

On the Methods for Studying the Mechanisms and Bioavailability of Iron

Gladys Oluyemisi Latunde-Dada, Ph.D., Maria de Lourdes Pires Bianchi, Ph.D., and Jose Eduardo Dutra de Oliveira, M.D., Ph.D.

Studies of the molecular mechanisms involved in the absorption and bioavailability of iron are important to attempts made worldwide to control the high incidence of iron-associated disorders. The ultimate objective of these studies is to develop methods that are relevant to iron bioavailability and interactions in humans. However, a comprehensive understanding of the chemical and physiologic mechanisms that influence iron bioavailability is necessary to achieve this goal. Initial studies using in vitro and animal models offer the potential for flexibility and manipulation of experimental variables that could provide valuable information toward the understanding and improvement of food iron bioavailability.

Introduction

The importance of iron nutrition and metabolism has been a subject of strong interest in both developing and developed nations.¹⁻⁴ The metabolic disorders of iron represent a double-edged sword of deficiency and overload and have stimulated numerous interdisciplinary studies of the various aspects of iron's nutrition, physiology, and biochemistry. Among these studies are two interrelated research interests. First, studies of dietary and physiologic factors that modulate the efficiency of iron absorption aim to formulate diets and dietary practices that enhance iron availability.^{5,6} Second, an intensive search is under way to unravel the molecules, mode, and mechanisms of intestinal absorption of iron.⁷⁻⁹

Despite many years of intensive studies, the pathways and general features of intestinal iron absorption are still speculative and hypothetical. Consensus has not

Dr. Latunde-Dada is with the Department of Chemical Sciences, Agriculture University, Abeokuta, Nigeria. Dr. Bianchi is with the Faculty of Pharmaceutical Sciences and Prof. and Dr. Dutra de Oliveira is with the Faculty of Medicine, University of São Paulo, Ribeirao Preto, Brazil.

yet been reached on the comprehensive molecular mechanisms involved in iron passage into, across, and out of the mucosal epithelial cells. The unfolding scenarios of the transit of inorganic iron into the mucosal cell include three possibilities: (1) a paracellular uptake that is non-specific, nonregulated, and has a low affinity for iron permeation,¹⁰ (2) transcellular passive and partially regulated diffusion,¹¹ or (3) a highly regulated transcellular transport that might involve an electrogenic energy-requiring carrier-mediated pathway,¹² a glycoprotein,¹³ fatty acid-mediated transport,¹⁴ and/or the recently elaborated mucin-integrin-mobilferrin-paraferitin complex.^{8,15}

This last theory proposes that inorganic iron in the diet is chelated in vivo to luminal mucin to maintain its solubility at the neutral pH of the small intestine.¹⁶ Iron is then transported across the mucosal microvillar membrane in association with a cell-surface integrin.¹⁵ It is subsequently transferred to a cytosolic mobilferrin¹⁷ in association with paraferitin,⁸ which is probably a ferrireductase. The emerging consensus, however, is that the reduction of Fe³⁺ to Fe²⁺ is a key regulatory step in the intestinal uptake of iron. The rate-determining reduction¹⁸ occurs in the intestinal lumen or at the mucosal surface. This might also be a characteristic feature of the transferrin-independent iron uptake pathway elucidated in other mammalian tissues.^{19,20}

Iron Absorption

The deluge of information generated thus far on the processes of intestinal iron absorption has been derived mostly from various in vitro model systems in animal cells or tissues.^{8,12,17,21} These include the use of enterocytes, duodenal biopsies, brush border membrane vesicles, vascular perfused intestine, and everted intestinal sacs, rings, or loops.²²⁻²⁴ These models have often been criticized on the basis of the disparities between the iron absorptive processes of laboratory animals, particularly rats, and humans.^{25,26} Such disparities include the observation that ascorbic acid and meat do not enhance iron absorption in the rat,^{27,28} whereas they do in humans, as studies have

consistently proven. Ascorbic acid in the free and natural form in fruits and vegetables is a potent enhancer of nonheme iron absorption.²⁹ It exerts its promoting effects by forming soluble chelates with iron in the stomach, converting ferric iron to the highly absorbable ferrous iron and maintaining the solubility of nonheme iron in the environment of the small intestine. These facilitating effects have been observed to counteract the inhibiting influence of dietary ligands, such as phytate in cereals and legumes³⁰ and polyphenols in vegetables.

