British Journal of Nutrition (2005), **93**, 65–71
DOI: 10.1079/BJN20041294

Iron availability of a fortified processed wheat cereal: a comparison of fourteen iron forms using an *in vitro* digestion/human colonic adenocarcinoma (CaCo-2) cell model

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(Received 14 August 2003 – Revised 3 September 2004 – Accepted 4 September 2004)

In this three-phase study we first compared the availability of fourteen Fe forms in a wheat-based ready-to-eat breakfast cereal using an *in vitro* digestion/human colonic adenocarcinoma (CaCo-2) cell model. We then investigated the effect of milk and/or coffee on those fortified cereals found in phase 1 to show promising increases in Fe availability. The Fe forms assessed in phase 1 were reduced (control), carbonyl, electrolytic, $FePO_4$, $FeSO_4$, $FeCO_3$, Na_2-FeEDTA, Ferrochel® (Albion Laboratories, Clearfield, UT, USA; ferrous bis-glycinate), encapsulated ferrous fumarate, $FeSO_4$, ferrous lactate and Biofer® (LipoTech, Britwell Salome, Oxfordshire; $FeSO_4$), SQM® (Sea-Questra-Min Iron; Quali Tech, Chaska, MN, USA; polysaccharide-complexed $FeSO_4$) and Sun Active® (Taiyo Kagaku, Yokkaichi, Japan). All these forms increased Fe uptake compared with the unfortified cereal. Relative to the control, the following increases in Fe availability were observed: electrolytic, 52 %; ferrous fumarate, 30–35 %; Sun Active, 78 %; Ferrochel, 125 %; Na_2FeEDTA, 291 %. Recent human studies have shown similar data with regard to Ferrochel, $FeSO_4$ and Na_2FeEDTA, with the latter being more bioavailable. Our phase-2 studies indicated that the addition of milk to $FeSO_4$-fortified cereal increased Fe availability, but this availability was markedly decreased by the addition of coffee to the digest. Conversely, a loss in availability from Na_2FeEDTA was observed with the addition of milk; however, the addition of coffee did not markedly affect Fe availability from this form. In phase-3 studies we observed increased Fe availability upon the addition of milk to cereals containing Ferrochel, $FeSO_4$, Sun Active, a mixture of reduced Fe and Na_2FeEDTA or reduced Fe. For these forms we did not assess the behaviour after the addition of coffee. In conclusion, when considering possible fortificants for optimizing Fe bioavailability within a foodstuff, it is of paramount importance to consider the interaction between the fortified foodstuffs and other components of the meal (such as milk and coffee with a breakfast).

Iron availability: *In vitro* digestion: CaCo-2: Wheat cereals: Iron fortification

Ready-to-eat breakfast cereals are a popular food and a good vehicle for Fe fortification. Elemental Fe powders are used widely in breakfast cereals as they are inexpensive and non-reactive. In general, these forms are regarded as low in bioavailability relative to other forms of Fe such as $FeSO_4$, ferrous fumarate and Na_2FeEDTA (Hurrell, 2002). Moreover, the bioavailability of elemental Fe is subject to naturally occurring inhibitory factors in cereal grains and in a varied diet, such as phytates and tannins. This and marketing strategies have spurred increased research into more available forms of Fe in specific food products.

Previous work has revealed that Na_2EDTA enhances the availability of Fe in breakfast cereals (MacPhail *et al.* 1994). Presumably this enhancement is due to the ability of EDTA to remain bound with Fe at the low-pH conditions in the stomach. Fe does not easily dissociate from EDTA at low pH and thus is not highly exchangeable with the inhibitory factors present in the stomach from dietary sources. However, upon delivery to the upper intestine (where the pH is near neutral) the Fe becomes dissociated from the EDTA molecule and can thus interact with the luminal contents (Lynch *et al.* 1993). Depending on the nature of the luminal contents, the Fe can become more or less available, particularly if binding does not occur with inhibitors such as phytic acid or polyphenolic compounds. It is thought that, by avoiding complexation to inhibitors in the stomach and upper intestine, Fe from Na_2FeEDTA results in more Fe being available for absorption. Na_2FeEDTA is organoleptically acceptable and is stable in cereals under normal storage conditions, making it an ideal candidate for cereal fortification.

