The Usefulness of *in vitro* Models to Predict the Bioavailability of Iron and Zinc: A Consensus Statement From the HarvestPlus Expert Consultation

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Abstract: A combination of dietary and host-related factors determines iron and zinc absorption, and several in vitro methods have been developed as preliminary screening tools for assessing bioavailability. An expert committee has reviewed evidence for their usefulness and reached a consensus. Dialyzability (with and without simulated digestion) gives some useful information but cannot predict the correct magnitude of response and may sometimes predict the wrong direction of response. Caco-2 cell systems (with and without simulated digestion) have been developed for iron availability, but the magnitude of different effects does not always agree with results obtained in human volunteers, and the data for zinc are too limited to draw conclusions about the validity of the method. Caco-2 methodologies vary significantly between laboratories and require experienced technicians and good quality cell culture facilities to obtain reproducible results. Algorithms can provide semi-quantitative information enabling diets to be classified as high, moderate, or low bioavailability. While in vitro methods can be used to generate ideas and develop hypotheses, they cannot be used alone for important decisions concerning food fortification policy, selection of varieties for plant breeding programs, or for new product development in the food industry. Ultimately, human studies are required for such determinations.

Key words: in vitro, Caco-2 cell, dialyzable iron, bioavailability, iron, zinc

Introduction

In many countries, low bioavailability of iron and zinc is one of the factors that limits dietary adequacy. The current demand for simple, inexpensive, valid models of dietary iron and zinc absorption in humans has arisen because of the rapid increase in large-scale nutrition programs to improve iron and zinc nutrition in at-risk populations. Such models of human iron and zinc absorption may be used widely to screen foods with increased mineral content or modified amounts of naturally occurring components that inhibit or enhance iron and zinc absorption that are achieved through food fortification, biofortification, or food processing techniques. Nonetheless, definitive conclusions as to the validity of various in vitro models to accurately predict bioavailability in humans have not previously been presented. This consensus statement was developed by a group of experts working in the field of iron and zinc nutrition to advise on the accepted uses and interpretation of results from in vitro models that are intended to inform the design of human nutrition intervention strategies.

Bioavailability of iron and zinc in humans is determined by a sequential series of events (stages) which can be influenced by both food and host factors.

1. Digestion and release of elements from the food matrix into the lumen of the gastrointestinal (GI) tract (availability).
2. Transport into intestinal enterocytes (uptake).
3. Efflux across the basolateral membrane of enterocytes into the circulation (absorption).
4. Retention, or endogenous excretion in urine and feces (retention).
5. Transport to tissues for use in normal body functions (utilization).
6. Transport to storage sites (body stores).

Food factors

Whilst it is possible that other, as yet unrecognized, dietary components influence the bioavailability of iron and zinc in humans, it is believed that the major food factors have been identified:

- The major recognized food components that inhibit iron absorption have been identified as phytic acid, pheno-

lic compounds, calcium, and certain proteins; the major enhancers are muscle tissues and ascorbic acid. In the short-term, these components operate primarily at the level of availability and uptake (stages 1 and 2) and have little effect on stages 3–6.

- The major recognized food component that inhibits zinc absorption is phytic acid, whose inhibitory effect may be further potentiated by calcium.

Host factors

There are a number of host factors that modify iron and zinc absorption. Regulation of iron absorption is modulated in response to erythropoietic activity, body iron stores, and enterocyte exposure to iron; a reduction in iron stores will result in increased efficiency of absorption. Conditions that adversely affect the health and function of the intestine will also affect absorptive processes. Diarrhea interferes with zinc absorption, and parasitic infections may cause bleeding, which will have an adverse effect on iron balance. Both iron and zinc absorption are up-
regulated during pregnancy and zinc absorption is increased during lactation, perhaps reflecting increased area of absorptive surface of the small intestine.

Stages of bioavailability simulated by *in vitro* models

- Dialyzability is designed to predict availability (stage 1).
- Caco-2 cells, in combination with a simulated digestion procedure, can be used to predict availability and uptake (stages 1 and 2).
- In the case of iron, it is also possible to use Caco-2 cells grown on transwell membranes to study various systemic effects on iron metabolism (absorption – stage 3) by growing the cells in different iron concentrations and/or manipulating the media in the basolateral well to simulate the effect of high iron stores. Evidence to validate the predictive accuracy of the *in vitro* transwell approach is required.
- Dialyzability and Caco-2 cells cannot be used to measure retention (stage 4), utilization (stage 5), or storage (stage 6).
- Algorithms, if accurately defined, may predict the composite effect of all 6 stages in population-based data. However, they are not generally designed to take into account host factors, with the exception of iron status.

In order for dialyzability or Caco-2 cells to be considered useful predictors of availability (stage 1) or for the Caco-2 cell method to be considered a useful predictor of uptake (stage 2), these methods must predict both the correct direction and magnitude of response to food components that inhibit or enhance iron or zinc absorption in human subjects. In order for algorithms to be considered useful predictors of bioavailability, they must consistently predict iron or zinc absorption in study populations of similar physiological status and across a wide range of combinations of food components.

