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Iron solubility compared with *in vitro* digestion–Caco-2 cell culture method for the assessment of iron bioavailability in a processed and unprocessed complementary food for Tanzanian infants (6–12 months)

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The Fe solubility test is a commonly used, easy and relatively cheap *in vitro* tool for predicting Fe bioavailability in food matrices. However, the outcome of a recent field trial comparing the effect on Fe status of Tanzanian infants of processed *v.* unprocessed complementary foods (CF), with otherwise the same composition, challenged the validity of this test for predicting Fe bioavailability. In the solubility test, significant more soluble Fe was observed in processed compared with unprocessed foods (mean 18·8 (SEM 0·21) *v.* 4·8 (SEM 0·23) %; $P<0·001$). However, in the field trial, no significant difference in Fe status was seen between processed and unprocessed CF groups after 6 months' follow-up. Therefore, twenty-four samples of these CF (twelve processed and twelve unprocessed batches) were analysed in triplicate for Fe availability using an *in vitro* digestion–Caco-2 cell culture method and results were compared with solubility results. Significantly more soluble Fe was presented to Caco-2 cells in the processed compared with unprocessed samples (mean 11·5 (SEM 1·16) *v.* 8·5 (SEM 2·54) %; $P=0·028$), but proportionally less Fe was taken up by the cells (mean 3·0 (SEM 0·40) *v.* 11·7 (SEM 2·22) %; $P=0·007$). As a net result, absolute Fe uptake was lower (not significantly) in processed compared with unprocessed CF (mean 1·3 (SEM 0·16) *v.* 3·4 (SEM 0·83) nmol/mg cell protein; $P=0·052$). These data clearly demonstrate that the Fe solubility test was not a good indicator of Fe bioavailability in these particular food matrices. In contrast, the results of an *in vitro* Caco-2 model supported the effects observed *in vivo*.

Iron: Bioavailability: Solubility: Caco-2 cells: Complementary food

Infants in developing countries still suffer on a large scale from stunted growth and Fe deficiency (World Health Organization, 2001; United Nations International Children's Emergency Fund, 2003). Children born with normal birth weight will generally satisfy their Fe needs during the first 6 months mainly through recycling of fetal Hb. The Fe in breast milk is highly bioavailable, but represents only a limited fraction of their Fe supply. During and after the weaning period, the infant becomes increasingly dependent on complementary food (CF) to meet physiological requirements for macro- and micronutrients (Michaelsen & Friis, 1998; Kramer & Kakuma, 2002). In developing countries, it has been observed that CF are largely cereal based with little variety. As a result, these foods are often low in energy density and nutrient content and contain significant quantities of micronutrient inhibitors, such as phytate and polyphenols. The consequence of a poor diet is that by the age of 12 months many children have stunted growth, are underweight and become increasingly Fe deficient (United Nations Standing Committee on Nutrition, 2004).

Traditional processing techniques have been used to improve energy density and Fe bioavailability of CF for young children in developing countries (Michaelsen & Friis, 1998; Mensah & Tomkins, 2003). Germination and fermentation are known to initiate starch hydrolysis and reduce levels of anti-nutritional factors such as phytate and tannins in cereal grains and legumes (Michaelsen & Friis, 1998). These techniques are usually combined with heat treatments to guarantee microbial safety of the porridges (Michaelsen & Friis, 1998; Kimanya *et al.* 2003). In a previous study, a CF was developed from local African ingredients that increased energy density and reduced the level of anti-nutritional factors. The aim was to improve growth and Fe status in infants (Mbithi-Mwikya *et al.* 2002). The product was evaluated in Tanzania in a double-blind, randomised, placebo-controlled trial of

Abbreviations: CF, complementary food; cpm, count per min.

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infants aged 6–12 months (Mamiro *et al.* 2004). The results of the trial did not show a significant difference in growth or in Fe status parameters between the processed and unprocessed CF groups after a 6-month follow-up. A first explanation for this apparently paradoxical observation put forward was that the CF feeding frequency was about twice as high in the control group, thus compensating to some extent for the lower energy (and Fe) density in the unprocessed CF after preparation of the porridges (Mamiro *et al.* 2004). However, even with this compensation, a difference in Fe parameters was expected.

