The use of Caco-2 cells in defining nutrient bioavailability: application to iron bioavailability of foods

R. Glahn, USDA-ARS Cornell University, USA

Abstract: In vitro models can be useful tools in developing objectives that warrant testing in animal and human subjects. For nutrient absorption studies, Caco-2 cell monolayers have been widely used for defining certain aspects of nutrient uptake and transfer across epithelial cells. For a practical example, Caco-2 cell monolayers have been applied as a screening tool to address the food factors associated with iron deficiency anemia (IDA). IDA is the most common micronutrient deficiency worldwide and is primarily due to poor dietary Fe bioavailability. Development of foods with improved iron bioavailability is essential to alleviating this critical deficiency. Human and animal studies of iron absorption from food are time-consuming, expensive and offer limited capacity to address luminal interactions of iron and food ingredients. These factors have led scientists to pursue rapid, high-throughput in vitro methods that enable analysis of food–iron interactions and prediction of Fe bioavailability for humans. As a result, a simulation of in vitro digestion combined with Caco-2 cell monolayers was developed. The primary reason for this evolution of methodology is the fact that Fe solubility does not always correlate with Fe availability. Addition of the Caco-2 cell monolayer adds a living component to the model system that in theory should reflect the key step in iron bioavailability (i.e. uptake of iron by the enterocyte). In vitro digestion and Caco-2 cell culture conditions can vary significantly, and if proper physiological and cell culture principles are not applied, then the in vitro method can generate results that are not a true reflection of in vivo effects. Therefore, it is critical that an in vitro model be assessed under a broad range of conditions and validated against known effects in humans. This review summarizes the development, validation, and application of currently the most broadly applied in vitro digestion/Caco-2 cell culture model, providing it as an example of how epithelial cell lines can be used in practical nutrition research.

Key words: Caco-2, bioavailability, in vitro, cell culture, food iron.

13.1 Introduction

In vitro models can be a useful and productive approach in many if not all of the life sciences. Furthermore, coupling in vitro studies with in vivo testing
The use of Caco-2 cells in defining nutrient bioavailability can be even more productive as *in vitro* studies are by nature less expensive, rapid, and often allow greater manipulation and simplification for mechanistic studies of physiological processes at the level of the cell. These cellular observations often give clues to *in vivo* effects and thus allow researchers to design more definitive animal and human trials. However, as with any *in vitro* model, care must be taken to accurately reproduce the physiological conditions relevant to the experimental objectives; otherwise, one runs the risk of generating information that is merely an artifact of the conditions, and not representative of the *in vivo* physiology it was designed to replicate.

For studies of intestinal absorption of nutrients, the use of intestinal cell cultures has facilitated our understanding of the mechanisms of absorption of many nutrients. Of the major cell lines used in nutrition research, the Caco-2 cell line has become widely used and characterized for a broad range of nutrient uptake studies. In most cases, the use of Caco-2 cell monolayers has proven useful for elucidating mechanisms of nutrient uptake; however, Caco-2 cell monolayers may not always be useful, necessary, or appropriate as a tool to predict bioavailability of nutrients. This is because the Caco-2 cell monolayer is merely a layer of cells, and there are a multitude of other factors that can regulate and influence absorption in the intestinal lumen. Not all of these factors such as the intestinal microflora, nutritional status, disease states, nutrient interactions, etc. can be replicated *in vitro*. There are definite limitations for intestinal cell monolayers and these should always be considered for each nutrient of study.

As stated previously, there is a multitude of literature demonstrating that this cell line is useful in defining mechanisms of uptake of various nutrients. Hence this chapter will focus on an example of the next level of use for cell culture models, which is the use of the cell line as a high-throughput screening tool for food iron bioavailability. Iron represents a unique challenge in nutrition as it is an essential nutrient but often poor in bioavailability and thus Fe deficiency affects more than a third of the world’s population. Given the complexity of iron bioavailability in foods, the use of an *in vitro* model is particularly essential for developing foods with more bioavailable Fe.

This chapter is organized into the following sections: (1) the origin of the Caco-2 cell line; (2) *in vitro* measurement of Fe bioavailability; (3) the physiology of the *in vitro* digestion/Caco-2 cell model; (4) validation of the *in vitro* digestion/Caco-2 cell model; and (5) the justification for using the cell line to measure Fe availability.

### 13.2 Origin of the Caco-2 cell line

The Caco-2 cell line was established from a human colon adenocarcinoma in the late 1970s.¹ The most common source of obtaining the Caco-2 cell line is the American Type Culture Collection (ATCC; Manassas, VA; see http://www.atcc.org). The primary clone of the Caco-2 cell line that is
available through ATCC is known as HTB-37; however, numerous other clones are known to exist in various laboratories worldwide, some of which are more useful or unique to the study of certain nutrients or pharmaceuticals. The Caco-2 cell line has been highly characterized for morphology, functional differentiation, and uptake of a broad range of nutrients and pharmaceuticals. Although colonic in origin, in culture the Caco-2 cell differentiates and closely resembles mature intestinal absorptive cells.