The human colonic adenocarcinoma (CaCo-2) cell line is a useful model for studies of intestinal Fe uptake in man (Alvarez-Hernandez *et al.* 1991; Han *et al.* 1994). In combination with *in vitro* digestion techniques, an effective model has been developed to study Fe availability of foods (Glahn *et al.* 1998). The method has been developed with sample homogeneity in mind; we believe the physical sample/digest mixing steps give adequate mixing to allow its application as a screening tool to

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pre-empt (and reduce costs of running) human feeding trials, for example by selecting the best fortificants of a breakfast cereal. In this model system, foods undergo simulated peptic digestion followed by intestinal digestion in the presence of CaCo-2 monolayers. This system provides a measurement of Fe uptake by living cells, in addition to the measurement of Fe solubility (Miller & Berner, 1989; Glahn *et al.* 1998, 1999, 2000). The system is unique in that it allows uptake to occur simultaneously with food digestion under the normal physiological pH conditions associated with the absorptive surface of the intestinal tract. Cellular Fe is stored in the form of the protein–Fe complex, ferritin. The formation of ferritin is a sensitive and proportional measure of Fe uptake as validated by numerous studies (Gangloff *et al.* 1996; Glahn *et al.* 1998, 1999, 2000). Use of this method negates the need for radiolabelling of the food Fe.

Although it is important that human and cell culture comparison studies are made, it is not prudent to rely on human–rat study comparisons. Rats have generally proved to be poor models for human Fe absorption as influenced by dietary enhancers and inhibitors, and thus we have not contemplated these. Directly comparative studies between human and CaCo-2 cell Fe uptake are currently few in number; however, the studies we have completed support our opinion that our model is a good indicator of Fe metabolism in man. Yun *et al.* (2004) showed that the model is able to predict the effects of ascorbic acid and polyphenolic compounds on Fe bioavailability in man. In addition, samples of duplicate diets from a human study conducted by Harvey *et al.* (2004) at the Institute of Food Research, UK were also analysed using our *in vitro* model. The results were comparable with those from the human data (Harvey *et al.*, unpublished results), mirroring changes in Fe bioavailability between vegetarian, poultry- and meat-based diets. Many other studies in the literature have not investigated the Fe compound within a whole food matrix, and thus comparisons between studies are not as straightforward as they could be. For example, other ingredients in the foodstuff such as Zn and phytic acid can interfere with Fe absorption. It has also been reported that particle size of the electrolytic Fe can affect uptake; up to a 50 % difference in uptake has been seen (Hoglund & Reizenstein, 1969). It may well be that the model system is more sensitive to some Fe forms than others, or that the effect of the food matrix is more sensitive within the system.

The present study was divided into three phases. In phase 1, we compared the bioavailability of Fe from cereals fortified with fourteen Fe forms. No milk was added to these experimental samples. In phase 2, we compared the bioavailability of cereal fortified with either $FeSO_4$ or $Na_2FeEDTA$ in the presence and absence of coffee and/or milk. In phase 3, we assessed the availability of six Fe-fortificant forms in the presence and absence of milk in the digest. The primary objective was to compare the availability of commercially available forms of Fe added to a wheat-based ready-to-eat breakfast cereal, and to provide a ranking of the Fe forms in this food matrix. This research represents the first systematic comparison of numerous forms of Fe in the same food product.

Materials and methods

Chemicals, enzymes and hormones

Unless otherwise stated, all chemicals, enzymes and hormones were purchased from Sigma Chemical Co. (St. Louis, MO, USA). A complete list of the Fe forms and suppliers is provided in Table 1.

Table 1. Iron compounds used to fortify ready-to-eat wheat-based cereals (prior to testing them using the *in vitro* digestion/human colonic adenocarcinoma (CaCo-2) cell model); form, supplier and iron concentration

Compound	Supplier	Iron concentration (mg Fe/g compound)
Reduced forms		
Reduced Fe (control)	ADM Arkady, Olathe, KS, USA	1009·6
Electrolytic Fe	ADM Arkady	967·9
Carbonyl Fe	BASF Corporation, Washington, NJ, USA†	964·9
Encapsulated forms		
SQM® Sea-Questra-Min Iron (ferrous sulphate and polysaccharide complex)	Quali Tech, Chaska, MN, USA	126·3
Biofer® (ferrous sulphate and vitamin C in soya lecithin liposomes	LipoTech, Oxfordshire, UK	205·8
Ferrochel® (ferrous iron with glycine matrix)	Albion Laboratories, Clearfield, UT, USA	204·2
Sun Active® (ferrous pyrophosphate in glycerol esters of fatty acids and enzymatically hydrolysed lecithin)	Taiyo Kagaku, Yokkaichi, Japan	81·0
Ferrous sulphate	Wright Nutrition, Crowley, LA, USA	205·8
(50 %) Ferrous lactate (vegetable protein encapsulation; Supercoat™)	Wright Nutrition	1·68*
(65 %) Ferrous fumarate (vegetable protein encapsulation; Supercoat™)	Wright Nutrition	178·8
(85 %) Ferrous fumarate (vegetable protein encapsulation; Supercoat™)	Wright Nutrition	251·6
Iron salt forms		
$FeSO_4.7H_2O$	Sigma-Aldrich, St. Louis, MO, USA	192·4
$FeCO_3$	Wright Nutrition	0·80*
$FePO_4$	Chemische Fabrik Budenheim, Budenheim, Germany	0·53*
Sodium ferric EDTA	Sigma-Aldrich	127·9

* mg Fe/g dough

† See Huebers *et al.* 1986.

