How In Vitro Screening of Iron Bioavailability Contributes to Biofortification of Staple Food Crops

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September 19, 2006
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Fe absorption:
Primarily occurs in the upper small intestine.
However, recent evidence suggests that the lower intestine (ileum and colon) may also be a significant site for absorption.

Prebiotics (i.e., nondigestible oligosaccharides such as inulin and partial hydrolysates of malto-oligosaccharides) selectively stimulate specific species of bacteria such as Bifidobacteria and Lactobacilli, that induce health benefits.

Biofortification

- Providing the nutrients via agriculture
- Linking agriculture to human health
- Relatively inexpensive, sustainable!!

In Vitro Digestion/Caco-2 Cell Culture Model: Fe Availability
(U.S. patent #6017713)

Food Preparation
- Pepsin Digestion
  pH 2, 1 h, 37 C (50 mL tube)
- Pancreatin-Bile Digestion
  pH 6.8 – 7.0, 2 h, 37 C

Soluble iron

Harvest cells for ferritin determination
24 h post start of Panc/Bile digestion

Caco-2 cells

Iron & Zinc Absorption

Hepcidin
Basolateral (Blood)

Apical (luminal)

Fe³⁺
Fe²⁺
Fe²⁺
Fe³⁺

Zn²⁺
Zn²⁺
Fe Bioavailability
Caco-2 vs. Human

Comparison of Absorption Ratios

- Absorption Ratio = Abs. Test Sample / Abs. Control

Cook and Munson, 1977
Concentration Effects of Ascorbic Acid
Brune et al. 1989
Fe Absorption and Phenolic Compounds

Validation of the In Vitro Digestion/Caco-2 Model:

Table 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>AR(a)</th>
<th>Caco-2 Predicted AR(b)</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>

(a) Absorption ratio, i.e. percent iron absorption from the meal with NaFeEDTA divided by that of the meal with FeSO4.
(b) The predicted AR based on the conversion equation of $\hat{A} = 0.6401 \times C$, published by (Yun et al., 2004), where $\hat{A}$ is the predicted Ln(AR) for humans and C is an observed Ln(AR) for Caco-2 cells.

Validation of the In Vitro Digestion/Caco-2 Model:

Table 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>AR(a)</th>
<th>Caco-2 Predicted AR(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human iron absorption (%)</td>
<td>5.5</td>
<td>9.0</td>
<td>1.64</td>
<td>1.73</td>
</tr>
<tr>
<td>Caco-2 cell ferritin (ng ferritin/mg cell protein)</td>
<td>2.66</td>
<td>6.02</td>
<td>2.45</td>
<td></td>
</tr>
</tbody>
</table>

(a) Absorption ratio, i.e. the percent iron absorption from the meal with NaFeEDTA divided by that of the meal with FeSO4.
(b) The predicted AR based on the conversion equation of $\hat{A} = 0.6401 \times C$, published by (Yun et al., 2004), where $\hat{A}$ is the predicted Ln(AR) for humans and C is an observed Ln(AR) for Caco-2 cells.

Validation of the In Vitro Digestion/Caco-2 Model:

Table 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.

<table>
<thead>
<tr>
<th></th>
<th>AR(a) (phytate:Fe molar ratio)</th>
<th>1:1</th>
<th>5:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Iron absorption (%)</td>
<td>0.31</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Caco-2 cell ferritin (ng ferritin/mg cell protein)</td>
<td>0.30</td>
<td>0.18</td>
<td></td>
</tr>
</tbody>
</table>

(a) 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.

Correlations Between Human and Caco-2 Studies

- Heme Fe uptake greater than nonheme Fe uptake in the presence of phytate (Glahn et al. J. Ag. Food Chem., 2002; 50:390-395)
- Fe bioavailability from FeSO4 greater than that from a polysaccharide Fe complex (Glahn et al., 2006; J. Nutr. Biochem. 17: 62-68)
- Fe from NaFeEDTA is more available than FeSO4 in the presence of polyphenolics (Worthy et al., J. Agr. Food Chem. 2002; 50:390-395)
- Tannic acid, phytic acid and ZnCl2 inhibit Fe uptake (Glahn et al. J. Ag. Food Chem., 2002; 50:390-395)


- **Objective:** To evaluate the benefits and the limitations of the different in vitro procedures used to measure iron and zinc bioavailability and to reach a consensus on their usefulness to predict iron or zinc bioavailability in man.
- 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 polyphenolics 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.
The HarvestPlus Bioavailability Screening Strategy

- In Vitro Digestion/Caco-2 Cell Model (n = 20 – 200 varieties)
- Piglet Feeding Trial (n = 2-5 varieties)
- Human Feeding Trials, Efficacy and Impact (n = 2 – 3 samples)