For many nutrients, the Caco-2 cell has been observed to be a useful model for measurement of uptake and perhaps transfer across the epithelial cell layer. In regards to Fe, early studies of Caco-2 cell monolayers documented greater Fe uptake and transepithelial transfer from Fe\(^{2+}\)-ascorbate versus Fe\(^{3+}\)-citrate or Fe\(^{3+}\)-NTA (nitrilotriacetic acid), and also observed greater iron uptake and transfer in iron-deficient versus iron-loaded cells. Subsequent studies demonstrated the need to utilize an iron removal solution to accurately quantify Caco-2 cell iron uptake. Han et al. demonstrated that inositol phosphates inhibit Fe and Zn uptake and transfer across Caco-2 monolayers; that ascorbate offsets the inhibitory effect of phytate on Caco-2 cell iron absorption; and that reduction of Fe\(^{3+}\) to Fe\(^{2+}\) dramatically increases Fe uptake. The first evidence of transporter-mediated uptake of Fe in Caco-2 cells was demonstrated by Gangloff et al. Subsequent studies identified the intracellular proteins and intestinal iron uptake transporters present in the brush border membrane of Caco-2 cells which contributed to more in-depth knowledge of the molecular mechanisms of iron absorption enjoyed today.

Iron balance is primarily regulated by absorption as there is no excretory mechanism for Fe, merely loss through fecal excretion. Given this fact, the first step in Fe bioavailability depends on whether the Fe is in a form that can be taken up by the intestinal epithelial cell. It is at this level that the Caco-2 cell line can be used as an effective, first-level screening tool.

As an example of how Caco-2 models can be applied to nutrient bioavailability, this chapter will also feature the development, validation, and application of a specific in vitro digestion/Caco-2 cell model of Fe availability. As evident in the literature, this model appears to be the most widely applied in vitro model in the field of Fe availability. It is a robust model as it has demonstrated the capability to be a high-throughput system with relatively low cost, has shown the capability to examine a broad range of foods and meal conditions, and produces results that, in general, agree with human studies. Indeed, with the recent increase in global biofortification programs such as HarvestPlus®, this model has the potential to offer key information to bioavailability studies and deserves an in depth evaluation and review.

13.3 In vitro measurement of iron bioavailability

In vitro estimation of iron bioavailability dates back to the mid-1930s and has been reviewed a number of times over the past 25 years. The first
widely used in vitro technique for measuring iron bioavailability was published by Miller and Schricker and co-worker and involved the use of pepsin, pancreatic, and bile extracts to simulate intestinal digestion of foods. The intestinal 'digests' were then incubated in the presence of a 'dialysis bag.' Iron bioavailability was estimated by the amount of Fe that dialyzed into the bag, hence Fe bioavailability from this method primarily reflects Fe solubility and molecular size.

The potential pitfall of simply using Fe dialyzability or Fe solubility as a marker of Fe availability is that it can significantly overestimate availability. For example, Fe—citrate is a highly soluble complex, yet it has relatively low iron availability as shown in both human studies and cell culture. Mono-ferric phytate is another highly soluble form of Fe, yet in single meal feeding studies it is clearly a poor source of available Fe in many foods, particularly when phytate exists in great molar excess relative to Fe. Therefore, given the potential pitfalls of using iron dialyzability as a marker of availability, it is clear that in vitro methods for measuring iron availability need to include a component that is more closely correlated with intestinal iron uptake.

The first publications on coupling in vitro digestion with culture of Caco-2 cell monolayers appeared in the mid-1990s. This methodology simply placed the radiolabeled dialysate from the Miller method on top of Caco-2 cell monolayers. This study and subsequent studies documented the enhancing effect of ascorbic acid and meat and demonstrated that the combination of in vitro digestion and Caco-2 cell culture offered significant advantages over measurement of iron dialyzability. However, these early attempts at combining in vitro digestion with Caco-2 cell culture were complicated by the need to use radioactive Fe to measure Caco-2 cell uptake. Radioisotopes are useful in the measurement of cell iron uptake and manageable when used at low volumes; however, there is a safety issue with radioisotope use, handling radioisotopes is inconvenient and tedious, and the extrinsic radiolabel may not fully equilibrate with all of the food iron. For example, it is very likely that extrinsic radioiron will not equilibrate with heme iron or ferritin iron under certain food conditions. Also, for the extrinsic radioiron to equilibrate with the intrinsic food iron, it must be subject to the same processing, cooking and in vitro digestion steps. This results in potential contamination of workspace and equipment, and for in vitro digestion studies, requires the use of relatively large volumes of radio-labeled solutions. Another concern of using radioisotopes is that the cell-associated Fe may not be truly taken up by the cell, but merely bound to the surface; however, use of a technique to remove surface bound Fe minimizes this concern.

As a result of these disadvantages, Glahn et al. developed a method whereby formation of ferritin, an intracellular protein that stores Fe to protect against oxidative damage, could serve as the marker of Caco-2 cell iron uptake (Fig. 13.1). This method negates the need for use of
Figure 13.1 Diagram of the _in vitro_ digestion/Caco-2 cell culture model developed by Glahn _et al._ (MWCO = molecular weight cut off).

