Original Article

Assessment of Iron Bioavailability in Ten Kinds of Chinese Wheat Flours Using an *in vitro* Digestion/Caco-2 cell Model*

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Abstract

Objective To compare iron bioavailability (Fe BV) from ten selected kinds of Chinese wheat flours in order to provide scientific basis for further human trials and enable plant breeding programs to screen biofortified wheat cultivars.

Methods An *in vitro* digestion/Caco-2 cell model was used to assess Fe BV of ten flour samples from six leading Chinese wheat cultivars and the stability of Fe BV in one cultivar was studied across three growing environments.

Results Significant differences were observed in both Fe BV and Fe bioavailability per gram of food (Fe BVPG) among cultivars (P<0.01) grown at the same location with the same flour extraction rate. Zhongyou 9507 and Jingdong 8 had Fe BV 37%-54% and Fe BVPG 103%-154% higher than the reference control. In the Anyang environment, Zhongyou 9507 had a higher wheat flour-Fe level and Fe BVPG. Differences in Fe BV were detected in cultivars with different flour extraction rates.

Conclusion Zhongyou 9507 and Jingdong 8 were identified as the most promising cultivars for further evaluation of efficacy by using human subjects. The growing environments had no effect on Fe BV, but did have a significant effect on Fe BVPG. Fe bioavailabilities in low-extraction (40%) flours were higher than those in high-extraction (78%) flours.

Key words: Iron biofortification; Bioavailability; Caco-2 cells

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INTRODUCTION

Iron (Fe) deficiency anaemia is the most widely prevalent nutritional problem in the world, involved in about 3.5 billion people, among whom over 90% are in the developing countries[1]. Although many factors are responsible for the onset of Fe deficiency, the most likely cause of this nutritional problem in developing countries is inadequate dietary intake of bioavailable Fe as a result of over-dependence on staple food crops including maize, rice, wheat, and cassava that are low in micronutrients[2]. The Chinese dietary pattern is predominantly plant based, and consumption of cereals and vegetables is much higher than in diets common in Europe and North America[3]. Cereals are

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low in Fe content and high in Fe absorption inhibitors. The bioavailability of Fe in a regular Chinese plant-based diet is as low as 2.57%, whereas in a diet including 144 g of meat the Fe absorption is 10.39%[2]. According to a nutrition survey carried out in 2002, the overall prevalence of anaemia in China was 15.2% in average, but was 24.2% among children under the age of two, and 21.5% among adults older than 60. More than 20% of women at reproductive age suffered from anaemia[4].

The problems associated with solving Fe deficiency by food fortification led to the suggestion of novel ideas such as cooking food in Fe pots and food fortification or supplements, but the success of such approaches remains questionable and unproven[5]. Recent understanding of plant metabolism has made it possible to increase the Fe content in staple foods by both conventional plant breeding and genetic engineering. Improving the micronutrient composition of plant foods may become a sustainable strategy to combat deficiencies in human populations[6]. For these reasons, the Consultative Group on International Agricultural Research (CGIAR) developed a biofortification challenge program, also known as HarvestPlus, with the objective of developing bio-fortified staple crops, such as common wheat (*T. aestivum* L.), with high micronutrient concentrations through plant breeding[7]. Wheat is not only one of the major crops in the world, but is also one of the major staple foods in northern China, especially in poor rural areas such as Gansu, Shaanxi, Inner Mongolia, and parts of Sichuan[7-8]. Therefore, it is important to enhance the concentration of Fe in wheat. With the assistance of the biofortification program of the HarvestPlus challenge project, the effects of genotype, environment, genotype by environment interaction, and milling extraction levels on wheat mineral element concentrations were evaluated in China[7-9]. However, Fe bioavailability (Fe BV) was not evaluated.

An *in vitro* digestion/human colonic adenocarcinoma (Caco-2) cell model system invented by R. P. Glahn, which mimics the gastric and intestinal digestion of humans, was shown to be a rapid and cost-effective means of studying Fe BV[10-11]. Among the target crops of rice, wheat, maize, cassava, potato and beans in the HarvestPlus program, the *in vitro*/ Caco-2 system was used successfully to screen and rank genotypes for Fe BV in rice, maize, potatoes and beans[3,12-16], but few reports have been found on wheat. Therefore, it was used in this study with the objective to (1) screen Fe BV in flours from six leading wheat cultivars with medium to high Fe concentrations, (2) assess the effects of growing environments on Fe BV of wheat flour, taking one leading cultivar as an example, and (3) evaluate the effects of flour extraction on Fe BV of wheat flour, taking two leading cultivars with high and low flour extractions as examples. The aim was to obtain information on wheat cultivars with high Fe BV for human trials in a rapid and inexpensive way.

