Advantages and disadvantages of the animal models v. in vitro studies in iron metabolism: a review

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Iron deficiency is the most common nutritional deficiency in the world. Special molecules have evolved for iron acquisition, transport and storage in soluble, nontoxic forms. Studies about the effects of iron on health are focused on iron metabolism or nutrition to prevent or treat iron deficiency and anemia. These studies are focused in two main aspects: (1) basic studies to elucidate iron metabolism and (2) nutritional studies to evaluate the efficacy of iron supplementation to prevent or treat iron deficiency and anemia. This paper reviews the advantages and disadvantages of the experimental models commonly used as well as the methods that are more used in studies related to iron. In vitro studies have used different parts of the gut. In vivo studies are done in humans and animals such as mice, rats, pigs and monkeys. Iron metabolism is a complex process that includes interactions at the systemic level. In vitro studies, despite physiological differences to humans, are useful to increase knowledge related to this essential micronutrient. Isotopic techniques are the most recommended in studies related to iron, but their high cost and required logistic, making them difficult to use. The depletion—repletion of hemoglobin is a method commonly used in animal studies. Three depletion—repletion techniques are mostly used: hemoglobin regeneration efficiency, relative biological values (RBV) and metabolic balance, which are official methods of the association of official analytical chemists. These techniques are well-validated to be used as studies related to iron and their results can be extrapolated to humans. Knowledge about the main advantages and disadvantages of the in vitro and animal models, and methods used in these studies, could increase confidence of researchers in the experimental results with less costs.

Keywords: experimental models, iron metabolism, in vitro studies, animal models

Implications
Iron deficiency is the most common nutritional deficiency around the world and it is considered a major public health problem. The mechanism and control of iron uptake by the gut has puzzled investigators in both in vivo and in vitro studies. A variety of experimental models have been used in studies related to iron, however, in vitro systems or animal models features different iron metabolism results compared with humans. This review covers the main experimental models commonly used in metabolic and nutritional studies of iron homeostasis, including their advantages and disadvantages.

Introduction
Iron deficiency is the most common nutritional disorder around the world and it is considered a public health problem (McLean et al., 2007). Because of iron’s insolubility and potential toxicity under physiological conditions, special molecules have evolved for its acquisition, transport and storage in soluble, nontoxic forms (Kolachala et al., 2007). The mechanism and control of iron uptake by the gut has puzzled investigators in both in vivo and in vitro studies (Latunde-Dada et al., 1998; Morgan and Oates, 2002; Latunde-Dada, 2009). Studies about the effect of iron are focused mainly in two ways; basic studies to elucidate iron metabolism, and nutritional studies to evaluate the efficacy of iron supplementation to prevent or treat iron deficiency and anemia (Lynch and Stoltzfus, 2003; Nadadure et al., 2008; Theurl et al., 2008; West and Oates, 2008; Lönnnerdal, 2009). There are many studies available in the scientific literature about iron absorption and regulation. Iron supplementation studies determine factors that affect iron absorption and oxidative damage (Beach et al., 2003; Casanueva and Viteri, 2003; Troost et al., 2003; Lönnnerdal et al., 2006; Nagababu et al., 2008; Jin et al., 2009).
A variety of experimental models have been used in studies related to iron through the time (Latunde-Dada et al., 1998; Srigriridhar and Nair, 2000; Morgan and Oates, 2002; Troost et al., 2003; Nagababu et al., 2008; Quintero et al., 2008; Jin et al., 2009; Latunde-Dada, 2009). Nevertheless, in vitro systems or animal models have different iron metabolism behaviour when compared with humans (Latunde-Dada et al., 1998; Vaghefi et al., 2005; Patterson et al., 2008; Quintero et al., 2008). Studies related to iron involve different procedures including isotopic techniques, simulated enzymatic gastrointestinal digestion or hemoglobin (Hb) depletion–repletion (Srigriridhar and Nair, 2000; Beach et al., 2003; Vaghefi et al., 2005; Kolachala et al., 2007; Quintero et al., 2008). This paper reviews the main experimental models commonly used in iron metabolic and nutritional studies, including their advantages and disadvantages.