Radioisotopes, and is a sensitive and clear marker of cell Fe uptake as it is well known that cells produce ferritin in response to increases in intracellular iron. Caco-2 cell ferritin is easily measured via radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) from commercially available kits, the same kits used for human ferritin measurements in clinical practice. Moreover, this method enables measurement of iron availability from foods direct from the producer or supermarket shelf and requires no special preparation for use in the cell model.

The _in vitro_ digestion conditions developed by Glahn _et al._ involve a dual chamber system that does not require a dialysis bag or centrifugation of the food digest. This model uses a simple method of attaching a dialysis membrane (15000 Da molecular weight cutoff) to a plastic insert, thus enabling digestion to occur simultaneously with uptake which is the condition present in the intestine. This methodology saves the time of centrifugation or handling of the dialysis bags, thus enabling very high sample throughput at low cost. As a result, this model has been the most widely applied model for foods. It has found applications in development of infant foods, in agriculture for screening plant cultivars for bioavailable iron, in the pharmaceutical industry for developing improved iron supplements, for studies of human milk and infant formula, in fortified ready-to-eat cereals, in assessing elemental iron powders baked into breads, in assessing the bioavailability of Fe from raisins and the effects of raisins on fortified Fe in foods, in food science for comparison of fortified iron in milk and breads, in assessing the effects of caseinates and casein phosphopeptides, and in fundamental studies designed to investigate factors in digests of animal tissue (i.e. meat) that promote iron uptake.
13.4 The physiology of the *in vitro* digestion/Caco-2 model

As an enhancement of the dialysis method to assess Fe bioavailability, Caco-2 cell monolayers have been added to the *in vitro* digestion. In designing an appropriate model for gastrointestinal digestion coupled with culture of intestinal epithelial cells, it is essential to reproduce the physiological conditions of the intestinal lumen as closely as possible. For studies of Fe availability, this involves reproducing the conditions of the duodenum where most Fe absorption is thought to occur. The amounts of pepsin, pancreatin, and bile salts used by Miller and Schricker and co-worker for *in vitro* digestion have generally been maintained throughout many studies and are well accepted by the nutrition community. Only minor variations in pH titration methods and treatment of the enzymes have been necessary to remove contaminant Fe and refine the titration of foods to more closely reproduce *in vivo* conditions. Indeed, the initial studies involving centrifugation steps, dialysis ‘bags’ and the use of PIPES as a buffer of the digest were certainly less physiological than the subsequent employment of a dialysis membrane insert, buffering with NaHCO₃, and the elimination of centrifugation of the digest.

Recent research using inductively coupled plasma mass spectrometry has shown that Caco-2 cell ferritin formation is highly correlated with cell Fe content, thus indicating that Caco-2 ferritin formation remains proportionate to cell Fe uptake over a broad range of Fe availability (Glahn *et al.*, unpublished observations; Fig. 13.2).

The use of a dialysis membrane attached to plastic insert fulfilled a couple of key conditions that facilitated the coupling of *in vitro* digestion with enterocyte culture and measurement of Fe uptake. It protects the cells from the digestive enzymes and bile salts; it allows digestion to occur simultaneously with uptake, which could be important given the low stability of promoters such as ascorbic acid. It protects the cells from significant microbial contamination thus accommodating the overnight incubation of the digest with the epithelial cells and allowing adequate time for ferritin formation to occur. The overnight incubation of the digests with the cells enables a comfortable schedule for laboratory personnel as it negates the need to work outside of normal working hours. Such a factor is essential if high throughput of samples is a goal of the research project.

There has been concern about the effect of the dialysis membrane on limiting the bioavailability of certain forms of Fe such as ferritin and possibly heme–Fe complexes. The ferritin molecule has a molecular weight well in excess of the molecular weight cutoff of the dialysis membrane. Thus, if ferritin can be absorbed intact by intestinal cells, then this form of Fe would not be absorbed under the conditions shown in Fig. 13.1. However, there are several physiological reasons why ferritin is not likely to be absorbed intact, at least not to any significant extent. Under *in vivo* conditions, the surface of the enterocyte is covered with a mucin layer that is permeable to
ions and molecules of relatively small size (i.e. a molecular weight of 1000 Da), but impermeable to large proteins such as pepsin and pancreatic enzymes. This permeability cutoff seems to occur around a molecular weight of 17,000, which makes it unlikely that the ferritin molecule could be absorbed intact by the enterocyte, as its molecular weight is well over 500,000. It is important to note that the molecular weight cutoff of the mucin layer is close to that of the dialysis membrane (i.e. 15,000) chosen by Glahn et al. and in this respect appears to be a good substitute for the mucin layer. Therefore, iron availability from ferritin most likely results from release of Fe from the molecule due to the presence of ascorbic acid, digestive enzymes, or other compounds in foods. Overall, the conditions originally developed for this method have remained unchanged and, at present, appear to be the most physiological and appropriate for in vitro screening.

