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Iron Bioavailability in Two Commercial Cultivars of Wheat: Comparison between Wholegrain and White Flour and the Effects of Nicotianamine and 2′-Deoxymugineic Acid on Iron Uptake into Caco-2 Cells

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ABSTRACT: Iron bioavailability in unleavened white and wholegrain bread made from two commercial wheat varieties was assessed by measuring ferritin production in Caco-2 cells. The breads were subjected to simulated gastrointestinal digestion and the digests applied to the Caco-2 cells. Although Riband grain contained a lower iron concentration than Rialto, iron bioavailability was higher. No iron was taken up by the cells from white bread made from Rialto flour or from wholegrain bread from either variety, but Riband white bread produced a small ferritin response. The results probably relate to differences in phytate content of the breads, although iron in soluble monoferric phytate was demonstrated to be bioavailable in the cell model. Nicotianamine, an iron chelator in plants involved in iron transport, was a more potent enhancer of iron uptake into Caco-2 cells than ascorbic acid or 2′-deoxymugineic acid, another metal chelator present in plants.

KEYWORDS: *iron, wheat, Caco-2 cells, phytate, nicotianamine, 2′-deoxymugineic acid, ascorbic acid*

INTRODUCTION

Iron deficiency anemia accounts for around 50% of all anemias,[1] with infants, children, and premenopausal women being most at risk. In both developing and developed countries the consequences of anemia result in significant health problems and economic cost.[2] One strategy for addressing the problem of iron deficiency is biofortification, with plant breeding or genetic engineering techniques being used to produce new types of staple foods (such as rice, wheat, maize, millet, and legumes)[3−6] with higher iron contents. This approach of increasing the micronutrient density of foods is particularly valuable in settings where food fortification or supplementation is not feasible.[7]

There is wide natural genetic variation between commercial cultivars of wheat grain with respect to total iron concentration, reported to range from 19 to 58 mg/kg.[8−10] This suggests that it should be possible to select iron-rich genotypes which can be exploited in breeding for biofortification. Furthermore, recent data show that the levels of iron in wholegrain are reflected in the white flour fraction, which is used to make many food products including white bread.[11] This is an important observation as the content and bioavailability of iron in food will be determined by the type of processing used. During milling of wheat, the outer layers of the grain and the embryo are removed to give the bran, leaving the starchy endosperm as the main component of white flour.[12] However, because the bran contains a higher concentration of iron,[13] the control of iron accumulation at sites within the grain is also of importance.[14]

The form in which iron is present within the grain affects its bioavailability, as does the presence of iron-binding complexes such as phytate. Most of the iron in cereal grain is bound to inositol hexakisphosphate (IP_6) or pentaphosphate (IP_5) and forms phytate salts.[15] Minerals bound to phytate are not accessible to iron transporters in the human gut due to the lack of specific enzymes that cleave the iron−phytate complexes,[16] but there is evidence that phytates may be either insoluble or soluble, depending on the nature of the bonding. Soluble salts in the form of monoferric phytate (MFP)[15,17] may be a bioavailable source of iron.[18]

In plants, the metal chelator nicotianamine (NA),[19] formed by NA synthase from three molecules of *S*-adenosylmethionine,[20] is involved in the intra- and intercellular transport of metal cations. The presence of elevated levels of NA in the grain has also been reported to enhance iron uptake in both cell and murine models.[19,21] Another chelator of iron uptake is 2′-deoxymugineic acid (DMA), which is synthesized via NA aminotransferase in graminaceous plants.[22,23] DMA is a phytosiderophore, and thus has a high affinity for ferric iron, and plays an important role in the solubilization and acquisition of iron(III) by the plant from the rhizosphere.[23,24] In a previous study using SEC-ICP-MS to measure the iron speciation in Rialto and Riband,[11] we found that iron complexed with NA/DMA accounted for 19.3 and 32.1% of the total iron in the

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white flour fraction in Rialto and Riband, respectively. In the whole grain, Fe-NA/DMA accounted for 5.3 and 7.3% of the total iron in Rialto and Riband, respectively. Our method did not allow a separation of Fe−NA from Fe−DMA as the two complexes are very similar. Therefore, increasing NA and/or DMA is another strategy for increasing bioavailable iron in plant foods, but this requires the use of transgenic technologies.

