Article to the Special Issue

The Use of Caco-2 Cells to Estimate Fe Absorption in Humans – a Critical Appraisal

Ann-Sofie Sandberg

Chalmers University of Technology, Department of Chemical and Biological Engineering, Food Science, Gothenburg, Sweden

Abstract: The Caco-2 cell model is widely used to assess the bioaccessibility/availability of iron from foods and diets. Analysis of iron uptake in this human epithelial cell line is usually preceded by a two-step digestion to simulate the conditions in the stomach and small intestine. Moreover, culturing the cells on inserts permits the measurement of iron transport. The cellular iron uptake is determined by direct measurements using radioisotopes, or indirectly by measurement of ferritin, the intracellular storage form of iron. There is a good correlation between Caco-2 cell uptake and human iron bioavailability for a number of dietary factors known to affect iron absorption. However, recent data suggest that in some cases there is no correlation. Possible reasons for such discrepancies, the benefits, and limitations of the Caco-2 cell model are discussed. In conclusion, *in vitro* experiments with Caco-2 cells are important tools for ranking foods with respect to bioavailability, for mechanistic studies of iron absorption, and for studies of dietary factors influencing absorption. The results need to be confirmed in humans.

Key words: Caco-2 cells, iron bioavailability, *in vitro* digestion

Introduction

Low bioavailability of iron is a major nutritional problem in developing countries, where the diet is based on staple foods. One strategy to improve the iron status is to biofortify staple food crops with iron and other micronutrients. For this reason, to rank the foods with respect to iron bioavailability, there is a need for a simple method to estimate iron bioavailability in humans. Such a method must be reproducible and reflect the *in vivo* conditions. It is thus of great importance that the *in vitro* methods are validated by *in vivo* measurements in humans. Traditionally a combined two step *in vitro* digestion and measurement of dialyzable or soluble iron is used to determine iron availability [1]; i. e., iron that is potentially available for absorption in the gut. Dialyzability methods are useful for screening purposes to identify major differences in availability, but it cannot predict the magnitude of response [2, 3]. For example, methods based on dialyzability exclude iron that is bound to large but not small molecules. However, some of these iron-bound large molecule complexes (e. g., ferritin) have substantial bioavailability whereas iron bound to certain small molecules is not bioavailable [4].

Combining *in vitro* digestion with uptake in Caco-2 cells is a step forward since it predicts both availability and uptake into the enterocyte and at times also the absorption. However, in a consensus statement of experts working in the field of iron nutrition, it was

DOI 10.1024/0300-9831/a000038

concluded that effects may be disproportionate to that observed in humans, but Caco-2 studies confer the advantage of assessing the impact of dietary modulators [2]. Conversion factors are needed in order to predict *in vivo* results in humans.

Bioavailability of iron is defined as the absorption and utilization of iron for normal metabolic processes. It includes several stages:

1. Digestion, which releases iron from the food matrix into the lumen.
2. The uptake of iron into the intestinal enterocytes.
3. The transepithelial transport of iron into the circulation.
4. The retention, utilization, and storage of iron in the body.

The use of the combined *in vitro* digestion Caco-2 cell model includes the two first stages, and if the cells are cultured on inserts this model also can be used to assess the third stage; i. e., the transepithelial transport into the circulation.

In the lumen, the solubility of iron and the formation of soluble ferrous complexes [5] are the main determinants of iron available for uptake into the enterocytes. This stage is influenced by food compounds such as phytate, polyphenols, ascorbic acid, and certain other organic acids. Recently it was reported in the literature that some food factors (iron, ascorbic acid, calcium) also exert intracellular effects in the enterocyte which influence the bioavailability of iron [6–8]. Furthermore, there are a number of systemic factors that influence the iron uptake; i. e., iron status, infection, and inflammation. The iron status affects the percentage of iron absorption but not relative absorption ratios between foods [9].

Caco-2 cell model for iron uptake and transport

The Caco-2 cells originate from an adenocarcinoma of a Caucasian 75-year-old male [10]. At confluence, the cells spontaneously differentiate to develop characteristics of small intestinal enterocytes and are therefore a useful tool to study events occurring in the small intestine. These cells also contain all the relevant uptake and transport proteins for iron. Cytochrome B reductase 1 (*CYBRD1* a.k.a. DCYTB) is expressed in Caco-2 cells and is responsible for the lumenal reduction of ferric to ferrous ion. The divalent metal transporter 1 (*SLC11 A2* a.k.a. DMT1 and NRAMP2) is also present and is responsible for ferrous iron uptake by a pH-dependent process. Iron efflux (basolateral) is mediated by ferroportin (S*LC40 A1* a.k.a. FPN1 and IREG1) and oxidized to ferric iron by ferrioxidase activity of hephaestin (*HEPH*) [for reviews see 6]. These facts suggest that Caco-2 cells are a suitable model to study the mechanisms of human intestinal iron absorption. However, the effects of hepcidin (*HAMP*) on iron status cannot be measured by the use of Caco-2 cells because the regulation of iron absorption by serum levels of hepcidin relies on hepcidin expression in the liver.