### 13.5 Validation of the in vitro digestion/Caco-2 cell culture model: comparison with human studies of Fe availability

For an in vitro model to be considered a useful tool, it should be consistent with the physiology of the human gastrointestinal tract and exhibit qualitative agreement with human studies. If such is the case, and if the model is
The use of Caco-2 cells in defining nutrient bioavailability

capable of handling a broad range of Fe concentration and availability in foods, then it is possible to develop conversion factors for quantitative prediction of human Fe absorption from the same meal. As the reader will see later in this chapter, a conversion equation for predicting human Fe bioavailability based on Caco-2 data has been developed.

Studies involving human subjects appear to support the use of Caco-2 cell ferritin formation as an index of Fe availability. For example, a positive correlation between ferritin concentrations in feces collected from human subjects and iron bioavailability has been reported. Presumably, fecal ferritin concentrations reflect enterocyte ferritin levels formed in response to uptake of bioavailable dietary iron, and it is this step that is reproduced by the model featured in this manuscript.

Many facets of iron bioavailability observed in humans have also been observed in this model system. For example, studies using the model showed that heme iron uptake is higher than non-heme iron uptake in the presence of phytate, that iron in human milk is more available than iron in bovine milk, the availability of iron from FeSO$_4$ is higher than from a polysaccharide-Fe complex, that NaFeEDTA is more available than FeSO$_4$ in the presence of polyphenolics, and that ascorbic acid and meat enhance uptake while phytic acid, tannic acid, and zinc inhibit Fe uptake. Qualitatively similar effects have all been shown in humans and the corresponding human studies are cited in the above-mentioned articles.

This model system has been compared with several human studies by reproducing the meals of the human trials. The most notable comparison involved two human studies that encompass a broad range of iron availability. The first human study documented promotion of iron absorption by ascorbic acid in a semi-synthetic diet. The second human study documented the inhibitory effects of tannic acid on Fe added to a simple bread meal. In the study by Cook and Monsen, increasing levels of ascorbic acid were added to an extrinsically radiolabeled semi-synthetic diet and iron absorption was measured from radioactivity in a blood sample 2 weeks after ingestion of the test meal. The study by Brune et al. appears to be the primary study used to generate the widely used algorithm developed by Hallberg and Hulthén to calculate the inhibitory effect of polyphenolics. This study added increasing amounts of tannic acid to a wheat roll meal and measured iron absorption by blood sampling 2 weeks post-consumption of the test meal. In order to compare the in vitro and in vivo results, the data were expressed as absorption ratios (AR). For the human studies, this calculation represents the percent absorption of iron in the presence of each ascorbic acid or tannic acid level, divided by the percent absorption of iron with no ascorbic acid or tannic acid. From the in vitro study, the corresponding Caco-2 cell ferritin formation values were used to calculate the absorption ratio as cell ferritin was used as the measure of Fe uptake. The in vitro results correlated well with the human studies (Figs 13.3 and 13.4).
348 Designing functional foods

**Fig. 13.3** Iron absorption ratios (AR) calculated from Caco-2 and human data for semisynthetic meals containing varying levels of ascorbic acid. Values represent the geometric mean of iron absorption at a given level of ascorbic acid divided by the geometric mean of iron absorption without ascorbic acid. Iron absorption in the Caco-2 cell model was indexed by ferritin formation. The natural log of the Caco-2 cell values and that of human studies were correlated at $R = 0.934, P = 0.012$. Adapted from Yun et al.61.

**Fig. 13.4** Iron absorption ratios (AR) from Caco-2 cell and human studies on the effects of tannic acid on iron absorption. Values represent the geometric mean of iron absorption at a given level of tannic acid divided by the geometric mean of iron absorption without tannic acid. The natural log of the Caco-2 cell values and that of human studies were correlated at $R = 0.927, P = 0.007$. Adapted from Yun et al.61.

Yun et al.61 also demonstrated the quantitative potential of this model to predict Fe absorption in humans. To do so, the authors observed that the combined data from the above-mentioned studies could be plotted on a single graph, thus encompassing a wide range of Fe availability (Fig. 13.5). The equation generated by this plot is $\hat{H} = 0.6401 \times C$. In this equation, $\hat{H}$ is the estimate of human iron absorption, expressed as $\ln(\text{AR})$, and $C$ represents the $\ln(\text{AR})$ of the corresponding Caco-2 cell data. The publication
The log-log relationship between absorption ratios (AR) from human and the parallel Caco-2 cell studies of this study. Caco-2 cell values represent the mean ln(AR), calculated from six replicates in the Caco-2 cell model. Human values represent the mean ln(AR), calculated from data for 12–13 subjects in a human study. Values were correlated at \( R = 0.986, P < 0.001 \). Adapted from Yun et al.\(^6\)

by Yun et al. can form the basis for applying this Caco-2 model as a quantitative predictor of human iron absorption; however, it is reasonable to expect that the conversion factors may change as the model is applied in future studies. The following comparisons are examples to illustrate the predictive power of this model.

