JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. **2010,** *58,* 3833–3839 **3833**
DOI:10.1021/jf904175d

Development of a Modified Caco-2 Cell Model System for Studying Iron Availability in Eggs

Ben Thompson,[†] Paul Sharp,[‡] Ruan Elliott,[§] Sharifah Al-Mutairi,[∥] and Susan J. Fairweather-Tait*,[†]

[†]School of Medicine, University of East Anglia, Norwich NR4 7TJ, United Kingdom, [‡]King's College London, London SE1 9NH, United Kingdom, [§]Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, United Kingdom, and [∥]Ministry of Health, State of Kuwait, P.O. Box 13416 Kaifan, Code No. 71955, Kuwait

A modified Caco-2 cell model system was developed for studying iron availability in mixtures of fresh and/or cooked foods subjected to a simulated gastrointestinal digestion. The effect of combining foods containing high levels of ascorbic acid with cooked eggs on ferritin expression in the cells was measured. There was no detectable increase in ferritin with eggs alone, indicating that none of the iron was available for uptake into the cells, but when mixed with orange juice or salad (lettuce, tomatoes, and red pepper) in ratios similar to those found in meals, there was a significant increase in ferritin concentration ($p = 0.0012$ and $p = 9.2 \times 10^{-10}$, respectively); the enhancing effect of salad was greater than orange juice ($p = 0.028$). These results suggest that the iron in eggs will be more readily absorbed when consumed with foods high in ascorbic acid.

KEYWORDS: Iron; ascorbic acid; Caco-2 cells; simulated digestion; ferritin

INTRODUCTION

Over a decade ago, a Caco-2 intestinal cell model system was developed for use as a rapid screening tool for measuring iron availability in staple foods (*1*,*2*). It includes a two-stage simulated digestion (gastric and intestinal phases) of dried, uncooked foods, tailored for high throughput of a large number of different varieties of cereals and legume crops, followed by measurement of iron uptake into a Caco-2 cell monolayer system that models the absorptive site for iron in the small intestine. Refinements to the model were introduced that more closely reflected in vivo physiological conditions and were used to examine iron availability in eggs, a relatively rich dietary source of iron providing approximately 1 mg iron/egg (*3*) but of low bioavailability because the iron is bound to phosvitin (*4*).

Ascorbic acid is a well-known enhancer of iron absorption (*5*); therefore, foods rich in ascorbic acid are prime candidates for improving iron availability from foods and, in the case of eggs, counteracting the negative effects of phosvitin (*6*). Typical Western diets include boiled, fried, poached, or scrambled eggs at breakfast, which may be accompanied by a drink of fruit juice. Eggs can also be made into omelets and consumed with salad as a lunch or evening meal. We selected orange juice and a salad of lettuce, red peppers, and tomatoes to test the hypothesis that the availability of iron in eggs could be improved when consumed with high ascorbic acid foods. The refined Caco-2 model system was applied to fresh foods in ratios that would be found in a meal.

*To whom correspondence should be addressed. Tel: +44 1603 591304. Fax: +44 1603 593752. E-mail: s.fairweather-tait@uea.ac.uk.

MATERIALS AND METHODS

Description of the Model System. The Caco-2 model is illustrated in **Figure 1**. Briefly, foods under examination were subjected to a 1 h pepsin digestion at pH 2 to simulate gastric digestion, followed by a 2 h pancreatin−bile digestion at pH 6.7 to simulate the intestinal phase of digestion. The resulting digestate was applied to a 15 kDa dialysis membrane placed above the Caco-2 monolayer. This membrane acts to protect the cells from the abrasiveness of the digestate, and the molecular weight cut off is similar to that of mucus (*7*) and provides a protective barrier for the cells against the enzymes used in the simulated digestion. After the digestate was added, the cells were incubated for 2 h to allow the iron to dialyze through to the apical compartment and to be transported into the cells by DMT1. The digestate was then removed, and the cells were incubated for a further 22 h to allow ferritin expression to take place, at which point they were harvested and the ferritin content was measured as a

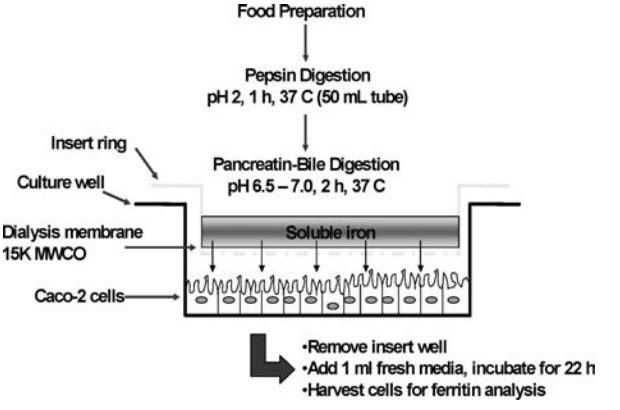

Figure 1. Schematic of the Caco-2 cell system.