Harvest Plus Food Crops

<table>
<thead>
<tr>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>Bananas</td>
</tr>
<tr>
<td>Beans</td>
<td>Barley</td>
</tr>
<tr>
<td>Rice</td>
<td>Cowpeas</td>
</tr>
<tr>
<td>Wheat</td>
<td>Groundnuts</td>
</tr>
<tr>
<td>Cassava</td>
<td>Lentils</td>
</tr>
<tr>
<td>Sweet Potato</td>
<td>Millet</td>
</tr>
<tr>
<td></td>
<td>Pigeon Peas</td>
</tr>
<tr>
<td></td>
<td>Plantains</td>
</tr>
<tr>
<td></td>
<td>Potatoes</td>
</tr>
<tr>
<td></td>
<td>Sorghum</td>
</tr>
<tr>
<td></td>
<td>Yams</td>
</tr>
</tbody>
</table>

Nutrients:
Fe, Zn, Vit. A.

Ecosystems – Different Selection Pressures

- Rainfed lowland
- Upland
- Flood prone
- Irrigated

Iron Bioavailability of Beans From Kenya
Iron Bioavailability of Beans From Kenya Using the In Vitro Digestion/ Caco-2 Model

No correlation with phytate concentration was observed.

Comparison With Humans: Great Northern (white) vs. Pinto Beans

Comparison With Humans: Maize with/without Ascorbic Acid

Caco-2 cell Fe Uptake from Beans

Caco-2 cell iron uptake from digest of sweet potato, with and without added FeCl₃ and ascorbic acid.

Sweet Potatoes and Fe Availability

Orange Flesh Sweet Potato: vehicle for Fe fortification?
From physiology to genetics via statistics

CIM identifies 4+ loci

Results: Fe Availability and QTL Mapping

• Loci associated with increased Fe bioavailability were identified on chromosomes 3, 6 and 9 while those associated with increased seed Fe content were identified on chromosomes 1, 2 and 5.
• The Fe concentration markers were found in two separate harvests. Fe bioavailability markers were only from one harvest. Additional experiments are underway to confirm the bioavailability markers.
Wheat Flour: Effects of Processing on Fe Concentration and Bioavailability

15 Wheat varieties

Whole Flour
Refined (80% extraction) flour

Bread Samples*:
Flat bread (whole and refined)
Leavened bread (refined only)
1, 2, 3 hr
*duplicate preparations of each

180 SAMPLES!

Fe Availability from Whole vs. Refined Wheat Flour
Caco-2 cell ferritin formation of 15 varieties of Mexico wheat flour samples (whole wheat flour vs refined flour)

<table>
<thead>
<tr>
<th>Wheat variety</th>
<th>Fe Availability from Whole Flour (ug/g)</th>
<th>Fe Availability from Refined Flour (ug/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grandin</td>
<td>42.2</td>
<td>27.3</td>
</tr>
<tr>
<td>BA</td>
<td>41.2</td>
<td>14.7</td>
</tr>
<tr>
<td>BA NORA</td>
<td>42.0</td>
<td>27.0</td>
</tr>
<tr>
<td>TOROPI</td>
<td>47.9</td>
<td>14.7</td>
</tr>
<tr>
<td>TOROPI (42.9)</td>
<td>41.7</td>
<td>14.7</td>
</tr>
<tr>
<td>BET-HAS</td>
<td>32.0</td>
<td>16.4</td>
</tr>
<tr>
<td>BET-HAS (32.0)</td>
<td>31.8</td>
<td>16.4</td>
</tr>
<tr>
<td>DURU</td>
<td>36.5</td>
<td>24.9</td>
</tr>
<tr>
<td>DURU M2656 (36.5)</td>
<td>36.3</td>
<td>24.9</td>
</tr>
<tr>
<td>OPA TA -485 (34.5)</td>
<td>34.3</td>
<td>16.4</td>
</tr>
<tr>
<td>OPA TA -85 (16.4)</td>
<td>34.3</td>
<td>16.4</td>
</tr>
<tr>
<td>BOB WHIT (31.8)</td>
<td>31.6</td>
<td>16.4</td>
</tr>
<tr>
<td>BOB WHIT (15.8)</td>
<td>31.6</td>
<td>16.4</td>
</tr>
<tr>
<td>NAINAR-60 (33.9)</td>
<td>33.7</td>
<td>16.4</td>
</tr>
<tr>
<td>NAINAR (33.9)</td>
<td>33.7</td>
<td>16.4</td>
</tr>
<tr>
<td>RAYON-F89 (29.8)</td>
<td>29.6</td>
<td>16.4</td>
</tr>
<tr>
<td>RAYON-F89 (14.8)</td>
<td>29.6</td>
<td>16.4</td>
</tr>
<tr>
<td>CMH81A.1261 (40.8)</td>
<td>40.6</td>
<td>21.2</td>
</tr>
<tr>
<td>CMH81A.1261 (21.2)</td>
<td>40.6</td>
<td>21.2</td>
</tr>
<tr>
<td>DGO95.123.13 (42.9)</td>
<td>42.7</td>
<td>17.6</td>
</tr>
<tr>
<td>DGO95.123.13 (21.7)</td>
<td>42.7</td>
<td>17.6</td>
</tr>
<tr>
<td>DURU M273 (31.5)</td>
<td>31.3</td>
<td>16.4</td>
</tr>
<tr>
<td>DURU M273 (23.1)</td>
<td>31.3</td>
<td>16.4</td>
</tr>
<tr>
<td>DGO95.3.4</td>
<td>42.9</td>
<td>21.2</td>
</tr>
<tr>
<td>DGO95.3.4 (21.7)</td>
<td>42.9</td>
<td>21.2</td>
</tr>
<tr>
<td>DURUM2812 (34.6)</td>
<td>34.4</td>
<td>16.4</td>
</tr>
<tr>
<td>DURUM2812 (23.7)</td>
<td>34.4</td>
<td>16.4</td>
</tr>
<tr>
<td>REBECA</td>
<td>28.9</td>
<td>15.6</td>
</tr>
<tr>
<td>REBECA (16.6)</td>
<td>28.9</td>
<td>15.6</td>
</tr>
<tr>
<td>PITIC-62 (28.4)</td>
<td>28.2</td>
<td>16.4</td>
</tr>
<tr>
<td>PITIC-62 (14.0)</td>
<td>28.2</td>
<td>16.4</td>
</tr>
</tbody>
</table>