Iron metabolism

Iron is complexed in the food, and the nature of the foodstuff determines its bioavailability. In most cases, dietary iron has to be dissociated and made soluble in order for the iron to be absorbed. The usually low pH of the stomach serves this function, releasing iron and maintaining it in Fe^{3+} state (Bothwell et al., 1979; Andrews, 1999). Once released, free iron comes into contact with absorptive cells of the proximal small intestine.

Iron absorption takes place in the duodenum and the proximal jejunum (Nadadur et al., 2008). Nonheme iron is rendered soluble in gastric secretion and remains soluble in the upper small intestine; its absorption is mediated by divalent metal transporter 1 (DMT1). Heme iron from Hb or myoglobin is transported by a protein namely PCFT/HCP1 and FLVCR, that acts also as a folate transporter and this seems to be its main role. This transporter protein appears to function independently of the putative heme receptor and receptor-mediated endocytosis and it acts as a direct heme iron transfer process across plasma membranes (Qiu et al., 2006).

Ferroportin (FPN) plays a role in iron transport enterocytes and in iron release from hepatocytes and macrophages. The diffusion of Fe^{2+} across the basolateral membrane is facilitated by FPN and Hephaestin, a membrane-bound protein that promotes oxidation of Fe^{2+} to Fe^{3+} prior to its release from transporter molecule. The cellular iron uptake, storage and efflux depend on the functional demands of the different cell types. In the majority of eukaryotic cells, cellular iron uptake occurs primarily by transferrin receptor (TFR)-mediated endocytic pathway. There are two TFRs, namely TFR-1 and TFR-2. The TFR-1 features a high affinity binding the complex transferrin-Fe^{2+}, and it has a key role involved on iron uptake in the majority of cells, while TFR-2 is expressed primarily in liver and binds the complex transferrin-Fe^{3+} with lower affinity (Nadadur et al., 2008). In addition to the cellular acquisition of iron by the classic transferrin-dependent pathway, there is another pathway, the uptake of nontransferrin-bound iron (NTBI) that requires iron reduction and subsequent cellular uptake of Fe^{3+} by DMT1 (Lane and Lawen, 2008). The iron reduction of NTBI and uptake is mediated by mucosal ferric reductases such as Duodenal Cytochrome b (Dcyt; Krause et al., 2000). In presence of catalytic concentrations of ascorbate, Dcyt may catalyze electron transfer from intracellular ascorbate to extracellular ascorbyl free radical to generate ascorbate, which could then directly donate a single electron to Fe^{3+} or Cu^{2+}. These results are supported by a novel model of NTBI reduction and uptake pathway in K562 erythroleukemia cells (Lane and Lawen, 2008). Figure 1 summarizes iron absorption pathways in the intestinal enterocyte.

The stored iron accounts for 20% to 30% of body iron and the majority is bound to ubiquitin and the highly conserved iron

![Figure 1](https://www.cambridge.org/core/core/terms, https://doi.org/10.1017/S1751731113001134).
binding protein, ferritin (Nadadur et al., 2008). Absorption is regulated according to the body's needs by hepcidin, a small cysteine-rich cationic peptide currently considered as the most important factor controlling iron absorption (Lynch, 2007). A study using human biopsies and rats concluded that the intestine iron uptake takes place through a sequential transfer involving interaction of luminal transferrin, transferrin–TFR and ferritin (Kolahchala et al., 2007).