Furthermore, iron absorption in the rat is dependent on serum iron levels rather than iron turnover or storage, as is the case in humans.³¹ The mucosal cell turnover rate is higher in rats than in humans; hence, significantly more iron is lost through mucosal exfoliation.³² Moreover, *in vitro* animal models lack the versatility and viability needed to mimic the intact viable intestinal mucosa.³³ Various attempts to culture human epithelial cells or to establish cell lines from enterocytes have proven unsuccessful.³⁴

A recent study³⁵ of iron uptake by freshly isolated enterocytes derived from human duodenal biopsies has its limitations. The authors highlighted the elimination of the physiologic influence of the luminal fluid and the mucus layer. In addition, the paracellular transport of iron was excluded in the measurement, thereby making possible an underestimation of iron uptake by the cells. The paradox was that the credence and veracity of the characteristics of iron uptake by the human enterocytes were based on similar observations in animal models.³⁵

The rate-limiting step of iron uptake from the intestinal milieu by enterocytes is influenced by, among many other factors, diet. The process of iron uptake by the gastrointestinal tract is thus a function of the interplay among gastric, luminal, mucosal, and systemic factors. Inorganic iron that is ingested within the complex matrix of a meal is solubilized and ionized in the acidic pH of the stomach. The various iron species are subject to physicochemical changes, including complex formation, chelation, or precipitation, as they move into the small intestine. A significant amount of iron is absorbed in the duodenum, where the pH still maintains its solubility. The pH of the intestinal milieu rises with the secretion of pancreatic buffer, bile, and enzymes in the small intestine,³⁶ thus predisposing iron to precipitation and rendering it insoluble. It is therefore in the duodenum that iron species are chelated to integral mucosal proteins located on the surface or traversing the mucosal cells. These processes are invariably impossible to simulate holistically and sequentially under a given set of *in vitro* conditions.

Animal Studies

Although the ultimate test of iron availability in humans is to conduct human studies, the use of human subjects for routine testing is practically impossible. Not only are clini-

cal studies expensive, time-consuming, and extremely difficult to perform with precision, but they require expensive equipment that is not within the capacity of most laboratories, even in developed countries.^{37,38} Animal assays should be employed to define dietary factors of primary importance, which may then be selectively studied in people. Initial studies with animal models provide the flexibility and opportunities to manipulate different variables involved in the mechanisms of food iron availability and absorption.

In Vitro Studies

The classic *in vitro* iron availability techniques³⁹⁻⁴¹ are based on simulated enzymatic digestion of food or meals and the estimation of soluble and/or dialyzable iron and are used to investigate the chemically available iron in a wide variety of foods.⁴² They are useful in predicting the trend, but not the magnitude, of the absorptive response in man.^{43,44} They serve as methods for ranking or categorizing foods with respect to the effect of variables such as species, processing, cooking, etc., on iron availability.⁴⁵ The only major exception is the dialyzability of nonabsorbable polyphenolic iron complexes in solution.^{46,47} Several attempts are being made to optimize and improve the efficiency of *in vitro* techniques, including a systematic modification of the work of Miller et al.³⁹ by Luten et al.⁴⁸ and the use of a multicompartmental model of the stomach and the small intestine.⁴⁹

Tissue culture systems have recently been employed to mimic intestinal iron transport systems. The most prominent, promising, and potentially versatile is the Caco-2 cell line model.^{50,51} Caco-2 cells are derived from human colonic adenocarcinoma cells and grown on microporous membrane in bicameral chambers to support differentiation into homogeneous epithelial monolayers.⁵² The monolayers are highly polarized,⁵³ with tight intercellular junctions and, hence, a high transepithelial resistance.⁵⁴ The cells express abundant intestinal microvilli, enzymes, and differentiation markers typical of human small intestinal enterocytes.⁵³ Caco-2 cells are thus potentially useful as an *in vitro* model for demonstrating vectorial epithelial passage by para- and transcellular routes. The cells have demonstrated several uptake characteristics observed in animal and human studies. Iron uptake by the apical surface is transported to the basolateral pole in a process that is saturable and facilitated not only by the valency of iron but also by the iron status of the cell.⁵⁵ In addition, Caco-2 cells resynthesize three important proteins involved in iron metabolism—apotransferrin, transferrin, and ferritin.