The aim of this research was to investigate whether there are differences in iron availability between unleavened bread made from wholegrain and white flours from two commercial wheat cultivars differing in the total iron concentration (Rialto, a hard wheat used for bread, which is high in iron; and Riband, a soft wheat used for biscuits and cakes, which is low in iron). Caco-2 cells are widely used as an in vitro model to study iron bioavailability,[25−27] and previous studies have shown good agreement between in vitro results (using this particular cell line) and in vivo determination of iron bioavailability from food in the presence of enhancers and inhibitors[28,29] (e.g., ascorbic acid (AA) and calcium[30,31]). We have therefore used Caco-2 cells, in combination with simulated gastrointestinal digestion of unleavened bread, to determine the availability of iron from MFP, iron(II) chloride, and iron(II) sulfate and the effects of NA, DMA, and AA on iron uptake into the cells.

MATERIALS AND METHODS

Preparation of Grain Materials and Unleavened Bread. Two commercial wheat cultivars (Rialto and Riband) that accumulate different levels of iron in their grain[10] were grown in field trials at Rothamsted Research in 2010, as described by Shewry et al.[32] Grains were milled in a Buhler mill (MLU-202) at Campden BRI, Gloucestershire, UK, to obtain whole wheat (100% extraction) and white flour (Riband, 38 ± 2.1% extraction; Rialto, 41 ± 3.4% extraction). Unleavened bread was made on a small scale: 20 g of flour was placed in a silica crucible, 5.76 mL of deionized water and 0.4 g of salt were added, and the mixture was kneaded into dough. The dough was left to proof at 30 °C for 30 min before being baked at 200 °C for 15 min. The breads were then broken up by hand into smaller pieces, approximately 0.5 cm^3, before flash freezing in liquid nitrogen and freeze-drying for 72 h. Samples were then homogenized and ground to a fine flour in an agate ball mill (Retsch PM400, Düsseldorf, Germany). The characteristics of the flours and unleavened bread samples are summarized in Table 1.

Table 1. Iron and Phytate Content of Rialto and Riband Flours and Unleavened Breads

flour type	iron (mg/kg) in flour[a]	phytate (g/kg dry wt) in unleavened bread[b]	phytic acid/iron molar ratio
Rialto white	11.9 ± 0.8	1.0 ± 0.1	7.13
Rialto wholegrain	46.7 ± 4.3	6.4 ± 0.2	11.60
Riband white	6.7 ± 0.5	0.4 ± 0.01	5.08
Riband wholegrain	30.3 ± 1.7	4.5 ± 0.2	12.57

[a]Total Fe (±SE, n = 3) determined by ICP-MS. [b]Analysis of phytate was performed using a commercially available kit (K-PHYT 12/12 Megazyme, Ireland), as per the manufacturer's instructions. Eiither 0.5 of 1.0 g of sample was extracted with 10 mL of HCl followed by an enzymatic dephosphorylation step with phytase and alkaline phosphatase and precipitation using a color reagent prepared from AA in sulfuric and ammonium molybdate. The absorbance (655 nm) of free phosphorus and total phosphorus (±SE, n = 3) was compared to phosphorus standards using a Varioskan spectrometer (Thermo, Finland).

Solutions of Ascorbic Acid, Monoferric Phytate, Nicotianamine, and 2′-Deoxymugineic Acid for Caco-2 Cell Studies. In Caco-2 cell studies, it is common practice to add AA to give an AA/Fe molar ratio in the range of 10−20:1, the amount being calculated on the basis of the concentration of iron in the samples.[26,33] However, as there was wide variation in total iron in the unleavened bread samples, ranging from 6.65 to 46.7 mg/kg, a standard amount of AA (880 μM, AA dissolved in Milli-Q water) was added to each gram of unleavened bread. Although this meant that some samples had a higher AA/Fe ratio than others (Table 2), the amount of AA added was high enough to ensure that it achieved maximal effect on iron uptake into Caco-2 cells, as observed in previous studies.[34]