Figure 1. Iron bioavailability from digests of whole and refined (80% extraction) wheat flour samples. Values are mean ± SEM, n=6, expressed as a percent of whole wheat flour Grandin. Numbers in parentheses are iron concentrations (ug/g, n=2). Although iron levels in refined samples were significantly lower than those of whole flour samples, iron bioavailability of refined wheat flour samples was significantly higher than that of whole flour samples except variety DGO95.3.4 which suggested that extraction did not only reduce iron contents but also removed some inhibitors of iron absorption.

Ferritin: Can it be used as a form of Fe for biofortification?

• Ferritin represents a potential form of Fe biofortification in plant foods, either by traditional plant breeding or biotechnology.
• Is the Fe in ferritin released during digestion?
• Does the ferritin molecule get degraded by gastric and/or intestinal digestion? If so, does it offer some protection of the Fe from inhibitors of uptake?
• Is the ferritin molecule absorbed intact by the enterocyte and subsequently degraded in the enterocyte?

Ferritin Fe: Equal to bioavailability of FeSO4 in humans

Fe Bioavailability From Ferritin
Observation: Ascorbic acid increases Fe bioavailability

<table>
<thead>
<tr>
<th>Ferritin type</th>
<th>FeSO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSF= horse spleen ferritin</td>
<td>4FeSO₄ + AA (1:20)</td>
</tr>
<tr>
<td>P-HSF = plant type ferritin</td>
<td>4FeSO₄ + PA (1:20)</td>
</tr>
<tr>
<td>AA=ascorbic acid</td>
<td>4FeSO₄ + PA (1:20)</td>
</tr>
<tr>
<td>PA=phytic acid</td>
<td>4FeSO₄ + AA (1:20)</td>
</tr>
</tbody>
</table>

Values in parentheses are molar ratios of Fe: AA or PA

Fe Bioavailability From Ferritin
Observation: Phytic acid inhibits, Ascorbic acid restores availability

<table>
<thead>
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</thead>
<tbody>
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</tr>
<tr>
<td>P-HSF = plant type ferritin</td>
<td>4FeSO₄ + AA (1:20)</td>
</tr>
</tbody>
</table>

Values in parentheses are molar ratios of Fe: AA or PA
Fe Bioavailability From Ferritin
Observation: Tannic acid inhibits, Ascorbic acid does not restore availability

Fe Bioavailability From Ferritin
Observation: Ascorbic acid and fish restore Fe availability

Conclusions
• Iron bioavailability from ferritin is equal to FeSO₄ in the in vitro digestion/Caco-2 cell culture model.
• Fe uptake from ferritin responds similarly to FeSO₄ in the presence of inhibitors (phytate and tannic acid) and promoters (ascorbic acid and meat).
• Unless other factors are involved in vivo, these results indicate that Fe from ferritin may not represent a form of Fe that escapes the chemical traps of phytate and polyphenolics.