The second explanation proposed was that the measured solubility (*in vitro*) did not estimate the more complex bioavailability (*in vivo*). The solubility method involves a simulation of the gastrointestinal digestion followed by a measurement of soluble Fe in the digest and thus covers only the first phase of the overall Fe absorption process. However, Caco-2 cell models offer a more physiological tool for screening Fe availability in food matrices, particularly when combined with a simulated digestion step (Fairweather-Tait *et al.* 2006).

The aim of the present study was to compare Fe availability determined by the *in vitro* digestion–Caco-2 cell culture method with Fe solubility for the processed and unprocessed CF and to examine these results in the context of an intervention study performed with the same foods in Tanzania.

Materials and methods

Food samples

CF samples were taken from twelve processed and twelve unprocessed production batches. The production process for both the processed and unprocessed CF was carried out under strictly standardised conditions. A detailed description of the CF is given by Mamiro *et al.* (2004). In summary, the processed CF consisted of germinated, autoclaved and dried finger millet (65·2 %) and kidney beans (19·1 %), roasted peanuts (8 %) and mango puree (7·7 %). The same ingredients in identical proportions were used for the unprocessed CF. The processed CF had a higher energy density (5·4 *v.* 1·6 kJ/g porridge) and a lower concentration of phytates (0·22 *v.* 1·15 % DM). The total Fe content was 4·74 mg/100 g DM for the processed samples and 5·67 mg/100 g DM for the unprocessed samples. The phytate:Fe molar ratios were 4·0:1 and 17·5:1 respectively for the processed and unprocessed samples.

Solubility method

The Fe solubility was determined using the pepsin–pancreatin method described by Miller *et al.* (1981) but without dialysis. The method involves simulating peptic digestion at pH 2 by adding pepsin and HCl to the food samples, followed by an incubation period of 2 h at 37°C. After incubation, the intestinal digestion is simulated by adjusting the pH to 7 using $NaHCO_3$ and then adding pancreatin and bile salt, followed by another incubation period of 4 h at 37°C. Finally, the samples were centrifuged and soluble Fe was measured in the supernatant fraction by atomic absorption spectrophotometry (Association of Official Analytical Chemists, 1995). The results are presented as percentage soluble Fe, which is calculated as follows:

$$\text{Soluble Fe}(\%) = (\text{soluble Fe}(\text{mg}/100\,\text{g DM})/\text{total Fe}(\text{mg}/100\,\text{g DM})) \times 100.$$

Cell culture

Caco-2 cells were obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK) at passage 40 and used in experiments at passage 46 or 47. Cells were seeded on collagen-treated six-well plates (Becton Dickinson Labware, Bedford, UK) at a density of 10 000 cells/cm^3 in collagen-treated wells. The cells were grown in Dulbecco's Modified Eagle Medium (Sigma Aldrich, Irvine, UK) with 10 % (v/v) fetal bovine serum, 2 % (v/v) penicillin–streptomycin–glutamine solution and 1 % (v/v) non-essential amino acid solution. The cells were maintained at 37°C in an incubator with a 5 % CO_2–95 % air atmosphere at constant humidity. The growth medium was changed every 2 d. The cells were used in experiments 14 d post-seeding.