Published on Web 02/19/2010

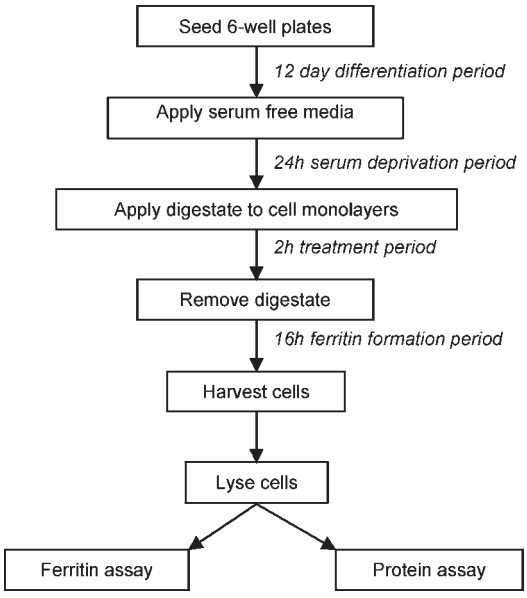

Figure 2. Outline of experimental protocol.

surrogate marker of iron status (*2*). A flowchart of the protocol is given in **Figure 2**.

Preparation of Six-Well Plates. Collagen-coated six-well plates (Greiner, United Kingdom) were seeded with 4.75×10^5 Caco-2 cells/well (P25-40) from ATCC and cultured for 12 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM glutamine, 5 mL 5000 μ/mL penicillin/streptomycin solution (Gibco, United Kingdom), 25 mM HEPES solution, and 10% fetal calf serum (FCS, Sigma, United Kingdom), and the media were changed every 3 days. Following this, the media were changed to minimum essential medium (MEM) supplemented with 5 mL of 5000 μ/mL penicillin/streptomycin solution (Gibco), 19.4 mM D-glucose, 10 mM PIPES, 26.2 mM sodium bicarbonate ($NaHCO_3$), 11 μM hydrocortisone, 0.87 μM insulin, 0.02 μM sodium selenate (Na_2SeO_3), 0.05 μM tri-iodothyronine, and 0.2 mg of epidermal growth factor (Sigma) in the absence of FCS for 24 h before treatment.

Simulated Digestion Technique for Freeze-Dried Samples. An iron-free pepsin solution was prepared by adding 0.8 g of pepsin (Sigma) to 20 mL of 0.1 M hydrochloric acid (HCl) and 10 g of Chelex-100 (Bio-Rad, United Kingdom). The resulting suspension was mixed for 30 min, the slurry was poured into a 35 mL volume Flexcolumn (VWR, United States), the eluate was collected, and the column was washed with a further 20 mL of HCl, which was also collected.

Iron-free pancreatin−bile solution was prepared by mixing 0.25 g of pancreatin with 1.5 g of bile extract (Sigma) in 125 mL of 0.1 M $NaHCO_3$ and adding 62.5 g of Chelex-100 (Bio-Rad). The suspension was mixed by shaking for 30 min, and the slurry was poured into a 53 mL volume Flexcolumn (VWR), the eluate was collected, and the column was washed with a further 50 mL of 0.1 M $NaHCO_3$, which was also collected.

Dried food (0.5 g) was weighed into a 50 mL plastic tube (Corning, United Kingdom), and 10 mL of a 140 mM sodium chloride (NaCl) and 5 mM potassium chloride (KCl) solution was added together with appropriate volumes of ferrous sulfate ($FeSO_4$), ferric chloride ($FeCl_3$), and/or ascorbic acid, all made freshly on the morning of the experiment and used within 2 h. The contents of the tube were mixed, and the pH was adjusted to 2 using 1 M HCl, pepsin solution (0.5 mL) was added to all tubes, and they were incubated for 1 h at 37 °C with agitation. The pH was adjusted to 5.5−6.0 with 1.0 M $NaHCO_3$, and 2.5 mL of the pancreatin−bile solution was added to each 10 mL of original sample. The pH was then further adjusted to 6.9−7.0 with 1 M $NaHCO_3$. Finally, the volume was adjusted (by weight) to the tube weight plus 18 g using 140 mM NaCl and 5 mM KCl, pH 6.7, solution. If the mass was greater than 18 g due to the buffering effect of the sample, then the volume of all of the tubes was adjusted to the same level with further 140 mM NaCl and 5 mM KCl, pH 6.7, buffer. At this point, 1.8 mL of the samples was applied to the cells and incubated for a further 2 h at 37 °C with agitation to allow the simulated digestion to continue and the solubilized iron to dialyze through the membrane.

Treatment of Cells. After the cells had been deprived of FCS for 24 h to remove iron from the system and thereby increase the sensitivity of response of the cells, a 1 mL MEM change was made, and inserts with a 15 kDa molecular mass cutoff dialysis membrane (Fisher, United Kingdom) were placed in the wells of the six-well plate. To each insert, 1.8 mL of the relevant sample was applied, and the plates were incubated at 37 °C with gentle agitation for a further 2 h. Each sample was applied in triplicate. The inserts were removed, a further 1 mL of MEM was added, and the plates were returned to the incubator for a further 16 h with no agitation.

Harvesting of Cells. The cells were harvested by aspirating the media and carefully rinsing the cells twice with 2 mL of buffer (130 mM NaCl, 5 mM KCl, and 5 mM PIPES pH6.7). Two milliliters of Milli-Q water was then added, and the monolayers were scraped using an inverted 200 μL pipet tip. The resulting lysate was suspended evenly, sonicated, and stored at −20 °C until analysis.

BCA Protein Assay. The total protein content of the cell lysates was analyzed using the BCA protein assay kit (Pierce, United Kingdom) according to the manufacturer's protocol. A standard curve was created using 0, 125, 250, 500, 1000, and 2000 μg/mL BSA, and 10 μL of the unknown samples was loaded into the 96-well plate in triplicate. After they were mixed on a rotating table and a 30 min incubation at 37 °C, the absorbance was measured at 562 nm using an ELx800 Absorbance Microplate Reader (Biotek, United States).