Cereal sample preparation

Ready-to-eat wheat-based breakfast cereals were produced, using a breakfast cereal formula, in the General Mills pilot facilities (Minneapolis, MN, USA). With the exception of the Fe fortificants, all other ingredients were as used in their regular product. Each batch of cereals contained cereals formulated either unfortified (no added Fe) or fortified with reduced Fe (reference control) or one of the compounds for investigation. A batch is defined as the cereals produced during a single day of formulation. After formulation, the samples were ground and homogenized and subsamples were ashed for subsequent Fe content analysis using inductively coupled plasma–emission spectroscopy (ICAP model 61E Trace Analyser; Thermo Jarrell Ash Corporation, Franklin, MA, USA). For this study a level of 0·3 mg Fe/g cereal was required, this being the level of Fe in fortified ready-to-eat cereals. Cereal samples exceeding this level were adjusted by dilution using the 'no added Fe' cereal samples from the same batch (i.e. the base cereal used to make the fortified cereals). Dilution factors were calculated taking into account the Fe naturally present in the 'no added Fe' cereals. Without fortification, the processed wheat sample contained 0·056 mg Fe/g cereal. Thus, with a target fortification value of 0·3 mg Fe/g cereal, the fortified Fe represented 81 % of the total Fe of the cereal. Post dilution (i.e. after correction to 0·3 mg/g) the samples were re-homogenized. All cereal samples were stored at −20°C.

Within batches the samples were randomly assigned to the uptake experimental runs and were spread evenly with regard to time from formulation, thus minimizing any time-related experimental influence. The batches were run sequentially. Batches 3 and 4 were analysed concurrently but were from different manufacture batches; phytate analysis showed no difference in their inositolphosphate 5 or inositolphosphate 6 content.

Cell culture

CaCo-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17, and maintained in a humidified chamber at 37°C and 5 % CO_2 in Dulbecco's modified Eagle medium (GIBCO Inc., Grand Island, NY, USA) with 10 % v/v fetal calf serum (GIBCO Inc.), 25 mM-HEPES and 1 % antibiotic antimycotic solution (GIBCO Inc.); the medium was changed every other day. For Fe uptake experiments the cells were used between passages 30 and 35 and at 14 d post seeding in collagen-treated six-well plates (Costar Corp., Cambridge, MA, USA) at a seeding density of 50 000 cells/cm^2. 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.

Experimental procedure

Sample digests were made following the procedure of Glahn *et al.* (1998, 1999), as summarized in Fig. 1. The digests contained 0·5 g fortified cereal and, if required by the design, 2·1 ml skimmed milk (Cornell Dairies, Ithaca, NY, USA) and/or 5 ml coffee (Folgers instant coffee, Cincinnati, OH, USA, 0·56 g/100 ml, 140 mM-KCl, 5 mM-1,4-piperazinediethanesulphonic acid at pH 2·0). All samples (excluding cereal with no added FE) had an equivalent amount of Fe (0·3 mg Fe/g cereal = 0·15 mg/digest). In phase 2, the control ($FeSO_4$) also contained 0·15 g/digest. After a 1 h

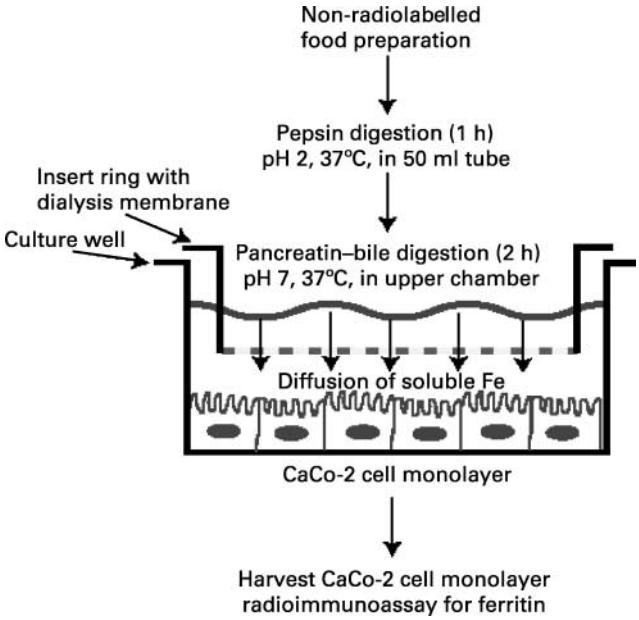

Fig. 1. Schematic diagram of the experimental procedure used with the *in vitro* digestion/human colonic adenocarcinoma (CaCo-2) cell model.