Earlier reports have raised a few questions about the metabolism of iron by Caco-2 cells. The oncogenic origin of the cells might predispose them to a higher cell turnover proliferation rate, thus exerting greater demand for

iron.³⁵ The synthesis of iron transit proteins, i.e., apotransferrin and transferrin, might be a physiologic response to cope with the higher iron demands for the cells' proliferation. Human enterocytes do not synthesize transferrin, and its involvement in iron absorption was jettisoned long ago.⁵⁶ Furthermore, human intestinal tissue contains a heterogeneous population of cells in which the goblet cells secrete mucus—an important chelate for iron uptake.¹⁶ The influence of ascorbic acid on iron uptake by Caco-2 cells has not been clarified.^{57,58}

However, the potential uses of the Caco-2 cells in vitro model is a step forward, particularly because it is being used in food iron availability studies.^{59,60} This recent development offers the potential for combining simulated peptic and intestinal digestion with the measurement of iron uptake.⁶⁰ It therefore contributes physiologic processes of absorption and transport into an in vitro digestion model. It is thus an attractive system for the studies of the mechanism of iron absorption and true determination of iron bioavailability.^{61–63}

Conclusion

With the urgent global quest to reduce and eradicate iron deficiency anemia and associated disorders of iron metabolism, it is imperative to develop tools that are not only simple, fast, and accurate but also reliable for studying the complex variables involved in iron bioavailability. These tools are potentially valuable in delineating and generating vast information on the chemical nature of food iron, its interactions with other food components, molecular mechanisms involved in its uptake by the intestinal cells, and, ultimately, its functional metabolism. The overall goal is the generation of information toward the improvement of food iron availability for humans. A combination of reductionist and comprehensive approaches is necessary to decipher and delineate the bioavailability and complex molecular mechanisms involved in iron passage into, across, and out of the mucosal epithelial cells.

1. Scrimshaw N. Iron deficiency. *Sci Am* 1991;265:24–30
2. Marx JM. Iron deficiency in developed countries: prevalence, influence of lifestyle factors, and hazards of prevention. *Eur J Clin Nutr* 1997;51:491–4
3. Freire WB. Strategies of the Pan American Health Organization/World Health Organization for the control of iron deficiency in Latin America. *Nutr Rev* 1997;55:183–8
4. O'Donnell AM, Carmuega ES, Durán P. Preventing Iron Deficiency in Infants and Preschool Children in Argentina. *Nutr Rev* 1997;55:189–94
5. Latunde-Dada GO. Sources and forms of iron in tropical diets and the effects of processing on availability. *Nutr News Bull* 1997;18:84–9
6. Reddy MS, Hurrell RF, Juillerat MA, Cook JD. The influence of different protein sources on phytate in-

hibition of nonheme iron absorption. *Am J Clin Nutr* 1996;63:203–7

7. Umbreit JN, Conrad ME, Moore EG, et al. Paraferriitin: a protein complex with ferrireductase activity is associated with iron absorption in rats. *Biochemistry* 1996;35:6460–9
8. Pountney DJ, Simpson RJ. Mucosal surface ferricyanide reductase in mouse duodenum. *Biochem Soc Trans* 1993;21:204S
9. Beard JL, Dawson H, Piñero DJ. Iron metabolism: a comprehensive review. *Nutr Rev* 1996;54:295–317
10. Peters TJ, Raja KB, Simpson RJ, Snape S. Mechanisms and regulation of intestinal iron absorption. *Ann N Y Acad Sci* 1988;526:141–7
11. Powell JJ, Whitehead MW, Lee S, Thompson RPH. Mechanisms of gastrointestinal absorption: dietary minerals and the influence of beverage ingestion. *Food Chem Toxicol* 1994;51:381–8
12. Raja KB, Simpson RJ, Peters TJ. Membrane potential dependence of Fe (III) uptake by mouse duodenum. *Biochim Biophys Acta* 1989;984:262–6
13. Teichmann R, Stremmel W. Iron uptake by human upper small intestine microvillus membrane vesicles. *J Clin Invest* 1990;86:214–53
14. Simpson RJ, Venkatesan S, Peters TJ. Brush border membrane non-esterified fatty acids, physiological levels and significance for mucosal iron uptake in mouse proximal intestine. *Cell Biochem Funct* 1989;7:165–71
15. Conrad ME, Umbreit JN. A concise review: iron absorption—the mucin-mobilferrin-integrin pathway. A competitive pathway for metal absorption. *Am J Hematol* 1993;42:67–73
16. Conrad ME, Umbreit JN, Moore EG. A role for mucin in the absorption of inorganic iron and other metal cations. *Gastroenterology* 1991;100:129–36
17. Conrad ME, Umbreit JN, Moore EG, et al. A newly identified iron binding protein in duodenal mucosa of rats. *J Biol Chem* 1990;265:5273–9
18. Raja KB, Simpson RJ, Peters TJ. Investigation of a role for reduction in ferric iron uptake by mouse duodenum. *Biochem Biophys Acta* 1992;1135:141–6
19. Jordan I, Kaplan J. The mammalian transferrin independent iron transport system may involve a surface ferrireductase activity. *Biochem J* 1994;302:875–9
20. Umbreit JN, Conrad ME, Berry MA, et al. The alternate iron transport pathway: mobilferrin and integrin in reticulocytes. *Br J Haematol* 1997;96:521–9
21. Muir AN, Hopfer U, King M. Iron transport across brush border membranes from normal and iron-deficient mouse upper small intestine. *J Biol Chem* 1984;259:4896–903
22. O' Riordan DK, Sharp PA, Epstein A, et al. Increased iron transfer in overnight fasted rats. *Gut* 1994;35:S52
23. Taylor EM, Raja KB, Simpson RJ, Peters TJ. Modulation of duodenal iron uptake by hypoxia and fasting in the rats. *Br J Nutr* 1997;77:459–73
24. Reddy MB, Cook JD. Assessment of dietary determinants on non-heme iron absorption in humans and rats. *Am J Clin Nutr* 1991;54:722–8
25. Dahm LJ, Jones DP. Secretion of cysteine and glutathione from mucosa to lumen in rat small intestine.