Identification of enhancers and inhibitors of iron absorption

Known enhancers from human absorption studies of single meals are ascorbic acid, certain other organic acids, and muscle tissue [11]. The major inhibitors identified in human absorption studies are phytate (inositol hexa- and pentaphosphates), calcium, tannic acid, and other iron-binding polyphenols. These food compounds have been studied in the Caco-2 cell model and found to predict the direction of response in humans. In several studies, ascorbic acid increased iron uptake in Caco-2 cells [12–15]. Muscle tissue also improved iron uptake in Caco-2 cells [16]. Phytate (inositol hexaphosphate) and inositol pentaphosphate had similar negative effects on iron uptake in Caco-2 cells as in human absorption studies [15, 17–20]. This was also the case for tannic acid and some other polyphenols [15, 19, 21] as well as calcium [7, 15]. The effect of organic acids was dependent on the amount, concentration, and chemical structure of the organic acids [22–23]. β-carotene enhanced iron uptake in Caco-2 cells according to Garcia-Casal *et al.* [24], while Bengtsson *et al.* [25] found no effect on ferritin formation in Caco-2 cells. Thus, studies show that Caco-2 cells reflect the expected relative bioavailability of iron in most cases, but it may overestimate or underestimate the effects of ascorbic acid. In addition, the expected bioavailability of iron-fortified compounds, shown in human trials, is not always reflected in the Caco-2 cell model [2]. The magnitude of response is dependent on the preparation steps, the digestion procedure and the use of dialysis membranes which differs between laboratories.

Intracellular effects of food factors

It has been shown that iron treatment decreased the Nramp2 expression in Caco-2 cells [26] and that the expression of intestinal iron transport proteins can

be altered shortly after changes in the lumenal iron concentration both in Caco-2 cells [27] and *in vivo* in rats [28]. This suggests that the duodenal enterocytes can modulate their nutrient absorption in response to the dietary intake. In fact both the activity of the influx transporter NRAMP2 and the efflux transporter ferroportin are influenced by iron. Sharp *et al.* [27] demonstrated that there is a dose-dependent decrease in expression of NRAMP2 protein in Caco-2 cells after exposure to iron. It was recently shown that increased iron reduced the apical uptake and basal efflux and increased basal uptake and apical efflux by the participation of NRAMP2 and ferroportin [29]. Thus the fluxes are regulated by the iron supply.

Other food factors known as enhancers and inhibitors of iron absorption; i. e., ascorbic acid and calcium, affect intracellular iron metabolism in the Caco-2 cell model. Ascorbic acid influenced the expression of proteins involved in uptake and transport of iron in the absence of iron [8, 30]. There was a transient (2 hour) upregulation of NRAMP2 , DCYTB, ferritin, and ferroportin proteins whereas longer exposure (24 hours) of ascorbic acid downregulated NRAMP2 and DCYTB protein expression. These results are in agreement with that found in human absorption trials, where the short-term effects of ascorbic acid intake improved iron absorption and long-term ascorbic acid supplementation did not affect iron status [31]. The effect of calcium on the protein expression of NRAMP2 and ferroportin was investigated by Thompson *et al.* [7]. The results indicate an inhibitory effect at the apical cell membrane by downregulation of NRAMP2 protein at the cell membrane, thereby decreasing iron uptake by the cell. No effect was found on ferroportin expression. However, Lönnerdal [32] observed that calcium decreased ferroportin at the plasma membrane, but not total ferroportin. This suggests that the inhibitory effect of calcium on iron absorption results from decreased serosal efflux. This may be a short-term effect of calcium on iron absorption.

Iron-binding polyphenols in legumes, tea, and grapes are other well-known inhibitors of iron absorption [33–35]. Bioactive polyphenols from green tea and grape seed extracts decreased both non-heme and heme iron transepithelial transport in the Caco-2 cell model rather than affecting iron uptake into the cell. However, there was no effect of polyphenols on expression of ferroportin or hephaestin protein [21,36]. It is possible that polyphenols forms complexes with iron in the cells and prevent iron exit via ferroportin. The mechanism by which polyphenols affects iron absorption is not yet known.