pepsin digest stage (in a 50 ml Falcon centrifuge tube; pH 2, 37°C and agitated at 110 oscillations/min), the samples were pH-adjusted and a pancreatin–bile mix added (final pH, 7). All the digest samples were adjusted to a final volume of 15 ml before addition to the cell culture model; this mixture was referred to as the 'intestinal digest'. The cell monolayer was washed with minimum essential medium (#41500; GIBCO Inc.) at pH 7 and 1 ml fresh medium added. To create a two-chamber six-well plate system, a sterile Transwell® insert (Corning Costar, Corning, NY, USA) fitted with a sterile dialysis membrane (15 000 molecular weight cut-off, Spectra/Por 2·1; Spectrum Medical Industries, Inc., Gardena, CA, USA) was placed in each well. A 1·5 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. After incubation, the insert ring and digest were removed, an additional 1 ml minimum essential medium was added to each well and the cell culture plate was incubated for an additional 22 h; the cells were then harvested for analysis of Fe uptake and mineral composition (Glahn *et al.* 1998). Samples were performed in duplicate on any given experiment day and averaged to reduce well variation, and each experiment day was repeated six times (*n* 6).

In phase 1, to determine accurately the amount of Fe 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 Fe.

Analyses

All glassware used in sample preparation and analyses was acid-washed. The protein content of the solubilized CaCo-2 cell homogenates was measured using a semi-micro adaptation of the Bio-Rad DC protein (colorimetric) assay kit (Bio-Rad Laboratories, Hercules, CA, USA). 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, USA). Analysis of the Fe content of the experimental solutions and digests was conducted by inductively coupled plasma–emission spectroscopy (ICAP model 61E Trace Analyser; Thermo Jarrell Ash Corporation). All data handling was performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

Statistics

Statistical analyses of the data were conducted according to the methods of Motulsky (1995) using the GraphPad Prism software package (GraphPad Software, San Diego, CA, USA). Where multiple series of experiments are shown on a single figure, ANOVA included only data within that series or 'batch', not the entire collection of experiments. Prior to analysis and where appropriate, data were log-transformed to achieve equal variance. ANOVA was performed with the Dunnett's post-test to compare the various means of each series of experiments. Means were considered significantly different if P values were less than or equal to 0·05.

Results

Analysis of ferritin formation in fortified cereals was performed on four different batches, as shown in Fig. 2; as our model determines relative differences the values are expressed as a percentage of the control (cereal fortified with reduced Fe). The data indicate that some intrinsic Fe from the cereal was available; upon feeding of batch 1 'no added Fe' samples, ferritin levels increased to 13·7 ng/mg cell protein, compared with baseline ferritin levels in the CaCo-2 cells of between 5 and 8 ng/mg cell protein. The control sample, fortified with reduced Fe, produced ferritin values of 30 ng/mg cell protein for batch 1; similar trends were observed with the subsequent batches. Addition of reduced Fe to the wheat cereal matrix did not produce a significant increase in ferritin formation (relative to the 'no added Fe' samples).

The data in Fig. 2 show the ferritin formation of the samples in phase 1 expressed as a percentage of the control (i.e. the reduced-Fe fortified cereals) as depicted by the horizontal line. Separate controls were produced for each batch of cereals, and used as such in the subsequent calculations. These results clearly indicate that Fe from $Na_2FeEDTA$ was significantly more available than reduced Fe. This was evident by ferritin values 200–300 % greater than that produced by reduced Fe. In addition, more Fe was dialysable from the EDTA form as indicated by consistently higher levels of lower-chamber Fe relative to the reduced Fe and all of the other Fe forms (Fig. 3). Without direct testing for the precise form of the Fe in the lower chamber, it cannot be determined to what extent the increased ferritin formation was due to greater amounts of Fe or to a more available form of Fe. Concurrently, higher levels of dialysable Zn were observed with the Fe from the $Na_2FeEDTA$ form; however, this was less pronounced and only significantly higher than that of the $FeCl_3$ and Biofer® (Lipo-Tech, Britwell Salome, Oxfordshire) cereals in batch 2, and the control and 'no added Fe' cereals in batch 3.