In addition, levels of key proteins in the absorptive enterocyte are influenced by oxygen tension in the cell, which, in turn, affects the transcription factor hypoxia-inducible factor 2α (HIF-2α) (Mastrogiannaki et al., 2009). This fact leads to subsequent changes in the transcription of DMT1 and FPN. In addition, the content of iron within the enterocyte regulates iron absorption through its effects on iron regulatory proteins (IRP) types 1 and 2 and their subsequent effect on mRNAs encoding DMT1, FPN, ferritin and HIF-2α. (Galy et al., 2008). The IRPs bind to specific sequences (iron-responsive elements) that influence mRNA translation (linked with FPN, ferritin and HIF-2α) or stability (linked with TIR-1 and DMT1; Sánchez et al., 2007). Consequently, presence of hypoxia or cellular iron deficiency, DMT1 and FPN are upregulated, promoting iron absorption from the diet.

Hepcidin is secreted into the circulation by hepatocytes and play a key role in the regulation of iron metabolism. Hepatic hepcidin levels are regulated by iron stores, hypoxia, erythropoietic rate and inflammatory status (Nicolas et al., 2002). Hepcidin acts by blocking the iron efflux into the blood circulation from the gut and the macrophages by binding to the iron transporter FPN, resulting in its ubiquitination and degradation. Low levels of circulating hepcidin are associated with many forms of genetic iron overload (Nemeth and Ganz, 2006). Various signaling pathways have been shown to regulate hepatic hepcidin levels with bone morphogenetic proteins (BMPs) which signal via intracellular pathways that transduce extracellular signals to the nucleus where they activate downstream gene transcription. These proteins are homologs of both the Drosophila protein, mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA (from gene sma for small body size). The name of this group of proteins (SMADs) is a portmanteau of this.

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Common methods used in Fe metabolism

In vitro experimental models

Because iron absorption takes place at the intestinal level, several in vitro studies have used different fragments of gut, such as enterocyte suspensions, brush border membranes and vesicles, perfused duodenal segment or everted gut sacs (Simpson et al., 1986; Goddard et al., 1997; Moshtaghie, 2006). To minimize the effect of the lack of a mucous layer in enterocyte suspensions, ascorbate was used as a quasi-physiological substitute for gut lumen iron chelators (Goddard et al., 1997).

Caco-2, derived from human colonic adenocarcinoma cells is a suitable model to study iron absorption. Although Caco-2 cells are originally colonic, they differentiate in culture, developing brush border membranes and exhibiting transport properties similar to intestinal epithelia. With regards to iron uptake, many investigators agree that Caco-2 cell monolayers are valid models which can be used to define the mechanisms of iron absorption as well as to investigate factors which affect iron availability (Glahn and Van Campen, 1997; Zhu et al., 2006; Anrondo et al., 2008). The cells express profusely abundant intestinal microvilli, enzymes and differentiation markers typical of human small intestinal enterocytes. Thus, Caco-2 cells are potentially useful as an in vitro model to elucidate vectorial epithelial passage by para- and transcellular routes. The cell cultures 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 iron ionization, but also by the iron status of the cell. In addition, Caco-2 cells resynthesize three important proteins involved in iron metabolism, apotransferrin, transferrin and ferritin (Latunde-Dada et al., 1998).

BeWo is a human placental cell line derived from a choriocarcinoma. These cells have been used as an in vitro model to study placental uptake a variety of nutrients including glucose, amino acids and iron. However, unlike Caco-2 cells, which are widely used in a similar culture system as a model of intestinal epithelial cell transport, BeWo cells do not feature the contact inhibition of growth. This fact makes more difficult to obtain them and sustain an intact cell monolayer that would be optimal for transport studies. Furthermore, the permeability of BeWo cells layers is dependent on the molecular size of the substrate applied (Heaton et al., 2008).