Table 2. Iron and Ascorbic Acid (AA) Concentrations of Unleavened Bread Digests

unleavened bread (1 g)	Fe content (μg)	Fe content (μmol/L)	AA (μmol/L)	Fe/AA molar ratio
Riband white	6.7	8.0	880	1:110
Rialto white	11.9	14.2	880	1:62
Riband wholegrain	30.3	36.2	880	1:24
Rialto wholegrain	46.7	55.7	880	1:16
Riband white fortified	36.7	43.8	880	1:20
Rialto white fortified	41.9	50.0	880	1:18
Riband wholegrain fortified	60.3	72.0	880	1:12
Rialto wholegrain fortified	76.7	91.6	880	1:10

Monoferric phytate (MFP) was prepared from a solution of 100 mL of 0.5 M acetic acid, 10 g of sodium phytate, and 1 g of ferric chloride. The mixture was stirred for 2 h until all of the sodium phytate was completely dissolved, 100 mL of 95% (v/v) ethanol was added, and the solution was left overnight. The precipitate was then collected by centrifugation (5000g for 10 min) (Medowrose Scientific Ltd., Oxford, UK), washed three times with acetone, allowed to air-dry, and stored in a desiccator.[35] The final product was analyzed by inductively coupled plasma atomic emission spectroscopy after digestion in ultrapure HNO_3 and $HClO_4$ (87:13% v/v).

Commercial sources of DMA and NA, with certified chemical composition (see TOC graphic), were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Both DMA and NA form 1:1 molar complexes with Fe. The F (II)−NA solution was prepared from ferric chloride (Sigma-Aldrich, Vienna, Austria), and Fe(III)−DMA was prepared from ferrous sulfate (Sigma-Aldrich). Each of the compounds was dissolved separately in Milli-Q water, and appropriate solutions were then mixed immediately to avoid oxidation. The molar concentrations are given in the captions of the figures.

Cell Culture Procedures. Caco-2 cells were grown in collagen-coated six-well plates (Greiner, Dungannon, UK) at a density of 4.75 × 10^4 in 2 mL of Dulbecco's modified Eagle's medium (DMEM) (LGC, Teddington, UK) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5 mL of 5000 u/mL penicillin/streptomycillin solution (Gibco, Paisley, UK), and 5 mL of 100× nonessential amino acids (Sigma, Dorset, UK). Media were replaced every 2 days. Cells between passages 27 and 34 were used for experiments at 13 days post seeding, and 24 h prior to experimentation, cells were switched to serum-free medium (minimum essential medium, MEM)[37] supplemented as above with the exception of fetal bovine serum. Experiments without a simulated digestion phase were initiated by the addition of fresh serum-free medium containing the appropriate treatment. After 24 h, the treatment medium was aspirated from the six-well plates, and the cells were washed twice with 2 mL of PBS. Deionized water was then applied to each well, and the Caco-2 cells were scraped off using an inverted 10 μL pipet tip. The cell suspension from each well was sonicated on ice, three times for 5 s, using a probe sonicator and stored at −20 °C.

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Initially, the simulated digestion procedures were identical to those reported by Glahn et al.[27,38] However, due to the low iron content of the flour samples, the sample volume was increased from 0.5 to 1 g and subsequently to 3 g, which produced a thick digestate, so the duodenal phase of the simulated digestion was performed in a test tube for 2 h and not above the Caco-2 monolayer (the usual procedure in Glahn's assay). Experiments with unleavened bread were initiated with iron deprivation of the cells by switching from supplemented DMEM to MEM. Twenty-four hours of simulated digestion[27,37] was then carried out with the following modification: the duodenal phase of digestion was continued for 2 h in test tubes (rather than over the cells) at 37 °C on a rotating table, and once completed all samples were centrifuged at 21000*g* for 10 min to remove any sample residue, which could have resulted in physical blockage of the dialysis membrane. After centrifugation, 10% of the resulting supernatant from each treatment was applied to the dialysis membranes and placed over the appropriate wells. Cells were incubated for 1 h with the supernatant at 37 °C on a rotating table at a speed of 20 oscillations per minute, after which time the digests were removed and the cells were incubated for a further 23 h. Then they were harvested, sonicated, and frozen at −20 °C. For the analysis sonicated cell lysates were defrosted at room temperature and kept on ice, and a spectroferritin ELISA assay (Ramco Laboratories, Stafford, TX, USA) was carried out 24 h post sonication, according to the manufacturer's instructions. Total protein was quantified using a BCA protein assay (Pierce, Rockford, IL, USA), and iron availability assessed from the ferritin content (expressed as ng/mg protein), a surrogate measure of iron bioavailability.[38,39]