Iron absorption from mutant low-phytate maize varieties has been measured relative to wild-type varieties in human subjects.\(^6\) In this study the maize varieties were harvested, the flour was ‘nixtamalized’ (i.e. soaked in lime) and prepared as tortillas. The tortillas were then extrinsically radio-labeled with Fe and fed to human subjects. Iron absorption was measured via blood collection. Iron uptake from the low phytate maize was 49% higher than from the wild-type maize. A close comparison of these maize samples has also been generated with the \textit{in vitro} digestion/Caco-2 cell model (Table 13.1).

In March of 2003, Dr Victor Raboy, the scientist who developed the mutant maize and co-author on the human study, sent samples to the laboratory of Dr Glahn at the US Plant, Soil and Nutrition Lab, Ithaca, NY. These samples were cooked (i.e. autoclaved), lyophilized, and ground to a powder, and tested for available Fe via the \textit{in vitro} model. The maize samples were from the same lines used in the human study and were of similar iron and phytic acid content. The primary difference between this experiment and the human study was that the maize samples were not nixtamalized (i.e. soaked in lime) and baked into tortillas. The nixtamalization process is essentially an alkaline solution of calcium hydroxide that softens the kernel and adds considerable calcium and other minerals to the samples. The added calcium and the alkaline conditions may certainly decrease the iron availability from the maize. This process could explain
Table 13.1 Comparison of iron availability from mutant low-phytate maize and wild type maize in humans and Caco-2 cells

<table>
<thead>
<tr>
<th></th>
<th>Wild-type maize</th>
<th>Low phytate maize</th>
<th>AR¹</th>
<th>Caco-2 predicted AR²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human iron absorption (%)</td>
<td>5.48</td>
<td>8.15</td>
<td>1.49</td>
<td>1.67</td>
</tr>
<tr>
<td>Caco-2 cell ferritin (ng ferritin/mg cell protein)</td>
<td>9.69</td>
<td>21.73</td>
<td>2.24</td>
<td>-</td>
</tr>
</tbody>
</table>

¹ Absorption ratio, i.e. the percent iron absorption of low phytate maize divided by that of the wild-type maize.
² The predicted AR based on the conversion equation of \( \hat{H} = 0.6401 \times C \), published by Yun et al., where \( \hat{H} \) is the predicted \( \ln(\text{AR}) \) for humans and \( C \) is an observed \( \ln(\text{AR}) \) for Caco-2 cells.

Table 13.2 Comparison of human and Caco-2 cell iron absorption from corn tortillas and black bean paste meals fortified with FeSO₄ or NaFeEDTA

<table>
<thead>
<tr>
<th></th>
<th>Test meal with FeSO₄</th>
<th>Test meal with NaFeEDTA</th>
<th>AR¹</th>
<th>Caco-2 predicted AR²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human iron absorption (%)</td>
<td>5.5</td>
<td>9.0</td>
<td>1.64</td>
<td>1.77</td>
</tr>
<tr>
<td>Caco-2 cell ferritin (ng ferritin/mg cell protein)</td>
<td>2.46</td>
<td>6.02</td>
<td>2.45</td>
<td>-</td>
</tr>
</tbody>
</table>

¹ Absorption ratio, i.e. the percent iron absorption from the meal with NaFeEDTA divided by that of the meal with FeSO₄.
² The predicted AR based on the conversion equation of \( \hat{H} = 0.6401 \times C \), published by Yun et al., where \( \hat{H} \) is the predicted \( \ln(\text{AR}) \) for humans and \( C \) is an observed \( \ln(\text{AR}) \) for Caco-2 cells.

why the human iron absorption (AR = 1.49) was lower than that predicted by the Caco-2 model (AR = 1.67). Alternatively, the human study could be different because of the extrinsic labeling method, as it may not have completely equilibrated with the intrinsic iron of the maize. However, despite these differences, the in vitro model prediction is reasonably close to the human results.

Davidsson et al.⁶⁶ measured iron bioavailability from iron-fortified Guatemalan meals based on corn tortillas and black bean paste. One comparison of this study involved the addition of FeSO₄ versus NaFeEDTA to a tortilla and black bean paste meal. Significantly more iron was available to the human subjects from the NaFeEDTA (9%) than from the FeSO₄-fortified meal (5.5%). These same meal conditions were reproduced as closely as possible for in vitro studies (Glahn et al., unpublished observations). A comparison of the results are summarized in Table 13.2. Clearly,
The use of Caco-2 cells in defining nutrient bioavailability

Table 13.3  Comparison of the dose-dependent effects of phytic acid on Fe uptake by human subjects and a similar study using the in vitro digestion/Caco-2 cell model.1

<table>
<thead>
<tr>
<th></th>
<th>AR² (phytate:Fe molar ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:1</td>
</tr>
<tr>
<td>Human iron absorption (%)</td>
<td>0.31</td>
</tr>
<tr>
<td>Caco-2 cell ferritin (ng ferritin/mg cell protein)</td>
<td>0.30</td>
</tr>
</tbody>
</table>

1 The cited studies are Hallberg et al.32 and Glahn et al.33

2 Absorption ratio; for human subjects this equals the percent iron absorption from the meal with phytate divided by that of the meal without phytate; for Caco-2 cells, this equals cell ferritin formation from an in vitro digest with phytate divided by that from a digest without phytate.

the Caco-2 cell model provides a slightly higher, but reasonable, estimation of the human results after use of the conversion factor reported by Yun et al.61.