Combined *in vitro* digestion Caco-2 cell model for estimation of iron absorption

The combined *in vitro* digestion Caco-2 cell model using two-step digestion according to a procedure described by Miller *et al.* [1], and exposing Caco-2 cells to radiolabeled dialysate for measurement of iron uptake in the cells, was first introduced by Gangloff *et al.* [37]. This method has been further developed, and the procedure commonly used today was described by Glahn *et al.* [13]. Briefly, the food is subjected to stomach digestion with pepsin (1 hour, pH 2, 37 °C) followed by intestinal digestion with pancreatin and bile acids (2 hours, pH 7, 37 °C). The digesta is transported to the apical compartment of the culture well and soluble iron is allowed to diffuse through a dialysis membrane with a cut-off of 15 kDa to become accessible for uptake by the Caco-2 cells. The dialysis membrane protects the cells from the digestive enzyme, but the problems previously identified with the *in vitro* dialyzability method remains.

Caco-2 cells of passage 30–40 and studies conducted at 14 days post-seeding are recommended. After 2 hours the digesta is removed and cells are washed, and after 24 hours the cell monolayer is harvested and ferritin formation measured. Some laboratories use iron isotopes for the uptake studies. There are also differences in what intestinal pH is used (pH 6.5 to 7.4) between laboratories. Cells are grown on inserts if basolateral iron transport is to be measured.

Comparisons between meal studies in Caco-2 cells and human iron absorption

The first study comparing iron uptake by Caco-2 cells with human iron absorption data was performed by Au & Reddy [36]. In this study, non-heme iron uptake in cells was compared with published human absorption data from Cook's and Hurrell's laboratories [39–43], using exactly the same meal composition, and showing a strong correlation between iron uptake in Caco-2 cells and absorption ratios in humans. Reduced uptake of iron was found with meals containing casein, bran, phytate, and soy added to semi-purified meals. The observed inhibitory effects were consistent with those identified in human absorption studies, although the effect on iron uptake of phytate was less pronounced and the effect of casein was more pronounced. Bovine serum albumin and ascorbic acid, having enhancing effects in humans, did not have similar effects in these cell experiments.

Later, Yun *et al.* [14] investigated the dose-response relationship of ascorbic acid and tannic acid in the Caco-2 cell model in replicate meals administered in human trials [34, 44]. They used ferritin formation as a marker of cell iron uptake, and found similar relative responses compared to human data. The absorption ratios were strongly correlated. From the natural logs of the absorption ratios, a conversion factor was formulated.

In some reports in the literature there are discrepancies between results from human and Caco-2 cell studies. Vitamin A (retinyl palmitate) added to a bread meal resulted in a 2.6-fold increase in iron uptake (^{59}Fe) [45] in Caco-2 cells, whereas there was no effect of vitamin A in clinical studies using meals labeled with ^{59}Fe or stable isotopes [46]. In the Caco-2 cell study, the meal consisted of white wheat bread, while corn bread was used in the human study. The effects of oxalic acid in spinach have also been investigated. Rutschke *et al.* [47] found a negative effect on iron uptake in Caco-2 cells with fresh spinach, in contrast to a clinical study by Bonsmann *et al.* [48] where no effect was observed in a meal of 150 g cooked spinach served with wheat bread.

To make comparisons between Caco-2 cell studies and human absorption studies, foods should be prepared exactly in the same way; i. e., identical meals must be used. So far, there is only one publication dealing with direct comparisons; in this case with two maize varieties, and white and colored beans [49]. Caco-2 cell iron uptake was measured as ferritin formation and compared to iron isotope absorption from single meals in humans. As the absorption was very low from the maize and beans, orange juice was added to improve absorption/uptake. Thus, meals with and without ascorbic acid were tested. Thirteen healthy women were served 100 g cooked beans extrinsically labeled with ^{59}Fe and ^{55}Fe using a 2 × 2 factorial design. White versus colored beans with/without 250 mL orange juice was studied, and 2-week isotope retention measured.

Frozen, prepared maize and bean meals were transported to Glahn's laboratory to assess iron uptake in the *in vitro* digestion Caco-2 cell model. Orange juice added to the meals was purchased at each location, and the ascorbic acid content matched by addition of this acid. Other organic acids in the juice; e. g., citric acid, were not analyzed and therefore not matched. The conversion factor described by Yun *et al.* [14] was used to predict human absorption ratios. The combined-digestion Caco-2 cell method accurately predicted the relative iron absorption from the maize meals with and without ascorbic acid. The Caco-2 cell data, however, inaccurately predicted lower iron bioavailability from colored beans (pinto beans) assuming higher polyphenol content than from white beans (Great Northern beans). It also predicted lower magnitude of enhancing response to the addition of orange juice.

