Plant Foods for Human Nutrition **41**: 299–307, 1991.

Determination of iron absorption by rat bioassay. Evaluation of methods of dosing ^{59}Fe on radioiron absorption from plant diets

MACIEJ S. BUCHOWSKI, ARTHUR W. MAHONEY*,
P.V. MRUDULA KALPALATHIKA & DELOY G. HENDRICKS
*Department of Nutrition and Food Sciences, Utah State University, Logan, UT 84322-8700, USA (*author for correspondence)*

Received November, 1990; accepted January, 1991

Key words: Iron bioavailability, iron balance, radioiron, extrinsic labelling, rats

Abstract. We studied the potential of an animal model to predict nonheme iron absorption in humans and tested a feasible and easy technique of dosing ^{59}Fe to evaluate bioavailability of dietary nonheme iron. Plant diets containing about 20 ppm or 75 ppm iron were the nonheme iron sources with ferrous sulfate (75 ppm) as a reference. Radioiron was administered by (a) gavaging in water, 1 h after a meal; (b) mixing with the meal; and (c) making a slurry with the meal and gavaging. No significant differences were found ($P \leqslant 0.05$) in ^{59}Fe absorption among the three methods of administering radioiron. Absorption of ^{59}Fe was similar to apparent iron absorption for all diets tested, whether the animals consumed the same diet as that of the test meal or a different diet. A high correlation ($r = 0.88$) obtained between the apparent iron absorption and ^{59}Fe iron absorption for different dosing techniques, indicates that extrinsic iron tag administered by any of these methods is valid to measure nonheme iron absorption. Apparent iron absorption values determined by rats fed 20 ppm or 75 ppm dietary iron from plant sources were similar to reported values for humans. It is concluded that the normal adult rat is an appropriate model to study iron bioavailability of human foods when iron status, maturity, iron intake relative to requirement, and method of measurement are similar to the human situation.

Introduction

Radioisotopic techniques are extensively used to evaluate bioavailability of nonheme iron in both humans and animals and are the most popular, new, convenient and accurate methods for such assessments. Recently, the use of extrinsic labelling technique in iron bioavailability studies has become popular owing to its simplicity and low cost over the intrinsic labelling techniques [1, 2]. Moreover, extrinsic radioactive iron behaves in a manner entirely analogous to the element intrinsic to most foods. Exogenous nonheme iron added to a vegetable or cereal food forms a common pool with

the endogenous food iron and is absorbed to the same extent as the endogenous iron [3, 4]. It is assumed that when the isotope is given in the meal or by gavage, it becomes uniformly distributed in the stomach with the native nonheme iron on consumption of the test foods.

Use of extrinsic radioiron labelling in human subjects has been validated. However, there is no information validating the extrinsic labelling technique in rats in which the experimental protocol is designed to mimic human iron bioavailability protocols with regards to iron status, maturity, iron intake relative to iron requirement or method of ^{59}Fe dosing. Therefore, the intent of this study was to develop an animal model paralleling the method used in humans to study nonheme iron bioavailability, to compare different methods of dosing ^{59}Fe for measuring nonheme iron availability, and to validate nonheme iron absorption determined by extrinsic ^{59}Fe absorption by comparison with total iron absorption determined by balance. A mixture of plant foods with varied iron complexes was used in the diets because these foods provide only nonheme. Thus, the validity of extrinsic ^{59}Fe absorption data as a measure of nonheme iron absorption could be evaluated with iron balance data. Also, ^{59}Fe absorption and iron balance data from animals acclimated to a casein-based diet were compared with animals acclimated to a diet containing a mixture plant foods.

Materials and methods

Animal care

Normal, adult female rats (Sprague-Dawley strain, Simonsen Laboratories, Gilroy, CA) weighing about 200 g, were housed individually in stainless steel metabolic cages equipped with stainless steel funnels and glass apparatus to separate and collect urine and feces. The animal room was maintained at approximately 25 °C, lighted between 0700 h and 1900 h and ventilated with complete air exchange every 3 min. Fresh demineralized water was provided ad libitum. Fresh Diet 1 (Table 1) was weighed into glass feeders and food consumption was measured daily. Blood was drawn from the retro-ocular capillary bed (5) using heparinized capillary tubes. At the end of the experimental period, animals were killed, livers removed and the carcasses were placed in tared glass canning jars with 25 ml glacial acetic acid and demineralized water, autoclaved for 1.5 h, and weighed. The cooked carcass was blended in the same jar which was fitted with a stainless steel blender head. Iron bioavailability was determined by apparent iron absorption using

Table 1. Comparison of diets used to evaluate the dosing of radioiron on the bioavailability of iron from plant diets[a]