MATERIALS AND METHODS

Wheat Samples

Six cultivars, namely Zhongyou 9507, Jingdong 8, Zhongmai 9, Zhongmai 175, Yumai 18, and Yumai 2, with medium to high Fe concentrations based on a previous report[8-9], were selected and planted at Anyang, Henan Province, in 2007. Additional samples of Zhongyou 9507 were obtained from Beijing and Urumqi in Xinjiang Province. A Buhler experimental mill (MLU 220, Uzvil, Switzerland) was used to produce flour from all the grain samples at a 78% flour extraction rate according to American Association of Cereal Chemists (AACC) approved method 26-21A[17]. Two additional flour samples with 40% flour extractions were obtained from Yumai 18 and Zhongmai 175. After milling, the ten flour samples were sealed in plastic bags and stored in a refrigerator (-20 °C) until used. The 78% flour extraction rate sample from Yumai 18 was always used as a 'reference control' because Yumai 18 had been a leading cultivar grown in Huanghuai wheat region of over 6.67×10^5 ha per annum for the 15 years before 2006. General information for all samples is shown in Table 1.

Chemicals, Enzymes, and Hormones

Unless stated otherwise, all reagents and materials were purchased from Sigma Chemical Co. and Costar Corporation.

Cell Culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at Passage 25, and used in experiments at Passage 30-40. Cells were seeded at densities of 50 000 cells/cm^2 in collagen-treated 6-well plates (Costar Corporation, Cambridge, MA, USA). The cells were grown in Dulbecco's modified Eagle's medium with high glucose (DMEM) plus 10% v/v calf serum

(Sijiqing, China), 25 mmol/L HEPES and 1% antibiotic antimycotic solution, and maintained at 37 °C in an incubator with 5% CO_2 and 95% air atmosphere at constant humidity. The medium was changed every 2 d. Fe uptake experiments were performed at 13 d post-seeding[16,18].

In Vitro Digestion

The preparation of digestion solutions (pepsin, pancreatin, and bile extract), *in vitro* digestion, preparation of the six-well culture plates with cell monolayers and harvesting of cell monolayers were performed following the procedure proposed by Glahn et al.[10,18]. An appropriate amount of each flour sample with digests containing 10 μg Fe was used[18]. Briefly, a pepsin solution was added to each sample at pH 2, followed by a pancreatin-bile acid solution at pH 5.5-6.0 one hour later. Ascorbic acid (AA) was added to each flour digestion with the molar ratio of added AA to Fe being 10:1[12]. A 1.5 mL aliquot of the resulting food digest (15 mL) was placed in the upper chamber of each cell-culture well created by attaching a dialysis membrane (15-kDa molecular weight cutoff, Spectrum Medical, Gardena, CA, USA) to an insert ring; Caco-2 cells had been grown on the bottom of the 6-well plates. Fe from food digests placed in the upper chamber for 2 h was dialyzed through the membrane and became accessible for uptake by the Caco-2 cells. The dialysis membrane was necessary to protect the cells from the digestive enzymes (pepsin, pancreatin, bile), similar to the protection provided by the mucus layer in the human intestine. Ferritin formation by the Caco-2 cells, a marker for cell Fe uptake, was used as the indicator of Fe BV[11].

To accurately determine the amount of Fe 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. The entire volume of solution in the bottom chamber was collected for measurement of total Fe at the end of the intestinal digestion period[12].

The cell monolayers were harvested 24 h after the start of the intestinal digestion period. To harvest the cells, the medium 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 cells were scraped from the plate surface, harvested along with the 2 mL volume of water in each well, then transferred into an eppendorf tube and sonicated 15 times at 100 w with 5 s sonication periods and 5 s pauses. The sonicated cells were stored at -20 °C[12].