Colorimetric Ferritin Assay. The total ferritin content of the cell lysates was analyzed using the Spectroferritin ELISA Assay kit (Ramco, United States) according to the manufacturer's protocol. A standard curve was created using 0, 6, 20, 60, 150, and 200 ng standard/mL, and 30 μL of the unknown samples was loaded into the 96-well plate in triplicate. The incubation steps were followed as in the protocol. The absorbance was measured at 490, 570, and 630 nm using an ELx800 Absorbance Microplate Reader (Biotek), and the results from 570 and 630 nm were subtracted from the 490 nm values.

Colorimetric Iron Assay. A range of iron(II) standards between 0 and 1.6 μg/mL were produced by diluting a 5 μg/mL stock solution of $FeCl_3$ (High-Purity Standards, United States) in 0.1 M HCl in the presence of 20 μL of 0.1 g/mL hydroxylamine hydrochloride (Sigma) dissolved in 10 M HCl. For the analysis of samples from the in vitro digestion, 1.5 mL of the digestate was centrifuged at 10000*g* for 5 min, 380 μL of the resulting supernatant was removed to a fresh tube, and 20 μL of hydroxylamine hydrochloride solution was added. One hundred microliters of each standard and sample was added to a 96-well plate, in triplicate, and 50 μL of chromagen solution [0.0156 g of bathophenanthroline disulfonic acid disodium salt (Sigma) dissolved in 50 mL of 2.0 M sodium acetate] was added. The plate was incubated for 10 min at room temperature before the absorbance was measured at 562 nm using an ELx800 Absorbance Microplate Reader (Biotek).

Experiment 1: Ferritin Time Course and Effects of Serum Deprivation and Ascorbic Acid Addition on Iron Availability. The original protocol for the rapid screening method was devised to comply with a normal working day in that treated cells were left overnight and harvested the next day for ferritin measurements. We examined the ferritin response to iron over time to gauge the best time to measure ferritin. Two other steps in the technique were also examined, namely, the effect of serum deprivation for 24 h prior to treatment (introduced to increase the sensitivity of the assay) and the addition of ascorbic acid to the digestate (to increase the response of the cells to iron).

Twenty-six collagen-coated six-well plates (Greiner) were seeded with Caco-2 cells (P39) from ATCC. Half of the plates were deprived of 10% FCS for 24 h, while the remainder were not. Prior to treatment of the cells, one plate with 10% FCS-supplemented media and one plate with media that had not been supplemented with 10% FCS were harvested for the baseline. The wells were rinsed twice with a wash buffer consisting of 130 mM NaCl, 5 mM KCl, and 5 mM PIPES (Sigma), which was then aspirated, and 2 mL of Milli-Q water was added. The monolayer was disrupted with an inverted pipet tip, suspended evenly, and transferred to a prelabeled 5 mL tube. The cell lysates were stored at −20 °C. Cells were treated using either 10 μM ferrous ascorbate or 200 μM ascorbic acid in supplemented MEM for 2 h. A 1 mM concentration of ferrous ascorbate

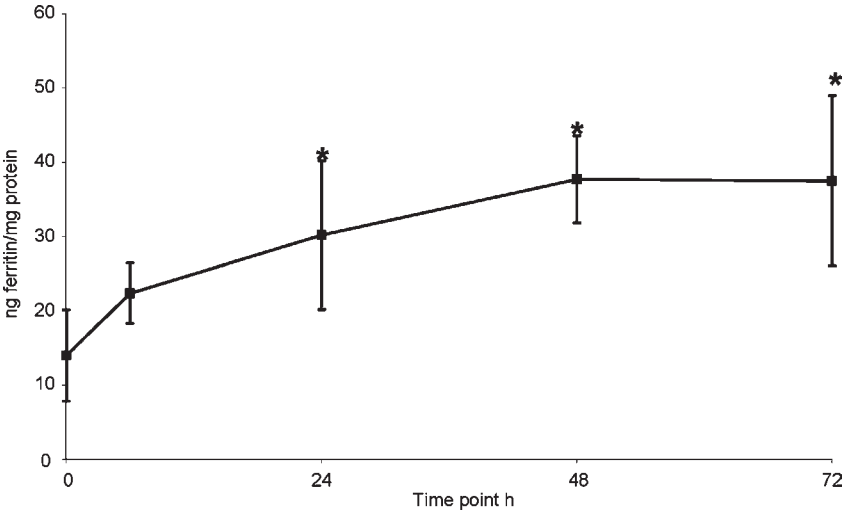

Figure 3. Time course of ferritin response when cultured in 10% FCS/DMEM over a 72 h period. The asterisk denotes the significant difference ($p < 0.05$) at the 0 h time point.

was prepared, using a 1:20 ratio of ferric chloride to ascorbic acid (Sigma), fresh on each day of the experiment, and this was added 1:100 (10 μM) to the supplemented MEM. The 40 mM ascorbic acid that was made fresh on the day and used to make up the ferrous ascorbate was added 1:200 (200 μM) to the supplemented MEM. Following the 2 h treatment, the media were changed back to the relevant supplemented MEM (either with or without FCS), which had not had ferrous ascorbate or ascorbic acid added. After a further 4 h, six plates were harvested: 10% FCS, serum-deprived, 10% FCS ferrous ascorbate-treated, serum-deprived ferrous ascorbate-treated, 10% FCS ascorbic acid-treated, and serum-deprived ascorbic acid-treated. This was then repeated 24, 48, and 72 h following the commencement of the treatment period. The cells were harvested, and the total protein content and ferritin content of the cell lysates were measured.