Iron source:	Ferrous sulfate	Plant Diet[b]	
Iron level, ppm:	75	20	75
Diet:	1[c]	2	3
Plant mixture	–	7.1	26.9
Casein[d]	34.6	32.9	28.2
Fat[d]	10.7	11.4	13.3
Minerals[e]	1.2	1.2	1.2
Vitamins[e]	2.0	2.0	2.0
$CaCO_3$	1.5	1.5	1.2
Na_2HPO_4	2.3	2.2	1.9
Cellulose	5.0	4.8	4.2
Dextrose	42.7	37.1	21.7
Iron, ppm[f]	77.0	17.0	76.0

[a] All values are expressed on the dry matter basis. All the diets were isocaloric (4.17 kcal/g) with 73.1% energy from carbohydrate, 15.4% from protein and 11.5% from fat.
[b] Plant diet contained a mixture of soy flour, wheat bran, potato flour, lyophilized spinach and parsley, all at 20% by weight.
[c] Diet 1 contained 0.375 g of freshly opened $FeSO_4 \cdot 7H_2O$.
[d] Casein and fat were adjusted to maintain the protein and energy ratio.
[e] Composition of the vitamin and mineral mixtures is given by Thannoun *et al.* [24].
[f] Measured iron level.

total iron intake and total fecal iron data and by the retention of extrinsic ^{59}Fe in the carcasses.

Diets

Reference diets supplemented with 75 ppm of iron (Table 1, Diet 1) was prepared with fresh ferrous sulfate to ensure consistent biovailability values. The iron level of 35 ppm is recommended for rats for growth and maintenance [6]. Iron level of 75 ppm was used to simulate the condition in human iron availability studies in which the subjects consume about 12 mg iron including the 3 mg in the test meal, but require only 1–2 mg daily. Plant diets consisting 20% by weight each of a mixture of soy flour, wheat bran, potato flour and lyophilized spinach and parsley, were adjusted to provide 20 and 75 ppm of iron. All the diets were supplemented with vitamin and mineral mixtures to meet the nutritional requirements of the rat for all nutrients except iron, the experimental variable. The formulations of all the diets are presented in Table 1. All the diets contained only nonheme iron so that apparent iron absorption could be used to evaluate radioiron absorption.

Administration of radioiron

The radioiron ($^{59}FeCl_3$, 2 μCi) was (a) gavaged in deionized water 1 h after being fed a 2.0 g test meal; (b) mixed with a 2.0 g meal and fed; or (c) mixed with a 1.5 g meal, made into a slurry and gavaged.

Experimental protocol

Fifty rats were fed Diet 1 (Table 1), ad libitum, for 7 days to become acclimated. On the 8th day, rats were assigned to 10 treatments (Table 2) after balancing for hemoglobin concentration (13.6 $\pm$ 0.8 g/dl) and body weight (198 $\pm$ 7 g) and were given the respective diets (Table 2). On the 11th day, after a 16 h fast, rats were put on the test diets and dosed with ^{59}Fe as summarized in Table 2. Rats in treatment 1 were given 2.0 g of Diet 2 and, 1 h later, gavaged with ^{59}Fe (2.0 μCi) in 0.5 ml of demineralized water. Rats were fed with the remaining allotment of diet for the day, and continued to receive Diet 2 for the remainder of the experimental period. In treatment 2,

Table 2. Evaluation of method of dosing on ^{59}Fe and apparent iron absorption from plant diets

Treatment	Regular diet[a]	Test diet	Method of dosing ^{59}Fe	Iron absorbed (%)[b]	
				^{59}Fe	Apparent
1	2	2	Gavaged in water[c]	23 $\pm$ 4	22 $\pm$ 3
2	2	2	Mixed with meal[d]	22 $\pm$ 2	20 $\pm$ 4
3	2	2	Gavaged in slurry[e]	20 $\pm$ 5	20 $\pm$ 2
4	3	3	Gavaged in water[c]	19 $\pm$ 3	18 $\pm$ 2
5	3	3	Mixed with meal[d]	16 $\pm$ 1	15 $\pm$ 3
6	3	3	Gavaged in slurry[e]	16 $\pm$ 3	16 $\pm$ 2
7	1	2	Gavaged in water[c]	22 $\pm$ 5	21 $\pm$ 2
8	1	2	Gavaged in slurry[e]	19 $\pm$ 1	18 $\pm$ 2
9	1	3	Gavaged in water[c]	17 $\pm$ 2	16 $\pm$ 3
10	1	3	Gavaged in slurry[e]	15 $\pm$ 1	16 $\pm$ 2
			LSD[f]	1.2	1.1

[a] The formulations and levels of iron in the diets are shown in Table 1.

[b] Mean $\pm$ standard deviation for five observations. Percentage ^{59}Fe absorption and apparent iron absorption was not significantly different ($P \leqslant 0.05$) for any of the treatments.

[c] Two grams of diet was given after 16 h fasting and 1 h later ^{59}Fe was gavaged in 0.5 ml demineralized water.

[d] The ^{59}Fe was mixed with 2.0 g of test diet.

[e] One and a half grams of test diet, 3.0 ml demineralized water and ^{59}Fe were made into a slurry.