Measurement of Ferritin, Fe, and Phytic Acid Phosphorus (PAP)

All glassware used in the sample preparation and analyses was acid-washed. Fe concentrations of wheat grain and flour were measured by using inductively coupled plasma atomic emission spectrometry (ICP-AES, OPTIMA 3300 DV) to quantify aqueous constituents following microwave digestion with HNO_3-H_2O_2 solution. Phytic acid phosphorus (PAP) was assayed according to Vaintraub and Lapteva[19]. Caco-2 cell protein was measured with a BCA protein assay kit (Shanghai Jierui Bioengineering Co.). An immunoradiometric assay was used to measure Caco-2 cell ferritin content (Iodine [125I] Ferritin Radioimmunoassay Kit, Beijing Kemeidongya Biotech Co.). Triplicates measurements of ferritin formation were made from each digest and averaged to represent a replication.

$$\text{Fe bioavailability (Fe BV)} = \frac{\text{Caco - 2 cell ferritin content (ng)}}{\text{Caco - 2 cell protein (mg)}} \quad (1)$$

$$\text{Fe bioavailability per gram (Fe BVPG)} = \frac{\text{Fe BV (ng/mg)}}{\text{Sample weight (g)}} \quad (2)$$

Experimental Design

Due to the low bioavailable Fe levels in staple food crops, ascorbic acid (10:1 AA/Fe, molar ratio) was added into the digest of flour to detect differences in bioavailable Fe between genotypes, following the suggestion of Glahn et al.[12], to enable greater and more consistent Caco-2 cell ferritin formation. Based on previous reports of no difference in Fe BV with cooking[3], samples were not cooked before *in vitro* digestion. Five independent replicates for each sample were used, together with the reference control Yumai 18. Samples were randomized in each six-well plate.

Statistical Analysis

The statistical analysis of the data was performed by one-way analysis of variance (ANOVA) by using SPSS package (Version13.0; Chicago, IL, USA). Means were compared by the least significant difference (LSD) test at $P<0.05$.

RESULTS

General Information of Wheat Flour Samples

The effectiveness of the milling process in decreasing Fe concentration varied among the genotypes tested (Table 1). The cultivars had medium to high grain-Fe concentrations, ranging from 40.0 to 52.0 mg/kg. The Fe concentrations were reduced by 39%-68% in milling, becoming 12.8 to 29.2 mg/kg. Significant differences in flour-Fe concentrations caused by location and flour extraction rate were indicated. There were large variations in grain and flour Fe concentrations among samples of Zhongyou 9507 from Anyang, Urumqi, and Beijing. The association of lower flour extraction rates and lower Fe concentrations in flour was also indicated for Yumai 18 and Zhongmai 175 (Table 1).

The levels of PAP in wheat flours ranged from 0.53 to 1.00 mg/g. Zhongyou 9507 from the Beijing location with 78% flour extraction had the highest value, whereas Yumai 18 with a 40% flour extraction rate the lowest.

Table 1. Fe and PAP Concentrations in Flour Samples from Wheat Grown at Different Locations and Milled at Different Flour Extraction Rates

Flour Sample	Location	Flour Yield (%)	Grain-Fe[*,†] (mg/kg)	Flour-Fe[*,†] (mg/kg)	Flour-PAP[†,‡] (mg/g)	Flour-Fe/Grain-Fe (%)
Yumai 18[§]	Anyang	78	40.0[a]	15.8[b]	0.78[c]	39.4
Jingdong 8	Anyang	78	52.0[f]	20.8[e]	0.82[cd]	40.1
Zhongmai 9	Anyang	78	47.7[d]	22.7[f]	0.87[d]	47.6
Zhongmai 175	Anyang	78	49.3[e]	20.9[e]	0.79[c]	42.4
Yumai 2	Anyang	78	43.0[bc]	16.0[b]	0.68[b]	37.2
Zhongyou 9507	Anyang	78	48.3[de]	29.2[g]	0.91[de]	60.5
Zhongyou 9507	Beijing	78	42.1[b]	16.7[c]	1.00[e]	39.6
Zhongyou 9507	Urumqi	78	44.7[c]	18.8[d]	0.94[de]	42.1
Zhongmai 175	Anyang	40	49.3[e]	15.8[b]	0.68[b]	32.1
Yumai 18	Anyang	40	40.0[a]	12.8[a]	0.53[a]	32.1

Note. Fe, iron. PAP, phytic acid phosphorus. [*] iron concentration in grain or flour sample. [†]Values (n=3) with no superscript letters (ie letters from a to g) in common are statistically significantly different (P<0.05). [‡]phytic acid phosphorus in flour sample. [§]reference control, leading cultivar released in the 1990s. Flour-Fe/Grain-Fe, the ratio of iron concentration in flour and in grain.