Fe from other sources such as electrolytic, encapsulated ferrous fumarate, Sun Active® (Taiyo Kagaku, Yokkaichi, Japan) and Ferrochel® (Albion Laboratories, Clearfield, UT, USA) resulted in ferritin values 30–125 % greater than that of reduced Fe. Of these four forms, Ferrochel displayed the highest ferritin formation value relative to the reduced Fe (+125 %), followed by Sun Active (+79 %), electrolytic (+55 %) and encapsulated ferrous fumarate (+30 − 40 %). All of these groups had lower-chamber Fe values

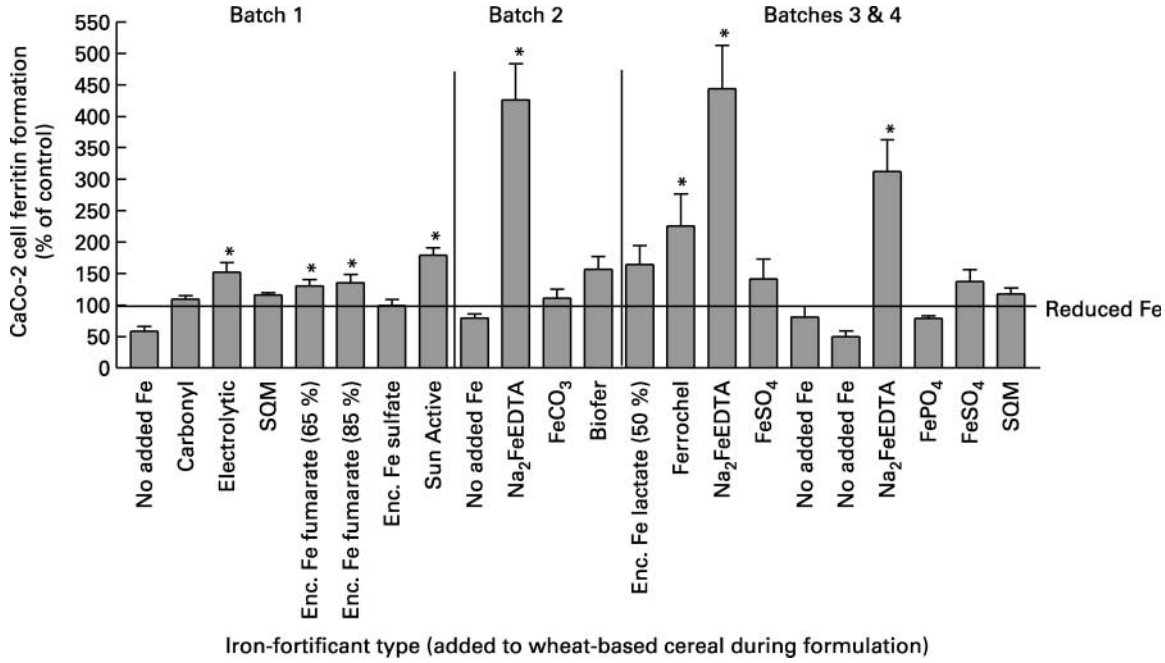

Fig. 2. Ferritin formation by human colonic adenocarcinoma (CaCo-2) cells in response to the iron from digests containing a wheat-based cereal fortified with a range of iron sources. Values are mean percentage ferritin formation compared with the control (cereal fortified with reduced iron) that was run simultaneously. Values are mean with their standard errors represented by vertical bars for six repeated digest–incubation procedures. Mean values were significantly different for the reduced iron control group: $^*P < 0{\cdot}05$. No inter-group comparisons are expressed here, as the objective was to compare iron bioavailability from the commonly used fortificant, reduced iron, with that from other potential iron fortifiers. The cereals are separated by batch, each batch being formulated from a common 'base cereal' that was split prior to fortification with the different iron forms. For details of iron-fortificant type, see p. 67.