Experiments Using Ascorbic Acid, Monoferric Phytate, Nicotianamine, and 2-Deoxymugineic Acid. A series of experiments were also performed without a simulated digestion. Solutions containing NA, DMA, and MFP were prepared in Milli-Q water. When appropriate, AA was added either at 1:9 molar ratio (Figure 4) or various ratios (Figure 5). The pH was adjusted to 2, and the samples were incubated for 1 h at 37 °C. The samples were diluted with MEM to the desired iron concentrations (1.6 or 3.11 μg Fe/mL) and incubated in a water bath at 37 °C for 1 h. Because small concentrated volumes of NA, DMA, or MFP solutions were used (600 μL were diluted in 6 mL), they did not markedly affect the pH of the treatment solutions (the final pH was 6.9−7.0). Before addition to the cell monolayers, all treatment solutions were filtered with 0.22 μm syringe filters (Merck Millipore, Watford, UK). The cells were incubated for 24 h and then harvested, sonicated, and analyzed as described above.

Statistical Analysis. Unless otherwise stated, all statistical analyses were performed using SPSS Inc. (Chicago, IL, USA; version 16.0.0). One-way ANOVA with Tukey's post hoc was used to examine pairwise differences on power-transformed data. Data are presented as the mean ± SD. Differences were considered significant at $p < 0.05$.

RESULTS

Caco-2 Ferritin Response to Unleavened Flat Bread Samples: Simulated Digestion Experiments. Data obtained from 1 g samples of unleavened breads (Figure 1) showed that significantly more ferritin was produced with white Riband flour than with wholegrain Riband and Rialto flours, in the presence of AA ($p < 0.005$ and $p = 0.004$, respectively). There were no significant differences in ferritin formation between the wholegrain and white flour breads from the two cultivars. When 3 g samples were used (Figure 2), the cells exposed to white unleavened bread (Rialto and Riband) produced significantly more ferritin protein than cells exposed to wholegrain breads ($p < 0.0005$ in both). Cells exposed to white bread from Riband produced significantly more ferritin than cells exposed to the other breads ($p < 0.0005$ in all comparisons).

To increase the cell response further, all flour samples were supplemented with ferrous sulfate with 30 mg Fe/kg prior to the production of the bread (Figure 3). There was a significantly higher cell ferritin response to unleavened bread made from white than from wholegrain flour, for both Rialto and Riband ($p < 0.0005$ for all comparisons). Cells exposed to Riband wholegrain bread produced significantly more ferritin than cells exposed to Rialto wholegrain bread ($p = 0.001$). The cell ferritin response to the positive control (53.54 μM $FeSO_4$) was not significantly different from the cell response to Riband white bread, but was significantly higher than the response to Rialto white and wholegrain and to Riband wholegrain breads ($p = 0.049$, $p < 0.0005$, and $p < 0.0005$, respectively). The cell response to unleavened bread made from Rialto wholegrain flour was not significantly different from the blank (17.7 ± 2.5 ng/mg total protein).