In vitro *digestion and iron uptake experiment*

The method used was based on Glahn *et al.* (1996). Approximately 3 g dried food sample, to which 8 ml NaCl (120 mmol/l) was added, was homogenised. The pH of the resultant solution was adjusted to 2 using HCl (5 mol/l). A volume of 500 μl (128·4 kBq) of $^{55}FeCl_3$–HCl solution was than added to the food homogenate and the volume was made up to 10 ml using NaCl (120 mmol/l). The peptic digestion was carried out by adding 500 μl pepsin solution (0·4 g pepsin in 10 ml HCl (0·1 mol/l) and incubating for 60 min at 37°C on a rocking shaker (180 oscillations per min). Before the intestinal digestion, the existing growth medium surrounding the cells was removed and the cells were washed twice with 2 ml 37°C Hank's balanced salt solution at pH 7. Then, 1·5 ml Hank's balanced salt solution was used to cover the cells during the experiment. A two-chamber system was created by a sterilised insert with dialysis membrane, which was placed above the cells. After the peptic digestion period, the pH of the food samples was adjusted to 6 using $NaHCO_3$ (1 mol/l). A pancreatin–bile solution (0·1 g pancreatin and 0·6 g bile extract in 50 ml $NaHCO_3$ (0·1 mol/l)) was then added and the pH adjusted to 7 using NaOH (0·5 mol/l). The volume was raised to 15 ml with NaCl (120 mmol/l) and the food samples were centrifuged (2 min at 800 rpm). A volume of 1·5 ml of the supernatant fraction was pipetted into the upper chamber of the two-chamber system. After an incubation period of 2 h on a rocking shaker (130 oscillations per min), 500 μl of the content of the upper chamber was placed in a scintillation vial for determination of ^{55}Fe. The insert was then removed and 500 μl of the content of the lower chamber was placed in a second scintillation vial. The remaining Hank's balanced salt solution above the cells was then removed and the cells were washed twice with 2 ml of a solution containing NaCl (140 mmol/l), KCl (5 mmol/l) and piperazine-N, N′-bis (2-ethanesulfonic acid) (10 mmol/l) at pH 7. Fe that was bound to the surface of the Caco-2 cells was removed by the same solution as mentioned earlier, but to which sodium hydrosulfite (5 mmol/l) and bathophenanthrolene

disulfonic acid (1 mmol/l) was added. After removing the surface-bound Fe, the cells were washed a last time and 1 ml NaOH (0·5 mol/l) was added to solubilise the cell monolayer. A 500 μl sample was then transferred to a third scintillation vial. Finally, a second 500 μl sample was transferred to a cryovial for protein analysis.

The experiment was performed four times to analyse all the twenty-four food samples in triplicate. Each time, a control in which no food sample was included was analysed simultaneously with the food samples using the same procedures.

The radioactivity of the solution pipetted into the upper chamber was not measured before incubation. Therefore, this initial radioactivity was calculated as the sum of the counts in the total content of the upper chamber, the counts in the total content of the lower chamber and the counts in the total volume of the cell monolayer.

Given the assumption of equilibration of the radioactive Fe and the food Fe, the amount of Fe (radioactive + food Fe) corresponding with 1 count per min (cpm) (nmol Fe/cpm) was calculated as the sum of the amount of radioactive Fe and the amount of food Fe in each sample before incubation (initial amount of Fe) divided by the initial radioactivity.

The amount of Fe (food Fe + radioactive Fe) that was left behind in the upper chamber (a), passed through the membrane (b), and was taken up by the cells after incubation (c), respectively, was calculated as follows:

(a) = counts in the total content of the upper chamber × nmol Fe/cpm;

(b) = (counts in the total content of the lower chamber + counts in the total volume of the cell monolayer) × nmol Fe/cpm;

(c) = counts in the total volume of the cell monolayer × nmol Fe/cpm.

The proportion of the Fe (food Fe + radioactive Fe) initially in the food samples that was left behind in the upper chamber (d), passed through the membrane (e) and was taken up by the cells after incubation (f), respectively, was calculated as follows:

(d) = (amount of Fe left behind in the upper chamber/initial amount of Fe) × 100;

(e) = (amount of Fe passed the membrane/initial amount of Fe) × 100;

(f) = (amount of Fe taken up by the cells/initial amount of Fe) × 100.

The amount of Fe (food Fe + radioactive Fe) taken up by the cells as a proportion of the Fe that passed the membrane (g) was calculated as follows:

(g) = (amount of Fe taken up by the cells/amount of Fe passed the membrane) × 100.

The absolute amount of Fe (food Fe + radioactive Fe) taken up by the cells per mg cell protein (thus adjusting for the number of Caco-2 cells in the well) was calculated as the amount of Fe taken up by the cells divided by the protein concentration in the cell monolayer.