- Am J Physiol 1994;267:G292-300
26. Scricker BR, Miller DD, Van Campen D. Effects of iron status and soy protein on iron absorption by rats. *J Nutr* 1983;113:996-1001
 27. Thannoun AM, Mahoney AW, Hendricks DG, Zhang D. Effect of meat-bread mixtures on bioavailability of total dietary iron for anemic rats. *Cereal Chem* 1987;64:399-403
 28. Scricker BR, Miller DD, Rasmussen RR, Van Campen D. A comparison of in vivo and in vitro methods for determining availability of iron from meals. *Am J Clin Nutr* 1981;34:2257-63
 29. Lynch SR. Interaction of iron with other nutrients. *Nutr Rev* 1997;55:102-10
 30. Davidsson L, Galan P, Kastenmayer P, et al. Iron bioavailability studies in infants: the influence of phytic acid and ascorbic acid in infant formulas based on soy isolate. *Pediatric Res* 1994;36:816-22
 31. Cook JD, Hershko C, Finch CA. Storage iron kinetics V. Iron exchange in the rats. *Br J Haematol* 1973;25:695-706
 32. Huebers HA, Finch CA. The physiology of transferrin and transferrin receptors. *Physiol Rev* 1987;67:520-8
 33. Hildalgo IJ, Raub TJ, Borchardt RT. Characterization of human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 1989;96:736-49
 34. Raul F, Keding M, Simon P, et al. Behaviour of isolated rat intestinal cells maintained in suspension on monolayer cultures. *Biol Cell* 1978;33:163-8
 35. Goddard WP, Coupland K, Smith JA, Long RA. Iron uptake by isolated human enterocyte suspension in vitro is dependent on body iron stores and inhibited by other metal cations. *J Nutr* 1997;127:177-83
 36. Carpenter CE, Mahoney AW. Contribution of heme and nonheme iron to human nutrition. *Cri Rev Food Sci Nutr* 1992;31:333-67
 37. Forbes A L. Use of animal studies in predicting human bioavailability: reply to ND Barnard. *Am J Clin Nutr* 1989;50:557-8
 38. Heuvel EG, van Dokkum HM, Schchaafsma G. Methods to measure iron absorption in man. *Food Rev Int* 1997;13:91-102
 39. Miller DD, Scricker BR, Rasmussen RR, Van Campen D. An in vitro method for estimation of iron availability from meals. *Am J Clin Nutr* 1981;34:2248-56
 40. Latunde-Dada GO. Iron contents and some physical components of twelve cowpea varieties. *Int J Food Sci Nutr* 1993;34:193-7
 41. Latunde-Dada GO. Effect of processing on iron levels and availability from some Nigerian vegetables. *J Sci Food Agric* 1990;53:355-61
 42. Forbes AL, Adams CE, Arnaud MJ, et al. Comparison of in vitro, animal, and clinical determinations of iron bioavailability: International Anemia Consultative Group Task Force report on iron bioavailability. *Am J Clin Nutr* 1989;49:225-38
 43. Hurrell RF, Lynch SR, Trinidad TP, et al. Iron absorption in humans: bovine serum albumin compared with beef muscle and egg white. *Am J Clin Nutr* 1988;47:102-7
 44. Hurrell RF, Lynch SR, Trinidad TP, et al. Iron absorption in humans as influenced by bovine milk proteins. *Am J Clin Nutr* 1989;49:546-52
 45. Van Campen D. Iron bioavailability techniques: an overview. *Food Technol* 1983;11:127-32
 46. Brown RC, Klein A, Simmons WR, Hurrell RF. The influence of Jamaican herb teas and other polyphenol-containing beverages on iron absorption in the rat. *Nutr Res* 1990;10:343-53