A human study by Hallberg et al.32 documented the dose-dependent effects of phytic acid on Fe availability from a wheat roll meal. The meal consisted of rolls made from white wheat flour and that were low in endogenous phytate and Fe. Various molar ratios of phytate:Fe were consumed by human subjects and the absorption ratios were calculated. A similar study was also done using the in vitro digestion/Caco-2 cell model.33 The in vitro study did not ‘feed’ a wheat roll to the cells, but merely added corresponding amounts of phytic acid and Fe to the in vitro digest. There were two molar ratios of phytate:Fe in common between the two studies, at 1:1 and 5:1. The absorption ratios are summarized in Table 13.3 and the values match almost exactly. In reviewing the recent literature, this author notes that there appears to be a discrepancy in the molar amounts of phytate:Fe as calculated by Hurrell67 where the Hallberg et al.32 paper is cited. The text of the Hallberg et al.32 paper states ‘Sodium phytate (sodium inositol hexaphosphate, Sigma Chemical Company, St. Louis, MO) was added in various amounts (2–250mg expressed as phytate phosphorous) to the rolls before the margarine was spread.’ The results in Table 13.3 are based on the interpretation that 2–250mg of Sigma sodium inositol hexaphosphate product (formula weight = 660) were added to the rolls. The publication by Hurrell67 presents different numbers for conversion of the ARs and phytate:Fe molar ratios of the Hallberg et al. data.32 This results in different molar ratios and higher AR values in the human subjects (i.e. approximately 0.35 and 0.30 at 0.7:1 and 4.5:1 phytate:Fe molar ratio, respectively). Still, even if the calculations by Hurrell67 are correct, then at worst case the in vitro pattern of inhibition by phytic acid remains qualitatively similar to the human results and a simple conversion factor may be needed.
A relatively recent study by Besiegel et al.\textsuperscript{68} demonstrated some differences in Fe uptake from beans between the \textit{in vitro} digestion/Caco-2 cell model and human subjects. In this study, extrinsically labeled samples of red and white beans were compared in a single meal feeding trial. The subjects were of varying levels of Fe status and absorbed Fe equally from both the red and white bean samples. These results were in contrast to the \textit{in vitro} results where the white beans gave significantly higher Fe uptake relative to the red beans. The interpretation of the results from this study was that the polyphenols present in the seed coat of the red beans, which are absent in the white beans, were the primary difference in Fe uptake from these samples. Also, the results suggest that the \textit{in vitro} model is more sensitive to the effects of the polyphenols. However, there is a key point that must be considered when examining this study. The samples were extrinsically labeled, and a follow-up study observed that the extrinsic radiolabel did not equilibrate well with the intrinsic Fe of the red bean sample;\textsuperscript{69} hence, it is questionable that the extrinsic label accurately measured Fe bioavailability from these samples. It should also be noted in this paper that corn samples were also compared \textit{in vitro} with the human subjects and the corn results were in agreement. Further research is needed to define these conflicting results between the \textit{in vitro} and \textit{in vivo} systems.

There are other striking similarities in results between studies in humans and the \textit{in vitro} digestion/Caco-2 cell model featured in this paper. Baech et al.\textsuperscript{70} documented the effects of adding pork meat to a phytate-rich, low ascorbic acid meal. They observed that relatively small amounts of meat (<50g) significantly enhanced non-heme iron absorption from the meal. Using the Caco-2 cell model, similar studies have been observed using a phytate and polyphenolic-rich meal. In this study, small amounts of beef increased Caco-2 cell iron uptake from a tortilla and bean paste meal (Fig. 13.6; Glahn et al., unpublished observations). The basal meals were obviously different in these two studies. The human study meal consisted of boiled polished rice, tomato sauce, pea purée, rapeseed oil, and a wheat roll that was made partially from whole wheat and presumably a refined wheat flour. The meal of the \textit{in vitro} study consisted of 50g maize tortilla and 50g black bean paste, the same meal used in a human study and compared previously with the \textit{in vitro} model.\textsuperscript{66} In both studies there were significant amounts of phytic acid; however, the meal for the \textit{in vitro} study likely contained greater amounts of polyphenolics. Also, the \textit{in vitro} meal used beef which increased the total Fe content of the meal by 200% at the full serving size. For the human study, the pork sample was relatively low in total iron content and only increased Fe content by approximately 8% at the highest level of meat. These contrasts between the studies negate the usefulness of closer comparison; however, both studies arrive at the same conclusion, that relatively low amounts of meat can improve iron absorption from a meal with low Fe availability.