Experiment 2: Effect of Freezing on Soluble Iron Concentration of Food Digestates. The use of fresh foods considerably extended the workload of the experimental day so that the effect of freezing the digestate, to be able to apply it to the cells on a different day, was examined. The concentration of iron in food digestates was measured before and after freezing to establish the effect that this process might have on the soluble iron, which would be predicted to have an impact on iron availability. The samples were frozen at −20 °C overnight, and the free iron concentration was quantified the following day. The samples studied were eggs, eggs plus orange juice, and eggs plus salad. All of the samples were digested in the presence and absence of ascorbic acid (from a 200 mM stock solution made up fresh on the day of the experiment) at 20× the calculated iron concentration (*3*) with the exception of orange juice where the value was obtained from the nutritional declaration on the pack.

Experiment 3: Refinement of the Model System To Study the Effect of Foods High in Ascorbic Acid on Iron Availability in Eggs. The in vitro Caco-2/simulated digestion model is mainly used for the analysis of iron uptake from dried food samples. This method was adapted to enable us to examine the effect of fresh foods on iron availability from eggs, in ratios similar to that found in typical meals, on a scale that can be used in the simulated digestion method. Each experimental day was repeated on three occasions with each sample being run in triplicate on each occasion (i.e., nine replicates per sample).

Large free range eggs were purchased the day before the simulated digestion. The eggs were cooked by adding them to boiling water, and they were left for 10 min before they were plunged into cold water to cool rapidly. They were left without removing the shell at 4 °C overnight. The next day, the shell was removed from the egg, and the weight was recorded. An equal weight of pH 2 140 mM NaCl and 5 mM KCl solution was added, and the egg was blended in a standard kitchen food blender for 30 s until a homogeneous solution was created. One gram (0.5 g of egg) of the resulting solution was taken for the digestion stage.

Smooth (not from concentrate) orange juice (Tropicana) was purchased the day before the simulated digestion. The orange juice was stored at 4 °C overnight, shaken, and opened, and 1.5 g was added to the relevant samples before the digestion stage. Red pepper, cherry tomatoes, and iceberg lettuce were purchased from the supermarket the day before the simulated digestion and stored at 4 °C overnight. On the experimental day, 25 g of red pepper, 40 g of tomato, and 20 g of lettuce were weighed, cut into small pieces, and immediately blended with 85 g of pH 2 140 mM NaCl and 5 mM KCl solution until a homogeneous solution was created. A 2.86 g amount (1.43 g of salad) of the resulting solution was used for the relevant samples for the digestion stage.

Statistical Analysis. All statistical analysis was performed with SPSS version 16.0.0 software (SPSS Inc., United States), using the analysis of variance general linear model with Tukey's posthoc analysis.

RESULTS

Experiment 1: Ferritin Time Course and Effects of Serum Deprivation and Ascorbic Acid Addition on Iron Availability. The expression of ferritin over 72 h is shown in **Figure 3**. Significant differences in ferritin levels are seen at 24, 48, and 72 h, as compared to the 0 h time point ($p = 1.4 \times 10^{-2}$, 2.4×10^{-4}, and 2.7×10^{-4}, respectively). The results from the 72 h time point were, however, not considered further as by this time the cells had begun to die and slough off the bottom of the six-well plate.

Following treatment with ascorbic acid (**Figure 4**), in the absence of serum, no significant differences were seen between the ferritin levels of the treated cells when compared to the $t = 0$ value ($p = 0.947$, 0.930, and 0.671 for 6, 24, and 48 h, respectively). Considering the time points individually, when the ascorbic acid-treated cells are compared to the control samples, there is a significant difference at $t = 6$ ($p < 0.001$) but not at any of the other time points ($p = 0.646$ and 0.134 for $t = 24$ and 48, respectively). In comparison, in the presence of FCS, an increase was observed as compared to $t = 0$ ($p = 0.003$ for $t = 6$ and $p < 0.001$ for $t = 24$ and 48). Significant increases were also observed in the ferrous ascorbate-treated cells when compared to the control sample at $t = 6$ ($p < 0.001$) and $t = 24$ ($p = 0.001$), although the increase at $t = 48$ was not significant ($p = 0.174$).

With the ferrous ascorbate-treated cells cultured in the absence of serum (**Figure 5**), there was no change in ferritin at $t = 6$ ($p = 0.919$) but an increase at both $t = 24$ and 48 ($p < 0.001$ in both cases). This pattern was mirrored by the ferrous ascorbate-treated cells grown in the presence of FCS where the decrease seen at $t = 6$ was not significant ($p = 0.702$), but at the subsequent time points, the increase as compared to the value at $t = 0$ was significant ($p < 0.001$ in all cases).