Conclusions (cont.)
• However, the present study was conducted under low pH (ie. pH = 2) conditions during the gastric phase of digestion. Thus, the protein cage of the ferritin molecule may have been destroyed, thus maximizing release of the ferritin Fe and potential interaction with the inhibitors.
• In the human GI tract, gastric pH in many meal conditions does not get to pH 2 prior to emptying, pH values of 3 – 6 are common.
• Hence, additional studies should be conducted at higher gastric pH to fully characterize the effects of inhibitors and promoters on ferritin-Fe.

Integrity and Digestibility of Reconstituted Horse Spleen Ferritin
Native gel electrophoresis. 7.5% Tris-HCl Bio-Rad ready gel. Running buffer: 25 mM Tris, 192 mM glycine. Sample buffer: 62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 0.01% Bromophenol blue. Coomassie Blue R-250 was used for total protein gel stains.

Integrity and Digestibility of Reconstituted Plant-type Ferritin
Native gel electrophoresis. 7.5% Tris-HCl Bio-Rad ready gel. Running buffer: 25 mM Tris, 192 mM glycine. Sample buffer: 62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 0.01% Bromophenol blue. Coomassie Blue R-250 was used for total protein gel stains.
Digestibility of Native Horse Spleen Ferritin (n-HSF) Under Different Conditions

Native gel electrophoresis. 7.5% Tris-HCl Bio-Rad ready gel. Running buffer: 25 nM Tris, 192 nM glycine. Sample buffer: 62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 0.01% Bromophenol blue. Coomassie Blue R-250 was used for total protein gel stains.

(a) Native HSF (b) Native HSF after pH 2.0 saline solution treatment (c) native HSF after pH 2.0 saline solution treatment plus 1.0 hr of pepsin digestion (d) native HSF after pH 2.0 saline solution treatment plus 2.0 hr of intestinal digestion with pancreatic and bile extract digestion (e) native HSF after pH 6.0 saline solution treatment (f) native HSF after pH 6.0 saline solution treatment plus 1.0 hr of pepsin digestion (g) native HSF after pH 6.0 saline solution treatment plus 2.0 hr of intestinal digestion with pancreatic and bile extract digestion (h) native HSF after pH 7.0 saline solution treatment (i) native HSF after pH 7.0 saline solution treatment plus 1.0 hr of pepsin digestion (j) native HSF after pH 7.0 saline solution treatment plus 2.0 hr of intestinal digestion with pancreatic and bile extract digestion.

Digestibility of Reconstituted Horse Spleen Ferritin (HSF) Under Different Conditions

Native gel electrophoresis. 7.5% Tris-HCl Bio-Rad ready gel. Running buffer: 25 nM Tris, 192 nM glycine. Sample buffer: 62.5 mM Tris-HCl pH 6.8, 25% glycerol, 0.01% Bromophenol blue. Coomassie Blue R-250 was used for total protein gel stains.

(a) Native HSF (b) Apo-HSF (c) HSF (d) HSF after pH 2.0 saline solution treatment (e) HSF after pH 2.0 saline solution treatment plus 1.0 hr of pepsin digestion (f) HSF after pH 2.0 saline solution treatment plus 2.0 hr of pepsin digestion and 2.0 hr of intestinal digestion with pancreatic and bile extract digestion (g) HSF after pH 6.0 saline solution treatment (h) HSF after pH 6.0 saline solution treatment plus 1.0 hr of pepsin digestion (i) HSF after pH 6.0 saline solution treatment plus 1.0 hr of pepsin digestion and 2.0 hr of intestinal digestion with pancreatic and bile extract digestion.

Co-culture of Caco-2 and Goblet Cells (HT29-MTX) vs. Caco-2 and Dialysis Membrane Method

Significance: The goblet cells produce a mucus layer, protecting the Caco-2 cells from the digestive enzymes, thus enabling more physiological conditions. Tests if ferritin is absorbed significantly as an intact molecule.

HT29-MTX cells

- Human colorectal adenocarcinoma cells adapted to 10^-6 M methotrexate
- Consist almost exclusively of mucus-secreting, differentiated cells
- Express gastric-like mucin
- Donated to our lab by Dr. Thécla Lesuffleur (INSERM U560, Lille, France)

Acknowledgments

- Ross Welch
- Dennis Miller
- Xingen Lei
- Larry Heller
- Mary Bodis
- Pei Pei Chang
- Zhaqiang Cheng
- Magnolia Ariza-Nieto
- Fuxia Jin
- Ying Hu
- Vincent Yeung
- Angela Mwaniki
- Owen Hoekinga
- Ed Buckler
- Mike Rutke
- Siow Ying
- Leon Kochian
- Cecilia Gonzalez

Thank you!