Conclusions regarding the usefulness and limitations of bioavailability models

*In vitro* dialyzability

*In vitro* dialyzability can be used to predict availability of non-heme iron and zinc. Dialyzability involves a two-stage simulated digestion process and the use of a dialysis membrane with a selected molecular weight cut-off. It usually, but not always, predicts the correct direction of response. The major limitations of the *in vitro* dialyzability model are:
- The measured magnitude of effect may differ from the magnitude observed in human studies.
- It assumes that small molecular weight iron complexes that pass through the dialysis membrane provide iron that is available for absorption and that iron bound to large molecular weight complexes that are retained by the membrane is not available. There are exceptions to both assumptions. For example, small polyphenol-iron complexes or organic acid-iron complexes may pass through the membrane and yet be of low availability. Ferritin, a large protein that is resistant to digestion, will not pass through the membrane, however there are recent data demonstrating that ferritin can be absorbed efficiently by mucosal cells, although the bioavailability of iron within the ferritin molecule remains unclear. Thus there is potential for misleading information from the use of *in vitro* dialyzability.
- It appears to be a useful method to rank iron fortification compounds in a food matrix with respect to availability but there is limited evidence that the magnitude of response is similar in humans.
- Although the direction of effect is usually the same, the relatively well-standardized methods still give responses of different magnitude between laboratories.
- To be most applicable to human bioavailability, foods need to be prepared in the manner in which they are normally consumed.

Caco-2 cells

If Caco-2 cells are combined with a simulated *in vitro* digestion procedure they may predict food factors that influence iron and zinc absorption in humans. However, the approach must be physiological and mirror the conditions of the intestinal tract as closely as possible. Deviations from normal physiological conditions resulting from technical considerations, such as the addition of high concentrations of ascorbic acid, must be taken into account when evaluating Caco-2 cell data.

Caco-2 cell methodology has the potential to identify undiscovered factors in foods that influence absorption or interactions between food components. However, if a dialysis membrane is employed, the caveats identified for *in vitro* dialyzability will apply. A combination of *in vitro* digestion and Caco-2 cells can discern differences between dialyzability or soluble iron and iron that is available for uptake by the enterocytes; not all soluble or dialyzable iron or zinc is readily available, as exemplified by the observation that ferrous ascorbate and ferric citrate have similar solubility/dialyzability but dissimilar bioavailability.
The major trans-membrane transporters for iron and zinc have been identified in Caco-2 cells. For iron, these same transporters have been identified in normal mammalian small intestinal enterocytes.

It has been demonstrated that Caco-2 cells predict the correct direction of response for all major iron absorption modifiers; whether or not they predict the same magnitude of response in humans needs to be established. In the case of iron uptake, the sensitivity of the Caco-2 cells to phytic acid and certain polyphenols appears to be similar to that in humans, but they may either overestimate or underestimate the effects of ascorbic acid and EDTA.

For zinc, it appears that Caco-2 cells can predict the correct direction of the response to phytate, but there are insufficient data to draw further conclusions.

When tested in Caco-2 cells, iron fortification compounds have not always reflected the expected relative bioavailability of these compounds as measured in humans. Differences in test meal preparation and prior in vitro digestion procedures are important factors and may be the cause of these discrepancies.

Caco-2 cell methodologies vary significantly between laboratories. The magnitude of response is influenced by prior enzymatic digestion procedures, inclusion of a dialysis step, the methods used to prepare and grow the cells, passage number, and the method used to detect the uptake of iron or zinc. Under certain conditions the expression of ferritin, which is both a surrogate marker of iron uptake and a cellular response to inflammation and oxidative stress, may be up-regulated. Although there are no data to date that demonstrate food-induced inflammation/oxidative stress on cell ferritin response, the physiological response of mucosal cells must be considered when using ferritin expression as the biomarker for iron uptake in cells. Inter-laboratory comparisons are needed to standardize the Caco-2 in vitro method for predicting iron and zinc absorption.

Caco-2 techniques require experienced technicians and good quality cell culture facilities to obtain reliable and reproducible results.

The more sophisticated models are dependent on the collection of accurate information for food intake and dietary absorption modifiers. In practice, reliable data on the content of these modifiers in foods often cannot be derived from food composition tables, and methods to measure them may vary between laboratories.

Algorithms have often been derived from isotopic absorption studies, and these limited data may not reflect the important variations that affect iron and zinc absorption from habitual diets.

Conclusions

- Dialyzability can be used to screen foods and iron compounds to identify major differences in availability relating to dietary composition, chemical form, and changes that occur during processing. However, as they cannot predict the magnitude of response in humans and sometimes predict the wrong direction of response, results must be interpreted with caution.
- Caco-2 cells can be used to study iron and zinc availability, uptake, and absorption from foods. When combined with an in vitro digestion, the impact of dietary modulators can be assessed, but the magnitude of different effects may be disproportionate to that observed in humans. Conversion factors for Caco-2 data are needed in order to predict in vivo results in humans.
- Caco-2 cell systems are a useful tool to explore the more fundamental aspects of iron and zinc absorption, including transport and signaling mechanisms.
- Dialyzability, Caco-2 cells, and algorithms make useful contributions to the development of new hypotheses. However, none of these methods can be used alone to evaluate the adequacy of dietary intakes, to guide food fortification and plant-breeding programs, or to develop dietetic products for the food industry. Policy decisions must always be based on evidence generated from human studies.

Algorithms

Several algorithms have been developed to predict the bioavailability of iron and zinc from mixed diets. Although these algorithms have not been independently cross-validated they can provide good semi-quantitative information, such as the classification of diets into high, moderate, or low bioavailability.