In conclusion, the above-mentioned comparisons between the \textit{in vitro} digestion/Caco-2 cell model and human studies should represent only the
beginning of this level of validation. More direct comparisons from the same meals should be pursued in order to refine the conversion factor for \textit{in vitro} predictions of human iron absorption via this model. One should expect that conflicting results will be observed in these types of studies, and recognize that in those differences may lie clues to the actual mechanisms of uptake that occur in the human intestine.

13.6 Justification for use of the \textit{in vitro} digestion/Caco-2 cell model as a screening tool

One of the primary factors that determines an experimental approach is cost. The costs associated with application of this \textit{in vitro} model are far below those of animal and human trials. A laboratory with one or two personnel trained in the use of this \textit{in vitro} model can easily conduct two sets of experiments per week, comparing eight to ten different samples with six independent replications, and process the cell samples for Fe uptake measurement within a few days after the experiment. The US dollar values associated with such costs are difficult to estimate due to potential differences in technician salary and overhead; however, it has been demonstrated in US Department of Agriculture (USDA) facilities that the \textit{in vitro}
approach can assess Fe availability of eight to ten samples for approximately $5000 which includes personnel salary, supplies, and overhead costs (R.P. Glahn, personal observations). Studies in humans comparing this number of samples or conditions are seldom done as it is cost-prohibitive. For example, at USDA facilities, a single or two meal feeding trial of two to four extrinsically radiolabeled food samples would cost $50000 and would require a minimum of 6–12 months to resolve paperwork, recruit subjects, and collect data (Dr Janet Hunt, USDA-ARS, Grand Forks, ND, personal correspondence). Obviously, performing human studies on more than a handful of samples is very costly for most research programs investigating Fe availability. Given the high cost of human studies it becomes imperative that these studies provide definitive results that advances the field. The in vitro digestion/Caco-2 cell culture model featured in this chapter has been developed for precisely that application. Used properly, it can accelerate knowledge of food Fe availability by refining experimental objectives and thereby increase the effectiveness of the human studies. Furthermore, in vitro studies can explore intralumenal interactions, effects, and mechanisms that are not possible or cost-effective to address in vivo.

The fact that concentration of Fe, the concentration of promoters and inhibitors of Fe uptake, and Fe solubility are not reliable predictors of Fe availability is also a key justification for use of this model. The coupling of Caco-2 cell culture model with in vitro digestion essentially makes in vitro Fe bioavailability screening plausible as it adds a living component to the process and completes the components necessary to model the initial steps in Fe uptake by the enterocyte. From a bioavailability standpoint, the primary step in determining availability is whether or not the form of Fe in the food is accessible for uptake by the enterocyte. After that step, the fate of the Fe is then dependent on the Fe status and physiology of the individual. The conditions defined by Glahn et al. were developed with this physiological approach, and were also designed to be cost-effective, easy to use, and able to accommodate a relatively large number of samples.

The following is an example as to how screening by concentration of Fe, promoters, and inhibitors alone can lead to costly mistakes and wasted resources. Mamiro et al. tested a complementary food for its effect on alleviating Fe deficiency anemia in 6–12-month-old infants in Tanzania. The objective of the study was to determine if a processed complementary food, estimated to provide more bioavailable Fe, improved the Fe status of infants relative to those consuming the unprocessed meal. The effects of processing were designed to lower antinutrient levels (i.e. phytate and polyphenolics), thereby improving Fe absorption from the meal. The meal had been previously analyzed and exhibited greater Fe solubility with marked decreases in phytate and polyphenolic concentration. After 6 months of feeding, the processed meal provided no additional benefit for growth, hemoglobin formation or Fe status, essentially indicating that no significant additional bioavailable Fe was present in the meal.
On a dry matter basis, this meal contained dried finger millet (65%), kidney beans (19%), peanuts (8%) and mango puree (8%). In the processed meal used by Mamiro et al., phytate levels were reduced by 34%, yielding phytate:Fe molar ratios of 16.6:1 and 11.8:1 in the unprocessed and processed foods, respectively. The nutritional analysis of the same foods conducted by Mbithi-Mwikya et al. observed phytate:Fe molar ratios of 16.7:1 and 3.3:1 for the unprocessed and processed foods, respectively. Mamiro et al. measured Fe solubility at 19% in the processed meal versus 5% in the unprocessed meal. Also, Mbithi-Mwikya et al. did not find any detectable tannins in the processed meal and tannin measurement was not reported in the Mamiro et al. study.

Clearly, the results of these two studies indicate that Fe solubility and content of inhibitors would not have been adequate predictors of Fe availability in these meals. One likely explanation for no improvement of Fe availability could be due to the presence of phenolic acids or flavanoids that constitute the tannins of the unprocessed ingredients. Tannins in foods are known to break down during cooking and storage of staple foods such as rice, yet the inhibitory effect on Fe availability remains, probably due to the phenolic acids. The assay used by Mbithi-Mwikya et al. may not have detected the phenolic compounds as certain methods for measuring tannins do not accurately measure the breakdown products. Hence, even though phytate levels were reduced in these foods, the polyphenolic effect remains and dramatically inhibited Fe availability. The in vitro digestion/Caco-2 cell model would likely detect the inhibitory effect of the phenolics and therefore prevent the waste of resources for an unproductive human study. Moreover, the in vitro model could be used to develop a low-cost complementary food that shows significantly higher Fe availability and thus has greater chance for success in the human efficacy trials. Such screening studies to define affordable and consumer-acceptable complementary foods are currently underway in this author's laboratory.