Fe BV and Fe BVPG

The data for Caco-2 cell ferritin formation (Fe BV), Fe BVPG of the six flour samples from Anyang with 78% flour extraction rates, and their values relative to the reference control are summarized in Figure 1. The mean Fe bioavailabilities (Fe BV) ranged from 14% below to 54% above Yumai 18. The analysis of variance indicated significant differences in Fe BV and Fe BVPG among cultivars (P<0.01). Jingdong 8 and Zhongyou 9507 had significantly higher Fe BV (37% and 54% above) and Fe BVPG (103% and 154% above) than the reference control, followed by Zhongmai 9, whereas there were no significant differences between Zhongmai 175, Yumai 2, and Yumai 18. As indicated in Figure 2, there was no significant effect of location on Fe BV, but there was a significant effect of location on Fe BVPG. Samples of Zhongyou 9507 from Anyang had significantly higher Fe BVPG than those from the other two locations.

The Fe BV and Fe BVPG values of Yumai 18 and Zhongmai 175 at two flour extractions are compared in Table 2. The varieties differed only in Fe BV of Yumai 18 at different flour extraction.

There were no significant correlations between dialyzable Fe as measured by the amount of Fe in the bottom chamber at the end of the intestinal digestion period and Fe BV (r=0.35, P>0.05). Significant differences in dialyzable Fe were observed among flour samples. Jingdong 8 had the highest dialyzable Fe among the flour samples, followed by Zhongyou 9507 from the Urumqi location, whereas Yumai 18 with 40% flour extraction rate had the lowest value (Figure 3).

Table 2. Comparison of Fe BV and Fe BVPG between Flour Samples of Yumai 18 and Zhongmai 175 Milled at Different Flour Extraction Rates. Values are expressed as $\bar{x} \pm s$ (n=5)

Index	Yumai 18		Zhongmai 175	
	78% Flour Yield	40% Flour Yield	78% Flour Yield	40% Flour Yield
Fe BV (ng/mg)	47.00±6.50	57.29±6.91*	46.32±9.72	50.18±8.86
Fe BVPG (BV/g)	74.12±10.26	73.56±8.87	96.75±20.29	79.43±14.02

Note. Fe BV, Fe bioavailability, as ng of ferritin/mg cell protein, based on the same Fe content in each flour digest (10μg Fe/15mL digest). Fe BVPG, Fe bioavailability per gram of food. *P<0.05 compared with Yumai 18 (78% flour yield) in the same line.

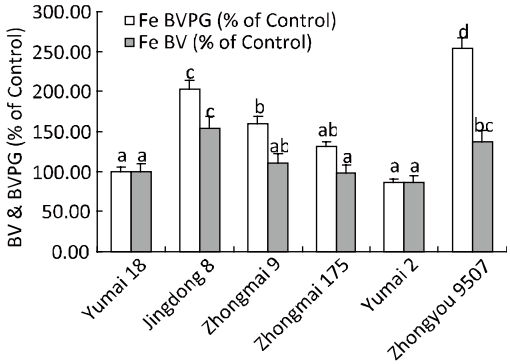

Figure 1. Fe BV and Fe BVPG (as percent of control, ie Yumai 18 with 78% flour yield from Anyang) in six flour samples with 78% flour extraction rates from Anyang. Fe BV, Fe bioavailability, as ng of ferritin/mg cell protein, based on the same Fe content from each flour digest (10 μg Fe/15 mL digest); Fe BVPG, Fe bioavailability per gram of food. Values ($\bar{x} \pm s; n = 5$) with no letters in common are significantly different (P<0.05).