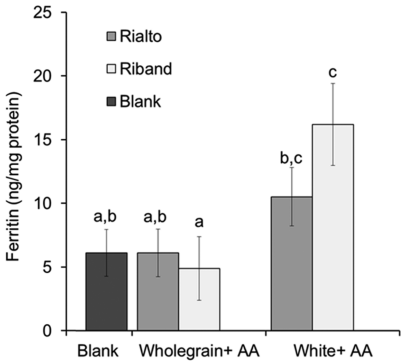

Figure 1. Ferritin concentration in Caco-2 cells exposed to digests prepared from 1 g of unleavened bread made from Rialto and Riband white and wholegrain flours in the presence of 880 μmol/L ascorbic acid (AA), expressed as ferritin concentration (ng/mg total protein). Data represent the mean ± SD ($n = 6$). Bars without a common letter (a−c) are significantly different, $p < 0.05$.

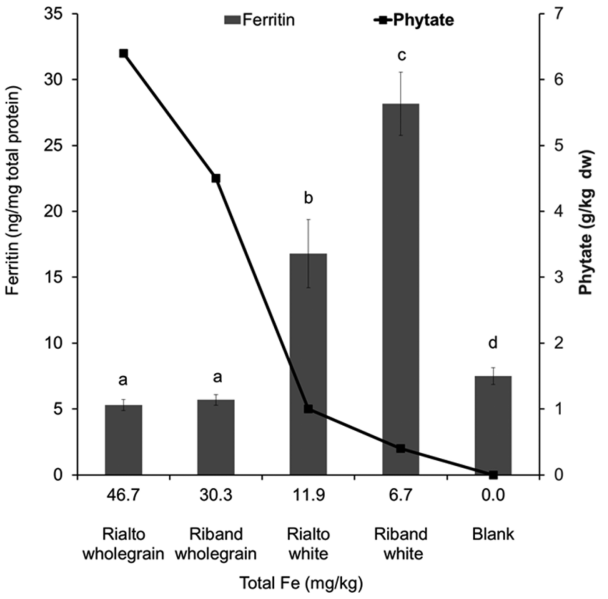

Figure 2. Ferritin concentration in Caco-2 cells exposed to digests prepared from 3 g of unleavened bread made from Rialto and Riband white and wholegrain flours in the presence of 880 μmol/L ascorbic acid (AA) expressed as ferritin concentration (ng/mg total protein, gray bars, *y*-axis). The phytate content (g/kg dry weight) of the unleavened bread is shown on the second *y*-axis and the iron content (mg/kg) on the *x*-axis. Data represent the mean ± SD ($n = 6$). Bars without a common letter (a−c) are significantly different, $p < 0.05$.

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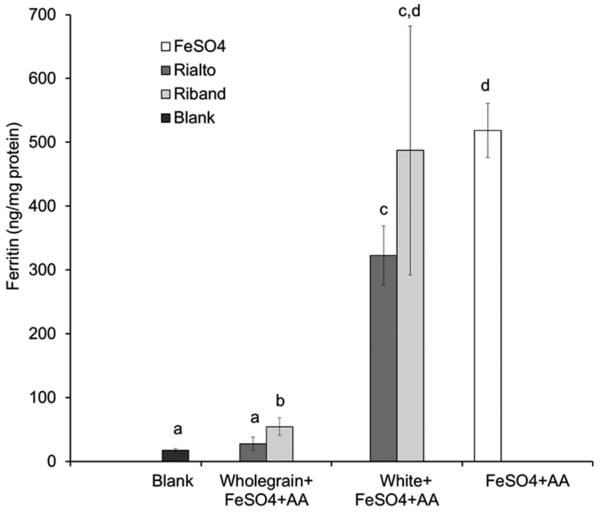

Figure 3. Ferritin concentration (ng/mg protein) in Caco-2 cells exposed to digests prepared from unleavened bread made from Rialto and Riband white and wholegrain flours in the presence of 880 μmol/L ascorbic acid (AA) and 53.54 μM ferrous sulfate ($FeSO_4$). Data represent the mean ± SD (n = 6). Bars without a common letter (a–d) are significantly different, $p < 0.05$.