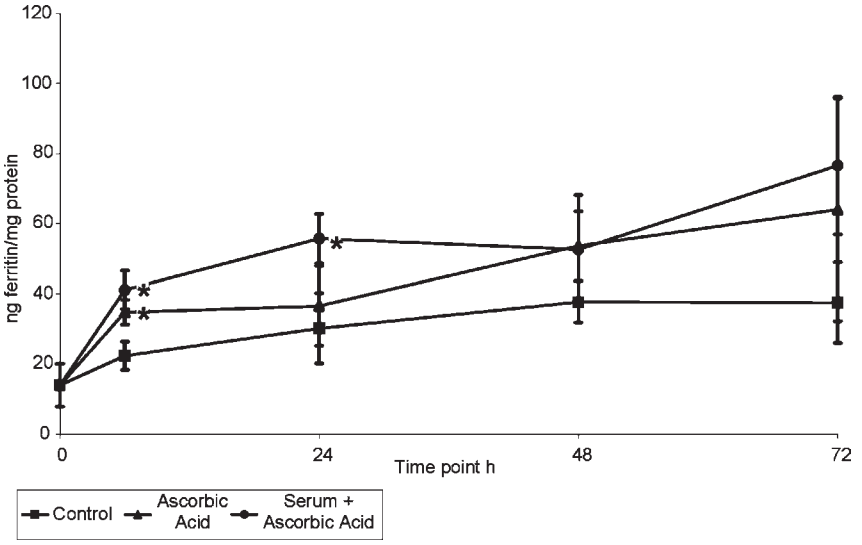

Figure 4. Effects of ascorbic acid treatment on ferritin protein levels over time in the presence and absence of serum. The asterisk denotes the significant difference ($p < 0.05$) to control at that time point ($n = 6$, ±SD).

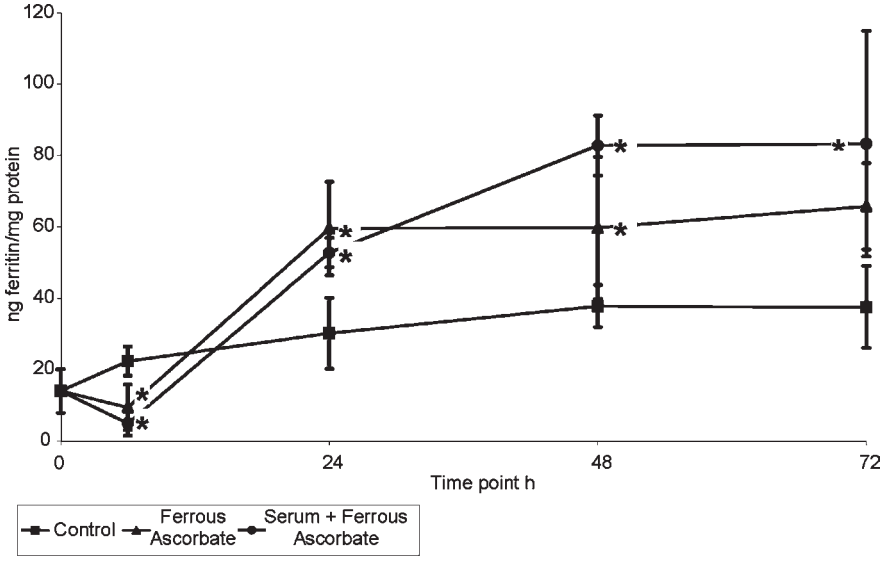

Figure 5. Effects of ferrous ascorbate treatment on ferritin protein levels over time in the presence and absence of serum. The asterisk denotes the significant difference ($p < 0.05$) to control at that time point ($n = 6$, ±SD).

When the time points were considered independently, at $t = 6$, there was a significant decrease in ferritin levels as compared to the control in both the FCS-replete and -deprived cells ($p < 0.001$ in both cases). At $t = 24$, the increases were also significant ($p < 0.001$ and $p = 0.001$ for serum-deprived and serum-replete cells, respectively). Finally, at $t = 48$, the increase was again significant in both cases ($p < 0.001$) in the serum-replete cells and $p = 0.024$ in the serum.

Experiment 2: Effect of Freezing on Soluble Iron Concentration of Food Digestates. Results are shown in **Figure 6**. In the control iron ($FeCl_3$) samples, there was no significant difference in the concentration of soluble iron after freezing, both in the presence and in the absence of ascorbic acid ($p = 0.95$ and 1.00, respectively). However, when native iron was present in a complex food matrix (as in the egg alone and egg plus orange juice samples), a significant increase in soluble iron was observed after freezing. In the case of the egg samples, both in the absence and in the presence of additional ascorbic acid, the up to 8-fold increase in soluble iron was highly significant ($p = 9 \times 10^{-12}$ and 5.4×10^{-10}, respectively). Similarly, in the case of the egg plus orange juice samples, the ~1.75-fold increase in soluble iron was also highly significant both in the absence and in the presence of ascorbic acid ($p = 2 \times 10^{-12}$ and 3×10^{-12}, respectively).

Experiment 3: Refinement of the Model System To Study the Effect of Foods High in Ascorbic Acid on Iron Availability in Eggs. The ascorbic acid content of lettuce, red pepper, and tomatoes is shown in **Table 1**. The levels of soluble iron in the orange juice and salad digestate samples were negligible (**Figure 7**). However, when egg was digested in conjunction with orange juice or salad digestate, a significant increase was seen as compared to the egg sample ($p = 1.2 \times 10^{-3}$ and 9.2×10^{-10}, respectively). Furthermore, a significant increase was observed between the egg plus orange juice samples and the egg plus salad samples ($p = 0.028$).