 47. Valdes DH, Gee JM, Fairweather-Tait SJ, Johnson IT. A comparison of methods for the in vitro determination of the effects of tea on iron availability. *Food Chem* 1992;44:331-5
 48. Luten J, Crews H, Flynn A, et al. Interlaboratory trial on the determination of the in vitro iron dialyzability from food. *J Sci Food Agric* 1996;72:415-24
 49. Larsson M, Minekus M, Havenaar R. Estimation of the bioavailability of iron and phosphorus in cereals using a dynamic in vitro gastrointestinal model. *J Sci Food Agric* 1997;74:99-106
 50. Halleux C, Schneider Y-J. Iron absorption by intestinal epithelial cells: Caco cells cultivated in cell free medium polyethylenetetrathalate microporous membrane as an in vitro model. *Cell Dev Biol* 1991;27:293-302
 51. Halleux C, Schneider Y-J. Iron absorption by Caco-2 cells cultivated in serum free medium as an in vitro model of the human intestinal epithelial barrier. *J Cell Physiol* 1994;158:17-28
 52. Pinto M, Robine-Leon S, Appay MD, et al. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *J Biol Chem* 1983;47:322-30
 53. Sergent-Engelou T, Halleux C, Farain E, et al. Improved cultivation of polarized animal cells on culture inserts with new polyethylenetetrathalate or polycarbonate microporous membranes. *Biotechnology Techniques* 1990;4:89-94
 54. Jumafie C, Maio C. Caco-2 cells in serum free medium as a model for the study of enterocytic differentiation. *J Cell Physiol* 1991;149:24-33
 55. Alvarez-Hernandez X, Nichols GM, Glass J. Caco-2 cell line: a system for studying intestinal iron transport across epithelial cell monolayers. *Biochim Biophys Acta* 1991;1070:205-8
 56. Pietrangelo A, Rocchi E, Casalgrandi G. Regulation of transferrin, transferrin receptor and ferritin genes in human duodenum. *Gastroenterology* 1992;802:120-9
 57. Han O, Failla ML, Hill AD, et al. Reduction of Fe (III) is required for uptake of nonheme iron by Caco-2 cells. *J Nutr* 1995;125:1291-9
 58. Halleux C, Schneider Y-J. Iron absorption by Caco-2 cells cultivated in serum free medium as an in vitro model of the human intestinal epithelial barrier. *J Cell Physiol* 1994;158:17-28
 59. Gangloff MB, Glahn RP, Miller DD, et al. Assessment of iron availability using combined in vitro digestion and Caco-2 cell culture. *Nutr Res* 1996;16:479-87
 60. Glahn RP, Gangloff MB, Van Campen DR, et al. Bathophenanthroline disulfonic acid and sodium dithionite effectively remove surface-bound iron from Caco-2 cell monolayers. *J Nutr* 1995;125:1833-40
 61. Glahn RP, Van Campen DR. Iron uptake is enhanced in Caco-2 cell monolayers by cysteine and reduced cysteinyl glycine. *J Nutr* 1997;127:642-7

62. Garcia MN, Flowers C, Cook JD. The Caco-2 cell culture system can be used as a model to study food iron availability. *J Nutr* 1996;126:251-8
63. Glahn RP, Wien EM, Van Campen DR, Miller DD.

Caco-2 cell iron uptake from meat and casein digests parallel in vivo studies: use of a novel in vitro method for rapid estimation of iron bioavailability. *J Nutr* 1996;126:332-9