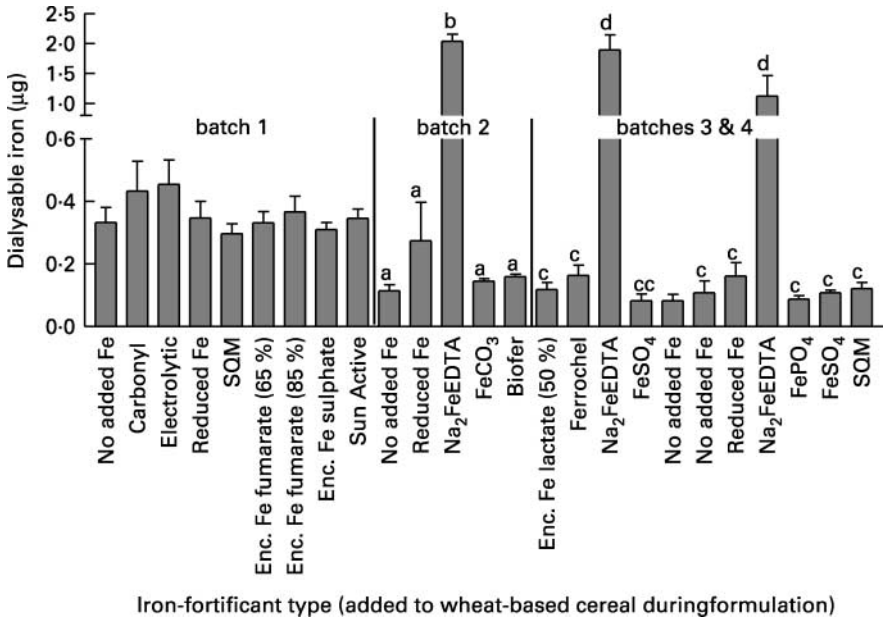

Fig. 3. The measurement of iron (from digests containing a wheat-based breakfast cereal fortified with a range of iron sources) that dialysed through a semi-permeable membrane and into the bottom chamber during the 2 h intestinal digestion period. Values are mean with their standard errors represented by vertical bars for six repeated digest–incubation procedures. [abcd]Mean values with unlike superscript letters were significantly different ($P<0·05$). The cereals are separated by batch, each batch being formulated from a single 'base cereal' that was split prior to fortification. For details of iron-fortificant type, see p. 67.

similar to that of reduced Fe, indicating that the Fe was more available and not merely present in greater amounts of dialysable Fe.

Three forms of $FeSO_4$ were studied. One was the standard salt $FeSO_4$ form; another was encapsulated with vegetable protein (enc. $FeSO_4$) and the third was encapsulated with vitamin C in a phospholipid membrane (Biofer). None of these forms showed significant differences in Fe availability relative to reduced Fe.

Other forms encapsulated with vegetable protein were ferrous lactate and ferrous fumarate. The encapsulated ferrous lactate showed no significant increase in availability relative to reduced Fe. The encapsulated ferrous fumarate was significantly more available relative to reduced Fe, exhibiting ferritin values 30–40 % greater than reduced Fe. Since we did not assay a non-encapsulated form of ferrous fumarate, we cannot determine if encapsulation or the fumarate provided this modest increase in Fe availability.

It was observed (Fig. 3) that the Fe dialysability from samples in batch 1 seemed higher than that of the subsequent batches. We therefore assessed the phytate levels of all the samples. We assessed levels of both IP5 and IP6; the mean average IP5, IP6 and total phytate were 1·02, 3·43 and 4·44 mg phytate/g cereal, respectively, and total phytate:Fe was 14·8. We found no significant differences between the batches, suggesting that the difference in the range of dialysable Fe is due to other unknown factors, possibly the levels of other inhibitors (such as polyphenols), which may vary from batch to batch. However, this difference in dialysable Fe was reproducible and did not affect the intra-batch variation of ferritin formation.

In the second phase of this study we observed that the addition of milk to a digest containing $FeSO_4$-fortified cereal increased ferritin formation (Fig. 4) from +92 to +158 % of the control ($FeSO_4$ without cereal; ferritin value of 62 ng/mg cell protein), where the control and fortified samples contained an equivalent of 150 μg Fe. The addition of coffee to the cereal digest with and without milk markedly decreased the Fe availability (between −73 and −62 % compared with the control). As shown previously, Fe availability from a digest containing Na_2FeEDTA-fortified cereal was greater than from the $FeSO_4$-fortified cereal (a further 250 % increase over the control compared with the increase already observed in the $FeSO_4$-fortified cereal). The addition of milk to this digest, however, decreased Fe availability to a similar level as that of the $FeSO_4$-fortified cereal with milk. The addition of coffee to the digest of the Na_2FeEDTA-fortified cereal containing milk did not further affect Fe availability, but there was a significant reduction in availability compared with the cereal digest alone (+342 % to +87 % for digest plus coffee and +39 % for digest plus milk and coffee). Also, all the Na_2FeEDTA-fortified cereal digests displayed an increase in availability over that of the control, whereas the digests containing $FeSO_4$-fortified cereal and coffee (with or without milk) decreased the availability below that of the control.

In phase 3 we further investigated the effect of milk on fortified wheat-based breakfast cereals. The control sample, fortified with reduced Fe, produced ferritin values of 23 ng/mg cell protein. The Na_2FeEDTA-fortified cereal also contained reduced Fe (at 1:1·25, reduced Fe:Fe in Na_2FeEDTA) to replicate better current fortification programmes in practice. As expected, the EDTA-fortified cereal increased availability over that of the control (+267 %). The Ferrochel-fortified cereal did not increase the availability significantly, unlike the phase-1 cereal; nor did the $FeSO_4$-fortified cereal. The cereal fortified with Sun Active did display significantly enhanced availability (+94 %). The 'no added Fe' group was included for completeness and as expected did not show marked Fe availability. The addition of milk to digests containing these fortified cereals (with the exception of the 'no added Fe' cereal) showed increases in availability (+180 to +280 %) over the control cereal.