Another key advantage of the in vitro digestion/Caco-2 cell model is that it can detect previously unknown or new interactions of foods or ingredients that can promote or inhibit Fe availability. An example of such is the observed effect of sweet potato on Fe availability. In this study orange flesh sweet potatoes were observed to have a strong promotional effect on Fe extrinsically added to a sweet potato sample. The implication of this research is: that developing sweet potatoes with higher Fe density could provide a better source of Fe as well as being an excellent source of pro-vitamin A. Alternatively, as sweet potato flesh is relatively low in Fe content, these observations suggest that fortifying a popular complementary food such as a sweet potato puree could provide an excellent source of Fe for infants.

Recent in vitro screening studies have also documented higher Fe availability in white beans relative to pinto, red, brown or black beans. In this study the levels of phytate varied significantly and was not correlated with
Fe availability. The primary correlation with Fe availability appears to be related to the polyphenolic profile of the bean samples. Significant differences in Fe availability were observed among the white bean genotypes and among the colored bean samples. The analysis of the bean varieties indicates that such differences would not be predicted based on nutritional content of Fe and inhibitors of Fe absorption. This represents another example as to how an in vitro model can provide useful insight to identify foods with improved Fe availability, as there are no published studies that suggest white bean varieties may be a better source of Fe. At present, studies are under way to confirm these observations in human subjects. If true, then white beans may represent a practical and effective component for complementary foods such as used in the aforementioned study by Mamiro et al.71

Further justification for the advent and application of the in vitro digestion/Caco-2 model is due to the specific needs of plant breeders and nutritionists in biofortification programs such as HarvestPlus. HarvestPlus is an international, interdisciplinary, research program that seeks to reduce micronutrient malnutrition by utilizing the powers of agriculture and nutrition research to breed staple food crops (e.g. rice, wheat, maize, beans, sweet potatoes, and cassava) with higher levels of Fe, Zn, and provitamin A. From the very beginning, bioavailability issues have been a major concern for HarvestPlus. Throughout the 10 years of planning, research, and development of HarvestPlus, it became apparent that measuring only the content of iron and zinc and the levels of inhibitors (i.e. phytate, tannins) would not be a viable option for this program for many reasons. First, as stated previously, concentration of Fe and Zn in seeds does correlate with the bioavailability of Fe or Zn in seeds. Second, there is a very large environmental effect on phytate concentration in cereal, grain and legume as the phytate concentration is greatly influenced by the levels of available phosphorous in the soil.77 This factor makes it practically impossible for plant breeders to use phytate content of breeding lines in their screening for genotypes with high levels of bioavailable iron and zinc. To do so as a breeding strategy would make the process enormously complicated and expensive as it would require development of numerous lines for numerous environments. Moreover, it does not appear that screening crop varieties via phytate content will be useful, as both animal and Caco-2 models show no correlation with Fe availability among genotypes.33,41,42,78 Although phytate is an inhibitor of Fe availability, these observations indicate that other factors in the seed appear to be responsible for the ultimate bioavailable levels of Fe and Zn. The most likely factors are polyphenolics and the nature of the complexes formed between Fe, Zn and this huge group of compounds. Relatively little is known as to how these compounds react with Fe and Zn to limit the bioavailability of these minerals; however, such studies are in progress within the breeding and bioavailability objectives of HarvestPlus. Finally, aside from screening numerous genotypes to identify those with
high and low bioavailability of these nutrients, HarvestPlus must also investigate how such foods interact within the typical diets and meals of the targeted regions; hence, there is a profound need in such a program for a screening tool that can assess the effects of processing, cooking and food interactions in typical meals. Such assessment is not possible using algorithms, as it would require a data set that has measured these effects over a broad range of foods and cooking procedures. A data set of this magnitude using human subjects would be enormously expensive to produce and thus is not likely to ever exist. An \textit{in vitro} model appears to be the only feasible approach to begin to explore these factors and interactions.

13.7 Conclusion

In conclusion, it is clear that over the past decade the advances in knowledge of molecular mechanisms of Fe absorption have been profound, and that the Caco-2 cell line has been a valuable tool in defining those mechanisms. Thus, it is logical to incorporate this cell line into applied research as a tool to measure bioavailable Fe in foods. As evident by the examples in this chapter, the conditions established by Glahn et al.\textsuperscript{21} have broad applications, from screening genotypes of staple food crops to development of improved forms of Fe for food fortification. However, although the degree to which these \textit{in vitro} conditions agree with human studies is remarkable, it is reasonable to suspect that this \textit{in vitro} system will have limitations on predicting Fe availability. Only direct testing of this model with human studies will enable further refinement and define the limitations of this \textit{in vitro} system.

13.8 References


360 Designing functional foods