Animal models used to study iron homeostasis

Most of our knowledge on iron homeostasis relies on studies performed on mice. Several genetic models are available, including both natural and gene targeting generated mutations of the main genes involved in iron absorption, recycling, storage and utilization. The mouse also represents a unique model to identify novel regulators of iron homoeostasis because its behavior is similar to humans in disorders such as hemochromatosis and anemia (Fiorito et al., 2012). For instance, the significant role of DMT1 in iron intestinal absorption was evident in studies in microcyt anemic
mice and Belgrade rat. A spontaneous mutation (G185R) found in both strains caused significant defects in intestinal iron absorption and assimilation by epithelial precursor cells (Fleming et al., 1998). Moreover, the targeted mutation of murine DMT1 gene (Slc11a2-/- mice) further confirmed its role in intestinal iron absorption (Gunshin et al., 2005). Embryonic lethality observed in TFR-1 knockout mice further reinforces the important role of TFR-1 in cellular iron uptake (Hentze et al., 2004). Severe anemia and rapid accumulation of iron in FPN deficient mice suggested that FPN is essential for iron recycling (Donovan et al., 2005). The study of iron-overload in the upstream stimulatory factor 2 (USF-2) knockout mice led to the serendipitous discovery of hepcidin (HAMP) gene (Nicolas et al., 2001). Targeted deletion of hepcidin gene in mice or mutations in human gene result in elevated body iron stores, presumably because of hyperabsorption associated with decreased iron in macrophages (Knutson et al., 2003).

Transgenic knockout mouse models of IRP1, IRP2 indicate that the double knockout is embryologically lethal (Smith et al., 2004). Observation of no overt phenotype for IRP1-/- knockout mouse is rather surprising and suggests that IRP2 can compensate for the loss of IRP1. Mouse models of IRP2 knockout exhibited increased iron content and expression of DMT1, ferritin and FPN. These observations suggest that other unidentified factors may participate along with IRPs in cellular iron homeostasis (Hentze et al., 2004). In mouse, a model of hereditary hemochromatosis was developed, which is an iron-overload disorder resulting from mutations in hemojuvelin, a protein involved in the maintenance of iron homeostasis (Huang et al., 2005).

Animal models used for nutritional studies

Many nutritional studies related to iron are performed in humans (Beach et al., 2003; Troost et al., 2003; Lonnerdal et al., 2006). Nevertheless, human research in basic metabolism is expensive and difficult to perform accurately. Animal studies are used in primary studies and sometimes followed with selective studies in people. Traditionally, rats have been the model of choice when performing nutritional studies. However, the rat model has a number of limitations which makes extrapolation to humans really questionable, including a significantly different energy and food intake, a different lifespan and body proportion, differences in intestinal morphology and enteric microbiota, as well as other distinct physiological differences. Another major problem with using rat models for mineral studies is their propensity for practicing coprophagy. Although this is an effective way for animals to recycle nutrients and maximize nutrient absorption, it may have a dramatic impact on the results of a nutritional study (Quintero et al., 2008). Some putative iron-binding proteins isolated from rats have also been demonstrated to have biological activity in humans. The absorption and metabolism of heme iron are known to occur in the rat mucosa similar to that in humans, even if the absorption of heme iron is lower in the rodent. Another difference between iron metabolism in rats and humans is the relatively high mucosal cell turnover in rats leading to higher iron losses. Iron absorption in rats is more dependent on serum iron levels than on internal iron turnover or iron stores as in humans. The expected increase of nonheme iron absorption when meat protein is added to the diet is also very difficult to demonstrate in rats (Hartmann and Bissel, 1982; Gordon and Godber, 1989; Conrad et al., 1992; Roberts et al., 1993).

Although no animal model will ever perfectly mimic the human condition, the pig has emerged as a superior nonprimate experimental model because despite some anatomic differences, the physiology of digestion and associated metabolic processes are very similar between humans and pigs (Milner and Ullrey, 1987; Swindle et al., 1994). Pigs are also the only widely utilized animal model that is truly omnivorous, and they have strikingly similar nutritional requirements to that of humans. Although the porcine model bears some remarkable similarities to humans, it is important to recognize that there are some differences between the two species, which may lead to a differing response to certain experimental regimes. Although the physiology of digestion and associated metabolic processes are alike between pigs and humans, it should be recognized that the absolute length and weight of the intestine does differ. Differences in body fat content between pigs and humans could translate into differences in nutrient absorption in cases of severe obesity. Incidentally, pigs practice coprophagy, which can be another confounding experimental factor, but this practice is quite rare in pigs, as compared with rats that frequently practice coprophagy (Quintero et al., 2008).