As shown in the previous experiment, Fe availability from Na_2-FeEDTA-fortified cereal decreased in the presence of milk (+267 to +200 %). The presence also of reduced Fe here seems to have

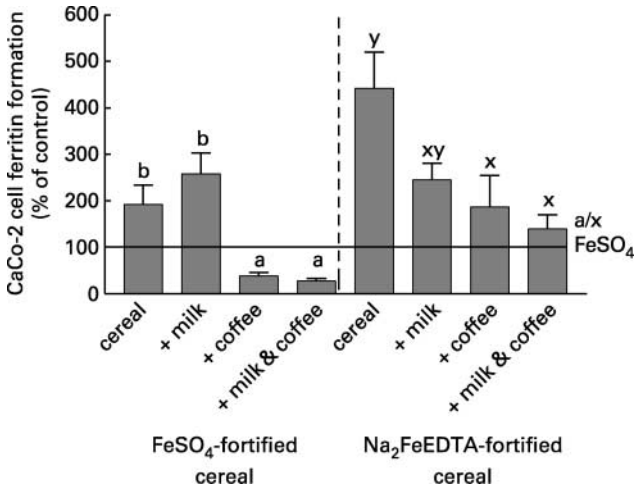

Fig. 4. Ferritin formation by human colonic adenocarcinoma (CaCo-2) cells in response to the iron from digests containing a wheat-based breakfast cereal fortified with either $FeSO_4$ or $Na_2FeEDTA$, with or without the addition of milk and/or coffee. Values are mean percentage ferritin formation compared with that formed in the presence of the control ($FeSO_4$, no cereal matrix) that was run in paired fashion as a reference standard. Values are mean with their standard errors represented by vertical bars for six repeated digest–incubation procedures. [abxy]Mean values with unlike superscript letters were significantly different ($P<0{\cdot}05$).

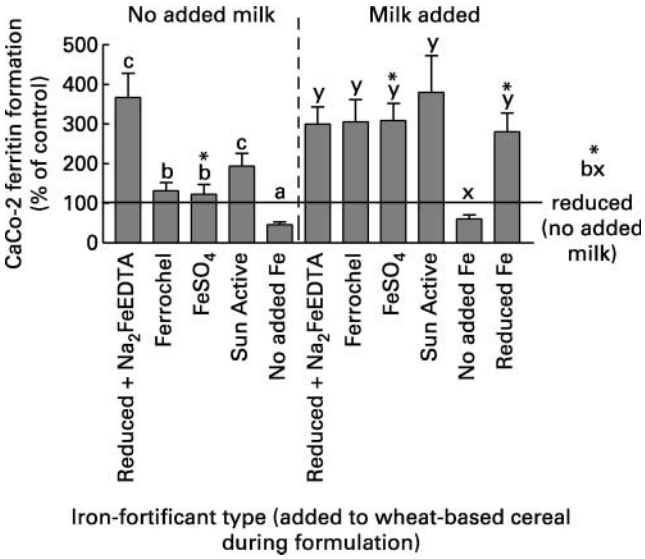

Fig. 5. Ferritin formation by human colonic adenocarcinoma (CaCo-2) cells in response to the iron from digests containing a wheat-based breakfast cereal fortified with a range of iron sources, with or without the addition of milk. Values are mean percentage ferritin formation compared with that formed in the presence of the control (cereal fortified with reduced iron) that was run in parallel. Values are mean with their standard errors represented by vertical bars for six repeated digest–incubation procedures. [abcxy]Mean values with unlike superscript letters were significantly different ($P<0{\cdot}05$). Mean values for ferritin formation of cereals fortified with $FeSO_4$ and reduced iron when milk is added to the digest were significantly higher than those for the absence of milk (*$P<0{\cdot}05$). For details of iron-fortificant type, see p. 67.

minimized the decrease in availability somewhat compared with the previous experiment where the fortificant was $Na_2FeEDTA$ alone. The Ferrochel, $FeSO_4$, Sun Active and reduced Fe forms showed increases in availability with the addition of milk (to +206, 209, 280 and 181 %, respectively). We did not assess the addition of coffee in this experiment.