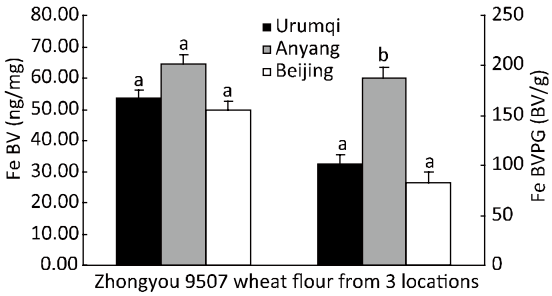

Figure 2. Comparison of Fe BV and Fe BVPG among Zhongyou 9507 flours milled at 78% extraction from three locations ie Urumqi, Anyang and Beijing in China, 2007-08. Left Y-axis, Fe BV, Fe bioavailability, as ng of ferritin/mg cell protein, based on the same Fe content from each flour digest (10 μg Fe/15 mL digest); Right Y-axis, Fe BVPG, Fe bioavailability per gram of food. Values ($\bar{x} \pm s; n = 5$) with no letters in common are statistically significantly different (P<0.05).

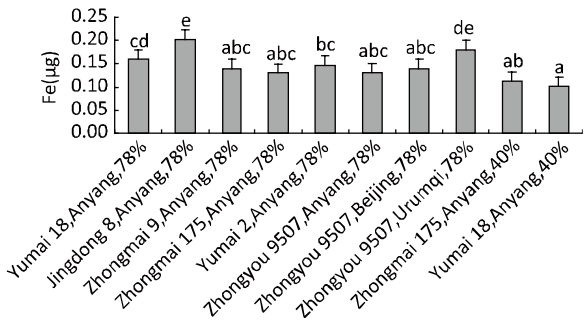

Figure 3. Total Fe in the bottom chamber at end of intestinal digestion period for the ten flour samples used in this study. Values ($\bar{x} \pm s; n = 5$) with no letters in common are significantly different (P<0.05). 78% and 40% are flour extraction rates.

Correlation of Fe Concentrations with PAP and Fe BV

There were no significant correlations among Fe concentration, PAP, and Fe BV (Table 3), whereas significantly positive correlations were observed between Fe in grain and flour samples and Fe BVPG.

Table 3. Pearson Correlation Coefficient among Fe and PAP Concentrations, Fe BV, and Fe BVPG in Ten Flour Samples

	Grain-Fe	Flour-Fe	Fe BV	Fe BVPG
Flour-Fe	0.62	-	-	-
Fe BV	0.48	0.47	-	-
Fe BVPG	0.64*	0.91**	0.78**	-
PAP	0.22	0.57	0.16	0.46

Note. PAP, phytic acid phosphorus. Fe BV, Fe bioavailability, as ng of ferritin/mg cell protein, based on the same Fe content in each flour digest (10 μg Fe/15 mL digest). Fe BVPG, Fe bioavailability per gram of food. * and ** indicate correlation coefficient significantly different from zero at P (probability of significance) <0.05 and 0.01 levels, respectively.

DISCUSSIONS

Genotype Screening, Relationships between Fe BV, Fe BVPG, and Dialyzable Fe

The comparison of Caco-2 cell ferritin formation, i.e., Fe BV, from each flour digest was based on the same Fe content (10 μg Fe/15 mL digest). However, there were large variations in Fe concentration among flour samples. Fe BVPG was a more convenient measure to compare the Fe BV of food and was thus used in previous reports by Glahn et al. in evaluations on maize and rice, in which 0.5 g maize or rice was used for each sample digestion[3,12]. Based on Fe BVPG of six cultivars from Anyang milled to the same flour extraction rate of 78%, Zhongyou 9507, was the most promising genotype for further evaluation with human subjects, followed by Jingdong 8. The flour samples of these two cultivars had higher Fe BVPG than the control by 103%-154%, and the flour-Fe concentrations were 32% and 85%, respectively, higher than the control. Based on agronomic data, Jingdong 8 also has high yield potential, resistance to powdery mildew, and tolerance to high temperatures during the grain filling stages[9], and it is therefore currently recommended as the most promising leading wheat cultivar for use as a donor parent in Chinese breeding programs targeting high Fe concentration.