The broiler chicken may be a useful model for initial in vivo screening of Fe bioavailability in foods because of its growth rate, anatomy, size and low cost. This model exhibited the appropriate responses to Fe deficiency and has potential to serve as a model for Fe bioavailability. Such a model should be most useful as an intermediate test of in vivo Fe bioavailability observations in preparation for subsequent human studies (Tako et al., 2010).

Animal models used for gestational iron studies

Animal models are capable of providing important information on the causal link between restricted dietary iron intake, induction of gestational iron deficiency anemia and adverse pregnancy outcomes. Gestational iron deficiency anemia has been studied in laboratory rodents. However, rodents do not provide an adequate model for the effects of third-trimester iron deficiency anemia on the fetus. Rodents are precocial, and the pups are born shortly after completion of organogenesis, whereas an extended period of postembryonic intra-uterine development occurs in human and nonhuman primates. Rodents, the most common laboratory animal model for human developmental experiments, complete embryogenesis at 15 to 16 days of gestation and are typically born at 18 to 21 days of gestation.

In addition, rhesus monkeys have single-offspring pregnancies and are known to display hematologic changes similar to those of humans in late pregnancy. Although the biology of pregnancy is similar in monkeys and humans, the environmental standardization possible in nonhuman...
primate studies, and the related lack of confounding factors markedly increases the sensitivity of small-sample experiments (Golub et al., 2006). Monkeys and rodents show us important evidence about the relation of iron deficiency anemia during gestation and lactation and a vulnerable period in early development that may result in long-lasting damage (Beard, 2007).

Advantages and disadvantages of in vitro and ex-vivo techniques

Researchers have developed in vitro techniques to study iron availability. One of the most ancient methods to estimate dietary iron availability included a simulated gastrointestinal digestion, followed by measurement of soluble and/or dialyzable low molecular weight iron to investigate the chemically available iron in a wide variety of foods (Miller et al., 1981). This work used radioisotopes of iron to measure available iron, but another group used the same in vitro method to study changes in ferritin iron and protein during cooking and gastric digestion by measuring ferritin concentrated by a gel filtration column (Hoppler et al., 2008). These methods could use iron availability quantification with spectrophotometric methods using an iron chromogen such as 1,10 Phenanthroline or Ferrozine because of the specific reaction with Fe$^{2+}$. They serve as methods for ranking or categorizing foods with respect to the effects of variables such as species, processing, cooking, etc. Simulated in vitro digestion is not a method on its own to measure iron availability. However, it may be used as a prestep, for example, to cell studies. These results cannot be extrapolated to absorption in the human intestine. They are useful in predicting the trends, but not the magnitude, of the absorptive response in humans.

Everted gut sac is an ex-vivo systems used since the middle of the past century in basic studies to elucidate the mechanism of intestinal iron uptake with contradictory results. In an attempt to resolve these divergent views, the use of this technique was examined in studies of iron absorption and used radio-iron isotopes of $^{59}$Fe (Pearson and Reich, 1965). This system proved to be susceptible to large errors at low iron concentrations. The methods of multiple transfers permitted precise estimation of uptake because the initial and final counts in the incubation medium could be determined easily, without having to transfer from the incubation vessel to the counting tube with the associated concomitant losses. In vitro gut sac systems have a relative insensitivity to physiological changes known to alter iron uptake in vivo, suggesting that mucosal participation in iron transport may have an extramucosal control. Nevertheless, researchers have continued to use everted gut sac systems in studies of iron interaction with other metals such as manganese and aluminium (Moshtaghie and Taher, 1993; Moshtaghie, 2006).