Discussion

We have shown in the present study that although there are many choices for an Fe fortificant to add to a food product, few offer a marked increase in Fe availability over that of the commonly used form known as 'reduced' Fe. Those forms assessed that showed a mild or moderate increase in availability were electrolytic, encapsulated ferrous fumarate, Sun Active and Ferrochel. $Na_2FeEDTA$ was also assessed and, without the presence of milk, was a highly available form in the breakfast cereal matrix of this study. The effect of EDTA on Fe availability observed in the present study agrees with data reported previously (MacPhail & Bothwell, 1992; MacPhail *et al.* 1994) that EDTA protects Fe from inhibitory ligands such as phytates and polyphenols. However, we have shown that the presence of milk can negatively affect the increase in bioavailability normally seen with $Na_2FeEDTA$ as a fortificant. The likely mechanism for this effect is a consequence of the pH of milk being near neutral or slightly alkaline. When milk is added to the cereal, at least some of the $Na_2FeEDTA$ complex dissociates prior to exposure or titration to the low pH of the stomach, where the $Na_2FeEDTA$ complex is more stable. This dissociation leads to interaction of the Fe with inhibitors and a decrease in availability. Thus, combination of the milk and Na_2-FeEDTA cereal prior to its being ingested and subject to low pH could decrease the availability of the Fe from this form. Ca in the milk may also play a role in that it may be in sufficient molar advantage to displace much of the Fe from EDTA, which would also result in more interaction of the Fe with inhibitors such as phytic acid. The practical conclusion to be drawn from observations in the present study is that in a breakfast cereal matrix with the presence of milk, $Na_2FeEDTA$ does not appear to have higher bioavailability relative to reduced Fe or $FeSO_4$; however, its advantage lies in its improved organoleptic quality and greater resistance to the inhibitory effects of phenolic compounds.

In this study we have also seen that the presence of milk in the digest of some fortified cereals enhances Fe bioavailability. It is unlikely that milk is stimulating the CaCo-2 cells; for example, the presence of milk reduced Fe uptake from EDTA-fortified cereal (Fig. 5), showing the opposite trend to other fortificants. The possible mechanism can only be speculated at, but we suspect the effect is probably a result of specific binding between Fe compounds and molecules within the milk being picked up by the model system. Perhaps the milk proteins prevent some of the binding of Fe to inhibitors such as phytates and phenolics, thus increasing the availability. These observations could be interpreted to contradict broader dogma perpetuated by studies where milk was observed to inhibit Fe uptake (Hurrell *et al.* 1989). However, one must note that the food matrix varies between studies past and present and this specific effect in a breakfast cereal matrix has not been studied before in man. Indeed, the presence of milk may explain why a greater increase in Fe availability was not observed in a similar human study where EDTA and $Na_2FeEDTA$ were added to a corn cereal matrix (Fairweather-Tait *et al.* 2000). Such subtle effects in food matrices are not likely to be tested in human trials due to the high cost; however, these effects can be extremely important to the better understanding of Fe availability in foods.

Interestingly, one of the fortificant forms used in this study, Ferrochel, showed good compatibility with milk, but a lower bioavailability when consumed with milk *v.* water (Olivares *et al.* 1997). In our study Ferrochel exhibited higher availability relative to reduced Fe in the absence of milk, and exhibited an increase in availability similar to that of $FeSO_4$ and reduced Fe in the presence of milk (Fig. 5). The study by Olivares *et al.* (1997) also noted that uptake of Fe from Ferrochel was increased in the presence of ascorbic acid. This is interesting as it suggests that the bis-glycine complex is accessible to promoter effects, yet shows some resistance to the inhibitory effects of phytic acid. We have not tested the effects of polyphenols on Ferrochel and have not found any published reports on this subject. However, it would seem reasonable that polyphenolics should exhibit a considerable inhibitory effect on Fe uptake from Ferrochel.

One factor that is often overlooked in fortification of cereal products is the effect of the genotype of the crop. In screening studies of numerous wheat, rice and maize genotypes using the same model as in the present study, we have observed considerable variability in the availability of the Fe intrinsic to the grain (Glahn *et al.* 2002; RP Glahn unpublished results). Wheat and rice show the greatest range of effect, presumably due to differences in phenolic profile and other unidentified factors. In general, food manufacturers tend to assume that the high fortification levels negate the effects of genotype; however, we caution that manufacturers should look into this possibility more closely, particularly for the less refined cereal products. If the intrinsic Fe availability is profoundly affected by genetics, the genotype could also influence the availability of the fortified Fe, both positively and negatively.

In summary, the present study documents similar findings as in human studies on the increased availability of Fe from $Na_2FeEDTA$ and Ferrochel in processed cereal foods. The study also ranks other forms of Fe, which could provide useful information to food scientists and nutritionists when formulating similar products for optimal availability and desirability. Furthermore, the results reinforce the concept that, in selecting the form of Fe, one must consider the foods likely to be consumed in the same meal to obtain optimal availability. The *in vitro* model used herein is inexpensive and ideally suited to such tests. Also, given the high degree of correlation with many human studies, it represents an extremely useful screening tool to refine objectives for definitive human trials.

Acknowledgements

The authors wish to thank Borys Polon, Mike Rutzke, Larry Heller and Jean Hsu for their technical assistance.

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