Analyses

The quantity of ^{55}Fe was measured by counting in a Packard liquid scintillation analyser (Pangbourne, Berkshire, UK). Protein was determined using a Pierce BCA protein assay reagent kit (Rockford, IL, USA). Statistical analysis was carried out with the SPSS software (SPSS Inc., Chicago, IL, USA). Food samples were measured in triplicate for both the Caco-2 cell culture method and the solubility method and means of the three measurements were used for the statistical analyses. A Kolmogorov–Smirnov test was used to test for normality. Both the percentage soluble Fe and Fe uptake by the Caco-2 cells were normally distributed in the processed and unprocessed samples, but because the number of samples was relatively small, a non-parametric test (Mann–Whitney U test) was used to compare the two groups of samples. A P value of 0·05 was taken as the threshold for significance.

Results

Table 1 presents the Fe availability of the processed *v*. the unprocessed CF determined by the solubility method and the *in vitro* digestion–Caco-2 cell culture method.

Solubility method

The mean percentage of soluble Fe was almost 19 in the processed samples, compared with approximately 5 in the unprocessed samples. This difference was statistically significant ($P<0·001$).

In vitro *digestion–Caco-2 cell culture method*

In the processed samples the mean percentage of the initial amount of Fe (food Fe + radioactive Fe) that was left behind in the upper chamber was approximately 89 compared with 92 in the unprocessed samples ($P=0·028$). Logically, a significant higher proportion of Fe had passed through the membrane in the processed samples. However, a mean percentage of 0·3 was taken up by the cells in the processed foods compared with 0·7 in the unprocessed samples. The latter was not significantly different ($P=0·114$).

The amount of Fe (food Fe + radioactive Fe) taken up by the Caco-2 cells, as a proportion of the Fe that passed the membrane, was significantly higher in the unprocessed samples in comparison with the processed samples ($P=0·007$).

The absolute amount of Fe (food Fe + radioactive Fe) that was taken up by the Caco-2 cells per mg cell protein was highest for the unprocessed samples (mean 3·4 (SEM 0·83) nmol/mg

Table 1. Iron availability of processed *v.* unprocessed complementary food (CF) determined by the solubility method and the *in vitro* digestion–Caco-2 cell culture method

(Mean values with their standard errors)

	Control (no food sample) (*n* 4)		Processed CF (*n* 12)		Unprocessed CF (*n* 12)		
	Mean	SEM	Mean	SEM	Mean	SEM	*P**
Solubility method							
Soluble Fe (%)			18·8	0·21	4·8	0·23	<0·001
In vitro digestion–Caco-2 cell culture method							
Fe left in upper chamber (%)†	80·1	1·77	88·5	1·16	91·5	2·54	0·028
Fe passed the membrane (%)†	19·9	1·77	11·5	1·16	8·5	2·54	0·028
Fe taken up by the cells (%)†‡	0·8	0·18	0·3	0·04	0·7	0·19	0·114
Fe taken up by the cells (%)†§	4·1	0·89	3·0	0·40	11·7	2·22	0·007

* *P* value for the difference between processed and unprocessed CF.
† Food Fe+radioactive Fe.
‡ As a proportion of the amount of Fe in the samples.
§ As a proportion of the amount of Fe that passed the membrane.

cell protein) compared with the processed samples (mean 1·3 (SEM 0·16) nmol/mg cell protein). This difference was, however, not statistically significant ($P=0·052$) (see Fig. 1).

Discussion

Fe bioavailability is the proportion of total Fe in a food or diet that is digested, absorbed and utilised primarily for Hb synthesis (Fairweather-Tait, 1987). It can be predicted by studying the behaviour of Fe at different steps (digestion, absorption and utilisation), depending on the research question and technical, economic and ethical constraints. In the present paper two methods to assess Fe bioavailability were compared: a solubility method, which measures the solubilisation of Fe following simulated gastric and duodenal digestion; an *in vitro* Caco-2 cell uptake method, which simulates Fe uptake in the intestinal tract.