There was no significant correlation between dialyzable Fe and ferritin values, consistent with previous reports[12,18,20-21]. It is likely that some of the soluble forms of Fe bound by phytic acid, i.e., monoferric phytate, could be soluble but not available[22]. Therefore, the initial *in vitro* method of simply using Fe dialyzability or Fe solubility as a marker of Fe availability could significantly overestimate the availability, and thus might predict the wrong direction of response[23].

Effect of Location, Flour Extraction Rate, and Other Potential Factors including PAP on Fe BV and Fe BVPG

The differences in grain-Fe and flour-Fe concentrations of Zhongyou 9507 at the three locations indicated that both the grain-Fe and flour-Fe concentrations were affected by planting sites, but no significant effect was detected on Fe BV (P>0.05) of samples from different locations. The results suggested that the growing environments have no influence on Fe BV, but a significant influence on Fe BVPG among cultivars from different environments was observed due to large differences in the Fe concentration[8]. Similar results were observed among early-maturing and late-maturing maize lines by Oikeh et al.[3,13]. The environment at Anyang appears to produce a higher wheat flour Fe BVPG in Zhongyou 9507. This merits further investigation. Thus high Fe content will be an important index to be considered along with Fe BV for selection of high Fe BVPG in wheat cultivars. It is important to test cultivars under representative environmental conditions to identify the most stable cultivars with high micronutrient concentrations due to the significant effects of environment on the wheat grain-Fe level and wheat flour-Fe level.

At present, little is known of the effects of the wheat flour extraction rate on Fe BV. Cultivars Yumai 18 and Zhongmai 175 at two different flour extraction rates (78% and 40%) were compared in the present study. Fe and PAP contents in both cultivars decreased with reduced flour extraction rates, whereas their Fe BV levels increased. This was particularly obvious in Yumai18 at the low-extraction rate; it had 22% higher Fe BV and 46% lower PAP levels than at the high-extraction level. The PAP content in Yumai18 at 40% extraction was only 0.53 mg/g, the lowest level among the ten samples and 34% lower than that of the experimental mean. These results indicated that low-extraction rates can decrease phytate contents in wheat flour and improve its Fe BV; however, such a strategy is commercially impractical and More research is therfore needed to confirm the results, because only two cultivars were used with two different extraction rates in the present study.

Both *in vivo* and *in vitro* studies have shown the inhibitory effects of phytic acid on nonheme Fe uptake[12,24]. Phytic acid is widely present in cereal grains and is the major factor for the low bioavailability of Fe from these foods. It accumulates in the bran layer of cereal grains and is significantly reduced during milling[25]. Clearly, phytate is one of the important Fe BV inhibitors, as reductions in phytate content in cereals lead to strong increases in Fe BV. However, phytate concentration was not consistently related to Fe BV in this study or in previous studies in rice[12,26]. This may be due to the limited range of variability in phytate concentrations among the ten wheat flour samples. The lack of a differential effect of phytate on Fe BV in these flour samples may also be due to phytate: Fe molar ratios (except for Zhongyou 9507 at Anyang, data not shown) in excess of 10:1 when maximum inhibition

of Fe uptake occurs *in vitro*[26].

Food processing methods are factors that need to be considered in reducing antinutrient concentrations, including phytate, and improving Fe BV. Fermentation, germination or soaking cereal grains before cooking improves the bioavailability of Fe[27]. The Fe BV in traditional Chinese steamed bread, one of the main end products of common wheat flour fermentation, is now being assessed in our lab by using the *in vitro*/ Caco-2 cell model.

In conclusion, this *in vitro* study indicates significant differences (P<0.01) in Fe BV among six wheat cultivars grown in a common location milled at the same flour extraction rate . Wheat flours from two varieties, namely Zhongyou 9507 and Jingdong 8, had Fe bioavailabilities 37%-54% higher than the Yumai 18 control. Their grain Fe contents ranging from 48 to 52 mg/kg, were 6%-14% higher than the experimental mean, and their flour Fe contents ranging from 20 to 29 mg/kg, were 10%-54% higher than the experimental mean. The growing environments had no effect on Fe BV, but did have a significant effect on Fe BVPG. Fe bioavailabilities in flour extracted at impractically low rates were higher than those in high-extraction flours. The promising cultivars will be further evaluated by human trials to determine their efficacy in improving Fe nutritional status in humans.

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