The effect of ascorbic acid-rich foods on iron availability from eggs is shown in **Figure 8**. Fresh samples, digested on each experimental day, were used for the egg studies. Previous data (not shown) indicated that the addition of ascorbic acid to the samples to improve iron availability (and hence increase the

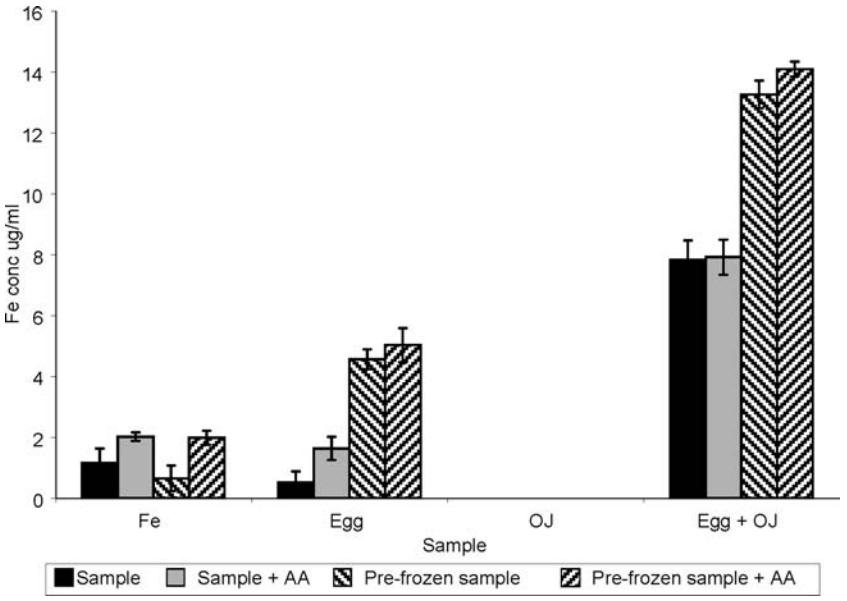

Figure 6. Effect of freezing the digestate on iron solubility.

Table 1. Ascorbic Acid Content of Different Components of Digestates

food	mg ascorbic acid/100 g	mg ascorbic acid/portion
red pepper, raw[a]	140	70.0
iceberg lettuce, raw[a]	3	1.2
cherry tomato, raw[a]	17	13.6
total salad		84.8
orange juice[b]	30	60

[a] Estimated from food tables (*3*). [b] Compositional declaration on a pack of Tropicana orange juice.

sensitivity of the system) introduced high levels of variability in the form of unacceptably large standard deviations. Although the samples that were analyzed here were replicated in the presence of ascorbic acid, the previous findings of high variability were confirmed so that the results from the ascorbic acid-supplemented samples were not used. The quantity of ferritin/mg total protein fell into two distinct groups (**Figure 8**): The egg, orange juice, and salad alone samples comprised one group, where the difference between samples was not significantly different, whereas the combinations of egg plus orange juice and egg plus salad both showed significantly increased levels of ferritin (~3- and 5-fold increase and $p = 2.8 \times 10^{-3}$ and 9.7×10^{-5}, respectively). Although an increase was observed in ferritin between the egg plus orange juice and the egg plus salad samples, the difference was not statistically significant (largely due to the high variation).

DISCUSSION

The in vivo enhancing effect of ascorbic acid has been recognized for many years (*8*) and is replicated in Caco-2 cells (*9*). In the experiments reported here, ferritin protein expression increased 6 h after ascorbic acid treatment in both serum-replete and serum-deprived cells, demonstrating that ascorbic acid on its own produced a ferritin response but that it was exacerbated by serum, most likely because the FCS contains iron. At all subsequent time points in the serum-deprived cells, there was no further increase in ferritin, whereas in the serum-replete samples, the levels continued to rise up to the 48 h time point. Ascorbic acid was therefore responsible for the initial rapid increase in ferritin protein expression, with the serum then providing iron for any increase beyond this time. Following the removal of ascorbic acid (2 h after the start of the experiment), the ferritin levels continued to rise, and when the level reached a plateau (in the serum-deprived cells), it remained elevated for at least 72 h, indicating little or no degradation of ferritin.

In the ferrous ascorbate-treated cells, a similar pattern of ferritin protein expression over time was seen in both the serum-replete and the serum-deprived cells, but in the serum-replete cells, as in the ascorbic acid-treated cells, the maximum level of ferritin protein expression was higher. At $t = 6$, there was significantly less ferritin in the ferrous ascorbate-treated cells, both in the presence and in the absence of serum, than in the control. Possibly ferrous ascorbate treatment caused an initial degradation of the ferritin protein relative to the control samples before the expected increase was initiated. It has been reported that oxidative stress can cause an increase in the activation of proteasome protease (*10*). While this could account for the initial decrease in ferritin levels in the cells, an alternative hypothesis is that when the cell detected high ferrous iron levels, it exported ferritin molecules from the cell while the cellular machinery produces more ferritin to act as a "sink" for the free iron.

At later time points, the ferritin levels continued to increase but to a greater extent in the serum-replete cells. As the initial iron dose period was only 2 h, the feedback mechanism that controls the translation of the ferritin mRNA does not appear to be regulated by the concentration of iron in the media surrounding the cells but by the previously absorbed iron. Another similarity with the ascorbic acid-treated cells is seen in the serum-replete samples where the additional iron in the serum contributes to a final maximum ferritin protein level that is higher than that seen in the serum-deprived samples.