Fe needs to be in solution before it can be taken up by the enterocytes. In the diet non-haem Fe is mostly present in the oxidised ferric form (Fe^{3+}), which is insoluble at intestinal pH, unless it is bound in a chelate (Conrad & Umbreit, 2002). The acid environment of the stomach removes Fe from food and helps to solubilise Fe by reducing ferric Fe to ferrous Fe (Fe^{2+}), which is soluble at intestinal pH. Ferrous Fe has a high tendency to become oxidised to the ferric form and therefore a reducing or chelating environment is needed in order to keep the Fe soluble (Beard *et al.* 1996; Conrad & Umbreit, 2002). In view of this, an assessment of soluble (reduced) Fe in the digest ('solubility method') is likely to represent a useful measure of the proportion of Fe that is effectively available for absorption, after the usual digestion processes in the human gastrointestinal tract. It is also generally assumed that this measurement correlates well with the fraction that is absorbed by the enterocyte. This method is moreover easy and rapid to perform and has a low cost. It is therefore not surprising that this method is widely used to evaluate the effect of different processing techniques on the solubility of Fe and other minerals (Duhan *et al.* 1999, 2002; Valencia *et al.* 1999; Mamiro *et al.* 2001; Yadav & Sehgal, 2002).

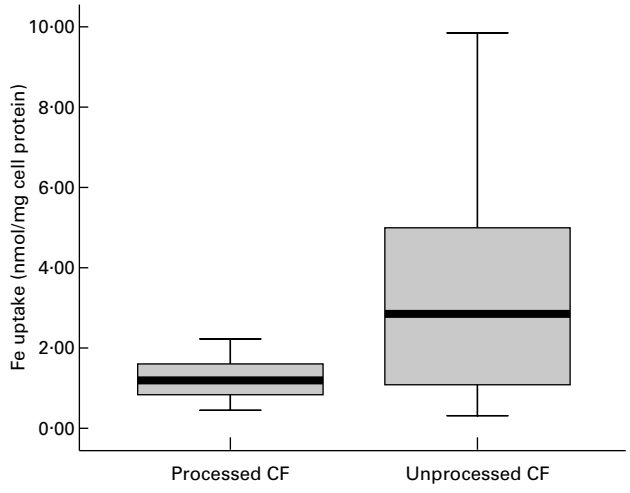

Fig. 1. Box and whisker plot of the distribution of the Fe uptake (food Fe + radioactive Fe) (nmol/mg cell protein) by the Caco-2 cells of the processed and unprocessed complementary food (CF) samples. Each box represents the interquartile range, which contains 50 % of values. The whiskers (|) extend from the boxes to the highest and lowest values. Medians are indicated by horizontal lines across the boxes.

In a previous study, the solubility method was used to measure the effect of processing on the Fe bioavailability in CF, especially developed for use in Tanzanian infants aged 6–12 months during their weaning period. The results of the solubility test predicted a much higher bioavailability in the processed matrix as compared with the unprocessed food. This contrasted sharply with the results of the field trial, in which no improvement in Fe status could be demonstrated in children who were fed with the processed food (Mamiro *et al.* 2004). An in-depth elaboration of the different elements that need to be taken into account in the interpretation of the discrepancy between expected and observed results in this experiment in Tanzanian infants can be found in the original publication (Mamiro *et al.* 2004). However, an overall conclusion from that field trial was that *in vitro* measurements of soluble Fe in a food matrix may be a poor predictor of changes in Fe status in populations with a high prevalence of Fe deficiency. This observation has led the authors to question the usefulness of the Fe solubility method for predicting absorption in the gastrointestinal tract.

It is known that Fe absorption is a complex process and the mechanisms by which this process is regulated remain

incompletely understood. It could be hypothesised, however, that in the overall context of the complexity of Fe absorption, the solubility test is potentially a poor predictor of the actual absorption.

Since the body cannot actively excrete Fe, body Fe content must be regulated at the point of absorption. Increased rates of erythropoiesis and low ferritin levels are important stimuli for Fe absorption with increased expression of receptor sites for Fe on the enterocyte. However, Hahn *et al.* (1943) and later Stewart *et al.* (1950) demonstrated that large doses of Fe can actually reduce the absorption of a smaller dose of Fe that is consumed later. This so-called 'mucosal block theory' has also been used to explain poor responses in Fe status in a trial in which Fe was given daily or once or twice per week. Frazer *et al.* (2003) demonstrated that this phenomenon is accompanied by changes in the expression of brush-border Fe transport proteins, which are regulated to some extent by enterocyte Fe levels.

