprising as it has been shown that solubility or dialyzability does not always equate with availability (*9*, *10*, *14*). It is quite likely that some of the soluble forms of Fe were bound by phytic acid (i.e., monoferric phytate), which can be soluble but not available (*26*).

Inositol pentaphoshate (IP5) and inositol hexaphosphate (IP6) have been shown to have the greatest inhibitory effect on Fe uptake in vitro relative to the lesser phosphorylated forms of inositol (*24*). These were the major forms of inositol phosphates present in the grain of all the genotypes studied. These compounds were found to be in great molar excess relative to the amount of Fe present in the rice grain (**Table 1**). The sum of IP5 and IP6, expressed as a molar ratio, was 13−80 times that of the Fe. Interestingly, the Azucena genotype demonstrated one of the highest Fe bioavailability levels and, also, contained the lowest ratio of phytic acid to Fe, a value of 13.8. The Azucena genotype may have greater Fe bioavailability under meal conditions as it would be easier for promoters such as meat and ascorbic acid to overcome the inhibitory effects of phytic acid. The Tong Lan Mo Mi variety had the next lowest phytate to Fe molar ratio but was relatively purple in color, a trait associated with low Fe bioavailability in these experiments.

Studies using this model have determined that maximal inhibition of Fe uptake from $FeCl_3$ occurred at a 10:1 phytic acid to Fe molar ratio in the absence of food (*26*). Obviously under meal conditions where other foods or drinks are present, Fe uptake could be affected by the presence of promoters or inhibitors in the meal. However, if phytic acid levels could be manipulated in a given genotype to a range in which inhibitors would be offset by promoters of Fe uptake, then the opportunity for more Fe absorption is possible. Glahn et al. (*26*) also observed that tannic acid produced maximal inhibition of Fe uptake at a 1:1 molar ratio or less in this in vitro model. Similar effects were observed in human studies, suggesting that at least certain polyphenols may be the dominant factor in determining Fe bioavailability from plant food sources (*27*).

The lower cost of using the Caco-2 cell in vitro model and the fact that the model does not require the use of intrinsic radioisotopes of Fe to determine bioavailable Fe from plant food sources support the contention that this model should become the method of choice for screening plant foods for bioavailable levels of Fe. However, direct comparison of the in vitro model to human studies should be vigorously pursued in the future to confirm that this model gives reliable results for use in ranking iron-enriched staple plant foods such as reported here.

Rice grains contain inherently lower amounts of micronutrients than many other noncereal staple food crops (*6*). The consumer preference for polished rice further reduces the Fe content of the rice grain by as much as 60%. At present, little is known of the effects of polishing on the bioavailable Fe content of rice. Although polishing removes Fe from the grain, it also removes inhibitors of Fe uptake such as polyphenols and phytic acid (*28*). From an experimental standpoint, we observed that preventing Fe contamination of the rice grain was very difficult. In pilot studies for this experiment we also found it possible to measure the relative Fe availability from the polishings separate from the endosperm or the unpolished kernel. These observations warrant further investigation as it may provide insight into breeding an improved variety, possibly by locating differences in bioavailable Fe from the parts of the rice grain. However, for this approach to be successful, contamination by the polishing device with Fe or other minerals must not occur.

In summary, the present study demonstrates that the in vitro digestion/Caco-2 model has the potential to identify varieties of rice and other crops that may result in sustainable plant-breeding improvements in Fe nutrition. Specifically, this study documents in vitro the low availability of Fe in rice and that

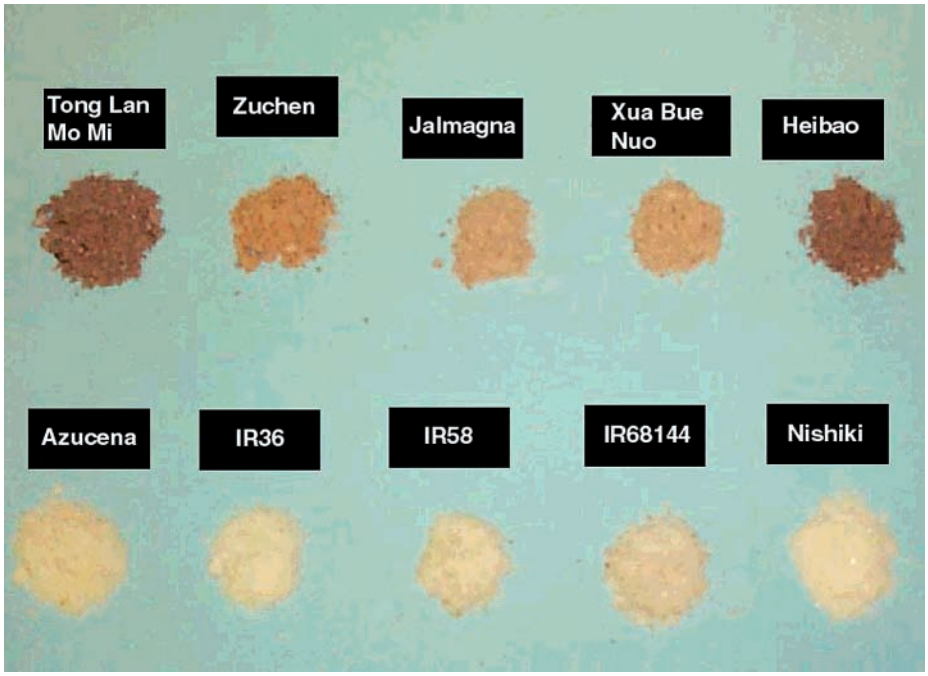

Figure 3. Photograph of rice samples used in the present study. Note the color of the top row of samples, which were relatively low in Fe availability.

comparisons in this model require additional ascorbic acid to offset inhibitory factors and thus expose a ranking of the genotypes. The question now is how closely this in vitro comparison predicts Fe availability to humans both in single-meal trials and in long-term studies. Given the relatively low cost of using this approach and the fact that both promotional and inhibitory effects documented in humans have been reproduced in this model (*9*, *10*, *12*, *26*), direct comparison of this model with human studies should now be conducted.

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