Freezing and adding ascorbic acid to the digestate had a significant effect on soluble iron concentrations. The addition of ascorbic acid to $FeCl_3$ samples caused a comparable increase in soluble iron concentration of the digestate both before and after freezing, which indicates that ferrous ascorbate was stable throughout the freeze−thaw process and remained intact, keeping iron in solution following thawing. In contrast to this, in the egg alone samples, there was an increase in soluble iron when ascorbic acid was added to the digestate before freezing but not after freezing, which suggests that although ascorbic acid initially complexed with iron to form ferrous ascorbate, during the

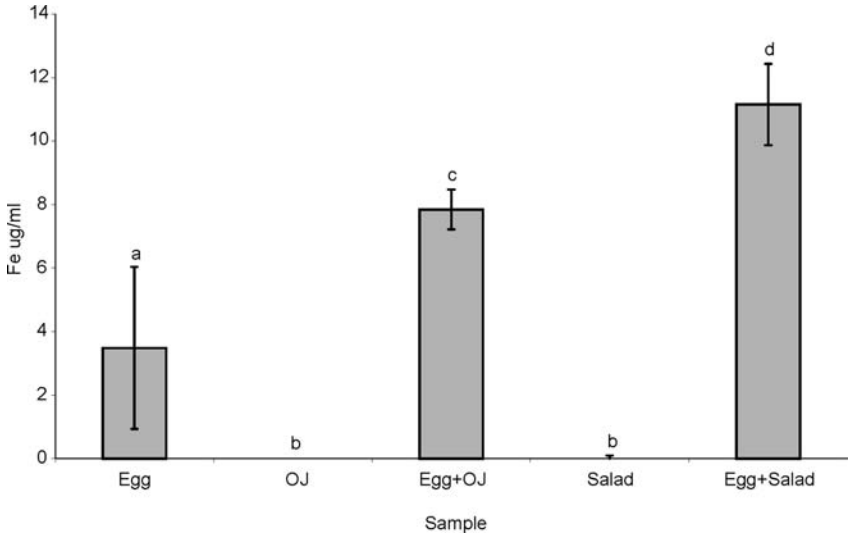

Figure 7. Quantity of iron solubilized from foods following simulated digestion. Letters a−d denote significant differences ($p < 0.05$).

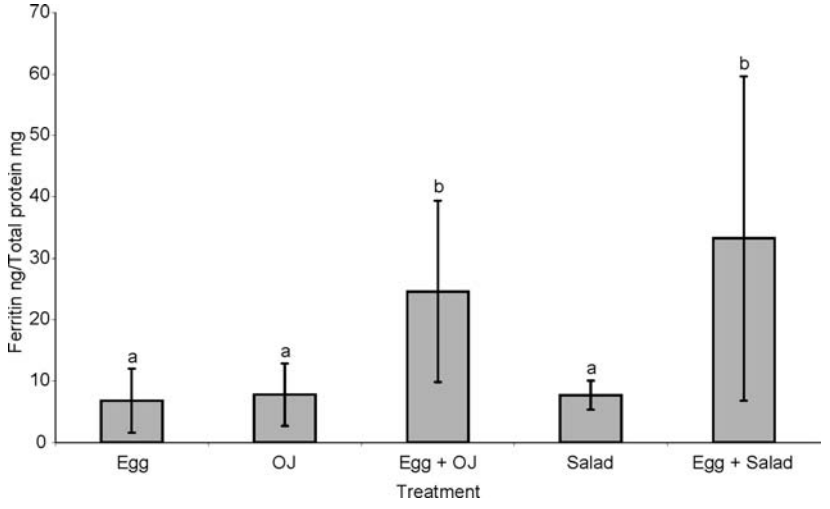

Figure 8. Effect of ascorbic acid-rich foods on iron availability from eggs. Letters a and b denote significant differences ($p < 0.05$).

freeze−thaw process, there were chemical changes whereby ascorbic acid lost its ability to complex with additional iron released from the food matrix. Finally, in the case of the egg plus orange juice samples, the addition of ascorbic acid made no difference to the soluble iron content in the fresh samples or following freezing, probably because of the high initial quantity of ascorbic acid in orange juice.

It has been reported that freezing and the freeze−thaw cycle increase nonheme-soluble iron content in meat and seafood (*11*). The results with eggs and orange juice support these observations, and the most likely explanation is that as the freeze−thaw cycle occurs, the associated contraction and expansion break down the cell structures in which a proportion of the iron is found, releasing it into the digestate as soluble iron. This theory is supported by the fact that freezing had no effect on the solubility of iron in the $FeCl_3$ samples in which there was no complex food matrix present.

Previously, the in vitro Caco-2/simulated digestion intestinal cell model has been used to measure iron uptake by Caco-2 cells from dried samples (*2*). This provides useful information that enables the ranking of iron availability from varieties of food crops, such as maize and beans, and there is also qualitative, albeit not quantitative, agreement about the enhancing effect of ascorbic acid on iron availability (*9*). Here, the previously described method has been adapted to use fresh/cooked food samples containing components in ratios that would be found in meals. A similar approach has been used previously to demonstrate that cooking improves iron availability in legumes and that the addition of ascorbic acid to ready-to-eat lentils increases the iron content of Caco-2 cells, measured in washed, lysed cells using atomic absorption spectroscopy (*12*). Cooked legumes were subjected to simulated digestion, and the digestate was applied to the cells, but the technique is less accurate and has lower sensitivity than the ferritin technique, requiring a high concentration of iron digestates, removal of extraneous iron from cells before lysing, and subtraction of baseline values of iron in the cells.

An enhancing effect of ascorbic acid on iron absorption from eggs has been reported previously in rats (*13*) and humans (*14*). Our data suggest that this may partly result from increased solubility of iron during digestion. Following simulated digestion, the egg alone sample contained approximately 3.75 μg/mL soluble iron, but when an ascorbic acid-rich food was added, the soluble iron concentration increased dramatically. In the absence of any ascorbic acid-rich food, ferritin formation following exposure of cells to digested egg samples was minimal, being

directly comparable to ferritin formation in cells exposed to digested orange juice and salad alone, both of which are relatively low in iron. However, once a source of ascorbic acid was added, either as salad components or as orange juice, the ferritin formation was increased significantly. These results show that, when digested in conjunction with egg in a typical meal-based ratio, orange juice and salad both enhanced iron uptake from eggs. These data support the results of the human study where orange juice was found to significantly increase iron absorption from eggs in human volunteers (*14*).