Caco-2 cells have been shown to be a useful screening tool for estimating bioavailability and are increasingly used to study molecular mechanisms of Fe transport and uptake. Significant correlations between Fe uptake by Caco-2 cells from semi-synthetic meals and human absorption data have well been described (Au & Reddy, 2000; Yun *et al.* 2004). *In vitro* digestion–Caco-2 cell culture models confirmed the inhibitory effect of phytic acid, tannic acid and Zn on Fe uptake successfully (Glahn *et al.* 2002*b*). Thus the Caco-2 cells have demonstrated their capacity to estimate bioavailability.

In contrast to predictions made from Fe solubility data, the results of the Tanzanian intervention study are in accord with the data generated from the Caco-2 cell model for Fe bioavailability described in the present paper. In the intervention study no significant differences were found for growth and Fe status parameters between the intervention group and control group. This *in vivo* observation fits well with the absence of a statistically significant difference for Fe uptake by Caco-2 cells between the processed and unprocessed CF samples, but contrasts sharply with the results of the solubility method that predicted a higher bioavailability in the processed samples. In the Caco-2 model, the results of the percentage Fe left behind in the upper chamber and passed through the membrane are as predicted from the results of the solubility test, since the greater the quantity of soluble Fe there is in the sample, the more Fe can be expected to pass through the membrane and the less will be left behind in the upper chamber. Apart from the unstirred layer, no such membranes are present in the human digestive tract. However, the mucus layer of the lumen has been shown to be permeable to ions and smaller molecules and impermeable to large proteins, and so protects the gastroduodenal mucosa from autodigestion (Allen & Carroll, 1985; Allen *et al.* 1993). *In vitro*, the membranes protect the Caco-2 cell monolayer from the enzymes used for the simulation of the digestive process. In contrast, the similarity of the results of the solubility test and the percentage Fe passing through the membrane conflicts with the observed Fe uptake by the Caco-2 cells. Since Fe solubility in the processed samples was significantly higher than in the unprocessed samples, significantly more soluble Fe is being presented to the Caco-2 cells in the processed samples. However, proportionally less Fe has been taken up by the Caco-2 cells in the processed samples compared with the unprocessed samples. The net result is that no significant difference is observed between processed and unprocessed samples for absolute Fe uptake in the Caco-2 cells. The reason for this differential result in proportional Fe uptake remains unclear. It may be possible that these changes relate to an alteration in the content and/or activity of specific compounds in the flours as a result of processing (Watzke, 1998). There is also the inhibitory effect of the polyphenolic polymers (tannins) in the food samples, which were decreased to undetectable levels in the processed samples (Mbithi-Mwikya *et al.* 2002). It could, however, be that the inhibitory effect of tannins was maintained after processing (Glahn *et al.* 2002*a*). Processing would break down polymers and the resulting monomer compounds may bind Fe, thereby inhibiting Fe absorption. Since phytate levels were reduced by processing but no significant increase was seen in Fe availability, polyphenolic compounds may be the primary inhibitory component, but further studies are required to confirm this.

There may be problems with the isotopic exchange in the extrinsic labelling technique used to measure Caco-2 cell Fe uptake, particularly with high levels of phytate and polyphenolics in the samples. Thus, caution needs to be taken when interpreting the results. The alternative method patented by Glahn *et al.* (1998) in which ferritin is used as a surrogate marker of Fe uptake over a more prolonged time-scale has different potential problems. Ferritin may be expressed in conditions of oxidative stress and inflammation, and not just a quantitative response to Fe (Fairweather-Tait *et al.* 2006). Nevertheless, the results of the present study clearly indicate that the solubility method can be misleading as far as absorption in the gastrointestinal tract is concerned, whereas the Caco-2 cell method concurs with *in vivo* data. This overall conclusion has important consequences for the assessment and evaluation of food-based interventions aimed at Fe deficiency and for clinical and epidemiological research that is carried out in this field.

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