Another of the ex-vivo systems is the Ussing chamber that has been used to elucidate the influence of heme iron and peptide release during globin hydrolysis and cysteine during iron absorption. This experimental system uses fully organized digestive membranes, including the mucus layer that affects the diffusion of iron from lumen to enterocytes. This is an advantage of an Ussing chamber in relation to a perfused duodenal segment, everted gut sac or enterocyte suspension where the wash buffer and manipulation damage the mucus layer (Vaghefi et al., 2005).

Isotopes provide an invaluable means for studying the metabolism of iron. Stable isotopes of iron are $^{56}$Fe, $^{57}$Fe and $^{58}$Fe. Because stable isotopes have virtually no health risk in their use, they can be used in measured amounts to trace how the micronutrients are metabolized by the body. This technique is considered the ‘gold standard’ for iron and other nutrient bioavailability studies in humans, including children. Isotopic methods were used to measure the effectiveness of fortification and supplementation programmes in several countries (Aggett, 1999; Walczyk et al., 2005; Moretti et al., 2006). There are two radioactive isotopes of iron suitable for use as biological tracers, $^{56}$Fe and $^{59}$Fe. In addition to the use of these isotopes individually, it is often desirable to introduce both $^{55}$Fe and $^{59}$Fe as a dual tracer in an experiment and determine the activity of each independently (Swindle et al., 1994; Pizarro et al., 2002). The hazards associated with the use of radioactive materials, the radioisotope could be replaced with a stable isotope (Morais et al., 1996; Zinn et al., 1999).

Iron absorption has been studied in mice by using the radioisotopes $^{57}$Fe and $^{55}$Fe in tied-off or dissected and everted duodenal segments. Owing to several drawbacks, the extended use of these approaches is discouraged because after oral administration of $^{57}$Fe-containing solutions, it is possible to measure both duodenal iron retention and duodenal iron transfer to specific organs using inductively coupled plasma mass spectrometry (ICP-MS). As $^{56}$Fe is administered orally, no surgery is needed before the end of the experiment, thus allowing the measurement of iron absorption under physiological conditions. Moreover, the use of ICP-MS for $^{57}$Fe detection ensures high sensitivity and provides quantitative data (Fiorito et al., 2012). A similar methodology was used to study the iron absorption and bioavailability from supplemented formula milk administrated to lactating rats (González-Iglesias et al., 2012). The Hb repletion assay is widely used in weaning pig model to assess serum iron and Hb regeneration. The animals are fed the experimental diets for a period of 1, 2, 5 or 20 weeks. Blood volume is estimated from BW and then hemoglobin regeneration efficiency is calculated (South et al. 2000; Quintero et al., 2008).

The broiler chicken was used as a model to study iron bioavailability with a unique duodenal loop technique for direct measurement of intestinal iron absorption. One-week-old chicks were allocated into iron-deficient v. iron-adequate treatment groups for 6 weeks. At week 7, birds were anesthetized and their duodenal loops were exposed. The loop was isolated and a nonocclusive catheter was inserted into the duodenal vein for blood sampling. A stable isotope solution containing $^{58}$Fe was injected into the loop. Blood samples were collected every 5 min for 120 min post-injection.
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for measurement of $^{58}\text{Fe}$ concentrations. Tako et al. (2010) evaluated expression of proteins involved in Fe uptake and transfer as DMT1, FPN1 and Dcytb as an indicator of iron bioavailability. Table 1 summarizes the main advantages and disadvantages of the models studied.

Conclusions

Many experimental models have been used since the past century in studies related to iron, most of which are still currently in use. In vitro models are usually simple, easy to handle and the results are obtained quickly with lower cost. These are some advantages in relation to studies in animal or humans. On the other hand, we have to consider the ethical aspect and only use animal and human studies when it is necessary.

Isotopic techniques are highly recommended in studies related to iron, but these are difficult in many routines worldwide because the experimental design requires a lot of special logistic conditions which are currently not feasible in many developing countries. Knowing about the main advantages and disadvantages of the in vitro and animal models used in studies related to iron is crucial to the researcher in the field of nutritional studies.

References

García and Díaz-Castro


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