In conclusion, the effects of a serum deprivation period and/or the addition of ascorbic acid on ferritin formation need to be carefully considered in relation to the research question, as previously reported (*15*). If the Caco-2 model system, coupled with the in vitro simulated digestion technique, is being used to rank iron absorption from similar foods in freeze-dried samples, then both can be used to enhance iron absorption and amplify any differences that are present. However, when used in this way, the techniques are far from physiological and when more complex food matrices are involved, as seen in the fresh food samples, the model should be refined so that it more accurately reflects nutritional practices, as a precursor to in vivo studies.

The Caco-2 cell model system, both alone and coupled with an in vitro digestion step, is a useful tool to study mechanisms of iron absorption and to screen foods and sources of iron for potential iron availability. Ideally, foods should be prepared as for human consumption before undergoing simulated digestion, and when part of a meal, the combined effect of all of the components should be measured. Using this modified in vitro system, it was demonstrated that the iron in eggs is more bioavailable when combined in a meal with ascorbic acid-containing foods. This technique can be used as either a preliminary screening tool prior to undertaking in vivo studies or for mechanistic research, but the data thus generated will not be sufficient to make firm predictions about iron bioavailability in humans. When combined with an in vitro simulated digestion stage, the Caco-2 cell system is a useful screening tool, provided that care is taken in interpreting the results in relation to the limitations of the system.

LITERATURE CITED

(1) Gangloff, M. B.; Glahn, R. P.; Miller, D. D.; VanCampen, D. R. Assessment of iron availability using combined in vitro digestion and Caco-2 cell culture. *Nutr. Res.* (*N.Y.*) **1996**, *16*, 479–487.

(2) Glahn, R. P.; Lee, O. A.; Yeung, A.; Goldman, M. I.; Miller, D. D. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. *J. Nutr.* **1988**, *128*, 1555–1561.

(3) Food Standards Agency. *McCance and Widdowson's The Composition of Foods*, 6th summary ed.; Royal Society of Chemistry: Cambridge, 2002.

(4) Ishikawa, S. I.; Tamaki, S.; Arihara, K.; Itoh, M. Egg yolk protein and egg yolk phosvitin inhibit calcium, magnesium, and iron absorptions in rats. *J. Food Sci.* **2007**, *72*, S412–S419.

(5) Lynch, S. R.; Stoltzfus, R. J. Iron and ascorbic acid: proposed fortification levels and recommended iron compounds. *J. Nutr.* **2003**, *133*, 2978S–2984S.

(6) Rossander, L.; Hallberg, L.; Bjorn-Rasmussen, E. Absorption of iron from breakfast meals. *Am. J. Clin. Nutr.* **1979**, *32*, 2484–2489.

(7) Allen, A.; Carroll, N. J. Adherent and soluble mucus in the stomach and duodenum. *Dig. Dis. Sci.* **1985**, *30*, 55S–62S.

(8) Brise, H.; Hallberg, L. Effect of ascorbic acid on iron absorption. *Acta Med. Scand. Suppl.* **1962**, *376*, 51–58.

(9) Beiseigel, J. M.; Hunt, J. R.; Glahn, R. P.; Welch, R. M.; Menkir, A.; Maziya-Dixon, B. B. Iron bioavailability from maize and beans: a comparison of human measurements with Caco-2 cell and algorithm predictions. *Am. J. Clin. Nutr.* **2007**, *86*, 388–396.

(10) Pandey, S.; Lopez, C.; Jammu, A. Oxidative stress and activation of proteasome protease during serum deprivation-induced apoptosis in rat hepatoma cells; inhibition of cell death by melatonin. *Apoptosis* **2003**, *8*, 497–508.

(11) Camara, F.; Amaro, M. A.; Barbera, R.; Lagarda, M. J. Speciation of bioaccessible (heme, ferrous and ferric) iron from school menus. *Eur. Food Res. Technol.* **2005**, *221*, 768–773.

(12) Viadel, B; Barberá, R.; Farré, R. Effect of cooking and legume species upon calcium, iron and zinc uptake by Caco-2 cells. *J. Trace Elem. Med. Biol.* **2006**, *20*, 115–120.

(13) Miller, J.; McNeal, L. S. Bioavailability of egg yolk iron measured by hemoglobin regeneration in anemic rats. *J. Nutr.* **1983**, *113*, 115–123.

(14) Callender, S. T.; Marney, S. R., Jr.; Warner, G. T. Eggs and iron absorption. *Br. J. Hamaetol.* **1970**, *19*, 657–665.

(15) Scheers, N. M.; Sandberg, A. S. Ascorbic acid uptake affects ferritin, Dcytb and Nramp2 expression in Caco-2 cells. *Eur. J. Nutr.* **2008**, *47*, 401–408.

Received for review December 2, 2009. Revised manuscript received February 11, 2010. Accepted February 15, 2010. This work was funded by a CASE studentship from the Biotechnology and Biological Sciences Research Council (industrial partner GlaxoSmithKline), with additional support from the British Egg Industry Council. It formed part of B.T.'s Ph.D. thesis at UEA and S.A.'s M.Med.Sc. degree at the University of Sheffield.