Benefits and limitations with the use of the Caco-2 cell model

The initial procedure in the combined digestion Caco-2 cell method is a two-step *in vitro* digestion to simulate gastric and intestinal digestion. The first question is whether the simulated digestion reflects the *in vivo* situation. There are obvious differences compared to *in vivo* digestion; for example, the lack of pH gradient, the use of a fixed stomach pH of 2, stomach digestion time set to 1 hour, and the intestinal pH adjusted to 7. However, according to recent data, the stomach pH should be 4 [50–51]. The intestinal pH used in the digestion is of outmost importance because it strongly influences the iron solubility, and furthermore NRAMP2 in the enterocytes is strongly proton-dependent [27].

A difference between the Caco-2 cells and the intestinal enterocytes is that the Caco-2 cells do not have an outer mucous layer. Instead a dialysis membrane with a cut-off of 15 kDa is used. Attempts have been made to co-culture a mucin-secreting cell-line (HT 29 - 5M21) [52] with the Caco-2 cell line, but it is quite complicated and does not give the anticipated response of all dietary factors influencing bioavailability. The mucous layer is substantial in the human colon but thin or absent in the duodenum (where the main iron absorption takes place), as well as in the ileum and jejunum [53].

Iron uptake in Caco-2 cells differs from the *in vivo* situation in that the iron is accumulated in the cells as there is no blood to be transported to. Hence, it is a closed system, which may affect kinetics. If the cells are grown on inserts, the transport across the basolateral membrane can be mimicked, but there is no hepcidin-controlled transport, which is the case *in vivo*.

One limitation of Caco-2 cells is that they are of colon cell origin. Compared to duodenal or small intestinal cells, the colon cell's transport rates of hydrophilic compounds paracellular are lower; colon cells are also less leaky, and there is less discrimination among the molecular sizes of compounds that are transported paracellularly [54].

Another limitation of using Caco-2 cells is that there is poor agreement in results obtained between

different laboratories. This could be due to the fact that quite experienced laboratories are needed to cultivate Caco-2 cells and perform studies in this model. Hence, a standardization of the digestion procedure (pH, etc.), cell passage to measure iron uptake, time considerations, and the choice of method to measure iron is needed. For example, to use ferritin formation as an endpoint for measuring iron uptake assumes that ferritin formation is proportional to iron uptake. This is not always the case as ascorbic acid can increase ferritin formation in the absence of iron [8]. In addition, the ferritin molecule can contain different amounts of iron [55]. The radiolabel studies of iron uptake are adequate for measures of intracellular iron uptake, but for measurement of transepithelial transport the radioisotope measurement gives an underestimation due to intracellular dilution of dietary iron.

The Caco-2 cell model often predicts the direction of response, but the magnitude differs from human studies.

There are still benefits of using the Caco-2 cell model to estimate the effects on iron absorption. A high-throughput system has been developed, which is useful for screening of a large number of samples [19]. Compared to *in vitro* dialyzability/solubility methods, the high-throughput system includes the iron uptake into the enterocyte, which is of importance since food factors also have been shown to have intracellular effects. This is so far shown in Caco-2 cells but needs be confirmed in animal studies. The Caco-2 cell model has also been useful in identifying potential inhibitors and enhancers of iron absorption, or at least in confirming those already identified in human studies.

The use of the Caco-2 cell model is furthermore an important tool to study molecular mechanisms of iron absorption; such studies have advanced the knowledge of regulation of iron absorption and metabolism.

Conclusions

The combined *in vitro* digestion Caco-2 cell method for estimation of iron bioavailability correlates in most cases with human iron absorption studies in prediction of direction of response, and is therefore useful for ranking foods with respect to bioavailability. But there are exceptions; e.g., white beans compared to colored beans. More studies are needed using direct comparisons of identical meals to measure response in Caco-2 cells and human trials.

To determine iron uptake in Caco-2 cells, direct measurements of iron with mass spectrometry is preferable to indirect measurement by use of ferritin formation. This is also the case for estimation of transepithelial transport of iron, where direct measurement using mass spectrometry would be the method of choice.

In vitro experiments with Caco-2 cells are nonetheless useful to understand the measurements recorded *in vivo* and to suggest future experiments that can be performed in the whole organism. The Caco-2 cell model has a potential for further development to more closely correspond to *in vivo* conditions.

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Prof. Ann-Sofie Sandberg

Dept of Chemical and Biological Engineering
Food Science
SE-412 96 Gothenburg
Sweden