Cell Ferritin Response to Iron(II), Iron(III), or MFP Treatments with Nicotianamine, Ascorbic Acid, 2-Deoxymugineic Acid, or Nicotianamine plus Ascorbic Acid. AA was present in the treatments (Figure 4) at a ratio of approximately 1:9 (Fe/AA), whereas DMA or NA was present at a ratio of 1:1 (Fe/NA or DMA). The cell ferritin response to iron with NA (Figure 4) was significantly higher than to iron with DMA ($p < 0.0005$), suggesting that NA is a more potent enhancer of iron uptake than DMA (at the tested molar ratio). The response was also significantly increased when AA was added to the iron with DMA ($p < 0.0005$), but further addition of NA had no extra effect.

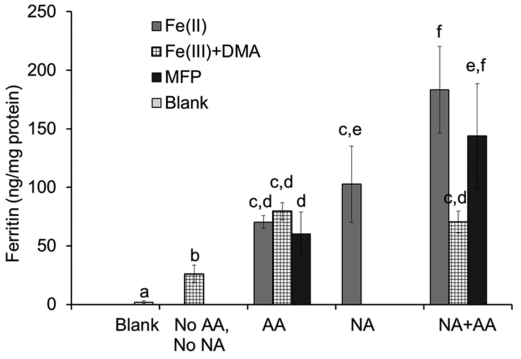

Figure 4. Ferritin concentration (ng/mg protein) in Caco-2 cells exposed to 1.6 μg of ferrous chloride (Fe(II)), ferric chloride (Fe(III))–DMA, or MFP in the presence of NA (at 1:1 Fe to NA ratio) or AA (at 1:9 Fe to AA ratio). Cell response is expressed as ferritin concentration (ng/mg total protein). Data represent the mean ± SD (n = 6). Bars without a common letter (a–d) are significantly different, $p < 0.05$.

The cell ferritin responses to MFP plus AA and iron(II) chloride plus AA were not significantly different, suggesting that the availabilities are similar. Also, further addition of NA to MFP plus AA or to iron(II) chloride plus AA significantly increased ferritin formation in cells ($p < 0.0005$ in both comparisons).

An experiment (Figure 5) was also designed to determine whether there was a dose–response effect of NA and to compare it with AA, the most potent known enhancer of iron uptake. Cells exposed to iron in the presence of increasing NA concentrations (from 1:0.25 Fe/NA molar ratio through 1:0.5 to 1:1 molar ratio) produced significantly more ferritin with increasing NA ($p < 0.0005$, $p = 0.001$, and $p = 0.004$, respectively). When a higher Fe/NA molar ratio of 1:4 was used, the ferritin levels were not significantly different from those in cells exposed to a 1:0.5 Fe/NA molar ratio. The cell ferritin response was positively related to AA concentration (from 1:0.25 Fe/AA molar ratio through 1:4 and 1:10 molar ratio; $p = 0.002$, $p < 0.0005$, and $p < 0.0005$, respectively). Cells exposed to the highest molar ratio of Fe to AA (1:10) produced ferritin at similar levels to cells exposed to the lowest molar ratio of Fe to NA (1:0.25), indicating that NA is a more potent enhancer of iron bioavailability than AA and evoked the highest ferritin response.

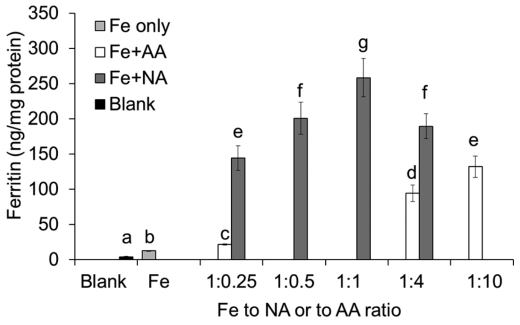

Figure 5. Ferritin concentration in Caco-2 cells exposed to 3.11 μg (13.88 μM) of ferrous sulfate ($FeSO_4$) with or without NA at Fe/NA ratios of 1:0.25, 1:0.5, 1:1, and 1:4 or AA at Fe/AA ratios of 1:0.25, 1:4, or 1:10. Cell response is expressed as ferritin concentration (ng/mg total protein). Data represent the mean ± SD (n = 6). Bars without a common letter (a–f) are significantly different, $p < 0.05$.

DISCUSSION

Unleavened bread made with white flour from the wheat cultivar Riband contained more available iron (when in the presence of AA) than the other breads tested, despite having the lowest iron content of all samples. A likely explanation for this is that it contained the lowest phytate content and lowest phytic acid/iron molar ratio (Table 1); there was an inverse relationship between cell ferritin response and phytate content (Figure 2). When unleavened bread was supplemented with iron (30 μg/g), together with AA (Figure 3), the same trend was observed.

Another contributory factor to the difference in iron availability between bread made from white and wholegrain flour may be the content of phenolic acids. Although not analyzed in our samples, limited data in the literature suggest that white flour has a much lower concentration of phenolic acids[40] as they are found primarily in the bran fraction.[41] Nevertheless, the difference observed between bread made from Riband and Rialto white flour is unlikely to be explained by their content of phenolic acids. Fernandez-Orozco et al.[42] analyzed flour from a number of locations and years and found no differences in the content of free phenolic acids between Riband and Rialto wholegrain flours (both contained 12 ± 7

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μg/g), but Riband (990 ± 87 μg/g) had a significantly higher total phenolic acids content ($p = 0.026$) than Rialto (833 ± 118 μg/g). As the ferritin response was higher in Riband, assuming typical phenolic acids concentrations in the wheat samples used in these experiments, this indicates that phytate is the main determinant of iron bioavailability.

The cell ferritin response was inversely proportional to the concentrations of phytate and iron in the breads tested. However, because the iron contents of the samples were not the same, the AA/Fe molar ratios in the samples differed (Table 2), with the highest AA/Fe ratio being present in the sample with the lowest iron content (Riband white unleavened bread). The rationale for using the same amount of AA for all samples is that when AA is used in excess/ it should have a similar effect across all iron concentrations.[34]

Since 1953, it has been mandatory in the United Kingdom to add iron to white and brown flours to restore the content to the levels found in wholegrain flour (16.5 mg kg^{-1}).[43,44] The iron present in wholegrain products is not bioavailable due to the presence of inositol phophates IP_6, IP_5, IP_4, and IP_3, which bind iron tightly. However, iron present in monoferric phytate, found in wheat,[35] appears to be more bioavailable (Figure 4). In dogs, monoferric phytate has been shown to be about 50% as available as ferrous sulfate at a low dose of iron (1.5 mg), although only one-seventh as available at a higher dose of iron (15 mg).[36]

NA had a clear enhancing effect on iron availability, being a more potent enhancer of iron uptake than DMA (Figure 4) and AA (Figure 5) (as also reported by Zheng et al.[21] for rice flour). The lowest molar ratio of Fe/NA that was tested (1:0.25) had an effect similar to that of AA at a 1:10 Fe/AA molar ratio (Figure 5).The most effective Fe/NA molar ratios observed in this study were 1:1 to 1:4 (Figure 5). Zheng et al.[21] also reported that a 1:1 Fe/NA molar ratio was the most effective for wild type rice and that a 1:4 Fe/NA molar ratio was most effective for a transgenic rice grain expressing an additional nicotianamine synthase (*NAS*) gene. However, these results cannot be directly compared with those presented here as the iron compounds were examined in the absence of a food matrix and the test materials were not subjected to a simulated digestion procedure.

In the samples studied the phytate levels had more influence on iron bioavailability than total iron, a finding that should be taken into account in the development of biofortification strategies to reduce iron deficiency anemia. Our results also demonstrated that MFP with AA provoked a similar cell ferritin response to iron(II) chloride plus AA, whereas endogenous phytate (mainly IP_6 and IP_5) in wheat flour prevented iron from being taken up into Caco-2 cells.

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Notes

The authors declare no competing financial interest.

‖T.E. and A.A.W. are joint first authors.

ABBREVIATIONS USED

AA, ascorbic acid; MFP, monoferric phytate; NA, nicotianamine; DMA, 2′-deoxymugineic acid; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium

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