[f] Mean differences must equal or exceed the LSD value to be significant ($P \leqslant 0.05$).

rats were treated as described above, except that ^{59}Fe (2.0 μCi) was mixed with Diet 2 (2.0 g) and fed on the 11th day. In treatment 3, rats were treated as above except that ^{59}Fe (2.0 μCi) was made into a slurry containing 1.5 g of Diet 2 in 3.0 ml of demineralized water and gavaged on the 11th day. Treatments 4, 5 and 6 were similar to that of treatments 1, 2 and 3 except that Diet 3 was used instead of diet 2. Treatment 7 consisted of continued feeding of Diet 1 until the 10th day when the diet was removed. On the 11th day, after a 16 h fast, rats were treated as in treatment 1 with Diet 2. They were then given the remaining allotment of Diet 1 for the day, receiving the same for the remainder of the experiment. In treatment 8, rats were treated as in treatment 7 except that the meal given on the 11th day was replaced with a slurry consisting of Diet 2 (1.5 g), demineralized water (1.5 ml) and ^{59}Fe (2.0 μCi) that was gavaged. In treatments 9 and 10, rats were treated as in treatments 7 and 8, respectively, except that Diet 3 replaced Diet 2. Fresh diet was weighed daily and fed in glass feeders. Spilled or uneaten diet was air-dried and weighed to determine total food intake. Total fecal collection was made from the 11th to 18th days for total iron and ^{59}Fe analyses. On the 18th day, rats were killed, placed in canning jars and treated as described above.

Analyses

Hemoglobin was determined by the cyanmethemoglobin method [7]. Total iron in the food, diet, and feces was determined spectrophotometrically, using ferrozine color reagent with wet-ashed samples [8]. Analyzed values for National Institute of Technology (formerly National Bureau of Standards) bovine liver (NBS 1577a) and wheat flour (NBS 1567) reference samples were 191 $\pm$ 3 ppm iron (certified value, 194 $\pm$ 20 ppm iron) and 17.2 $\pm$ 0.7 ppm iron (certified value, 18.3 $\pm$ 1.0 ppm iron), respectively.

Activity of ^{59}Fe in the carcass, liver and feces samples was determined with a Hewlett Packard gamma counter. Standard curves with 0.002, 0.010, 0.020, 0.040, 0.050, 0.100, 0.200, and 0.400 μCi of ^{59}Fe per tube were prepared from the original stock solution and used with every test sample to correct for any errors in counting and nuclide decay. The quantity of ^{59}Fe dosed was equal to the sum of the radioactivity in the carcass, liver and feces. Urine was found to have negligible radioactivity in a few animals and, therefore, was not counted.

Total iron bioavailability was determined by apparent iron absorption as follows:

$$\text{Apparent iron absorption} = [(\text{Fe intake} - \text{Fe feces}) \div \text{Fe intake}] \times 100.$$

The data were analyzed by analysis of variance, analysis of covariance and correlation. Whenever F was statistically significant ($P \leqslant 0.05$), least significant difference (LSD) values were calculated to identify statistically significant differences among treatment means [9].

Results

Total iron absorption as determined by apparent iron absorption and radioiron absorption was evaluated by three methods of administering extrinsic ^{59}Fe (Table 2) using rat bioassay. Apparent iron absorption in rats fed twice their iron requirement ranged between 15–18%, while the iron absorption in rats fed 20 ppm iron ranged between 20–22%. A similar trend was observed for radioiron absorption. At the 20 ppm iron level, the radioiron absorption was 20–23% while at the 75 ppm iron level the radioiron absorption was 16–19%. The radioiron absorption and apparent iron absorption were not significantly different ($P \leqslant 0.05$) within any of the dosing methods or treatments. The correlation between these was high ($r = 0.88$ for data in Table 2). The ^{59}Fe absorption and apparent iron absorption values were similar between treatments (7 *vs* 1; 8 *vs* 3; 9 *vs* 4; and 10 *vs* 6) receiving similar test diets and dosing methodologies regardless of the regular diet. Absorption of both radioiron and total iron decreases slightly (4–6%) as iron level in the plant diets increased.

Discussion

Nonheme iron absorption from ^{59}Fe extrinsically-labelled plant foods was studied by rat bioassay mimicking the human model. In this study, we set the experimental conditions to be similar to human studies in which normal adult subjects are given iron over and above their requirements. We used normal adult rats and fed them with diet containing iron level twice their dietary requirement. Although, the general physiology of the rat does not exactly imitate the human absorption of iron, iron absorption by human beings and rats is ranked similarly [10]. This persisting similarity under a variety of conditions adds support to the use of an animal model in the preliminary assessment of iron absorption [11, 12]. In this study, the apparent iron absorption in rats fed 20 or 75 ppm dietary iron varied between 20–22% and 15–18%, respectively. Iron absorption in normal rats fed diet containing as much as twice (75 ppm) the recommended iron level was similar (15–18%) to that of human subjects (0.1–46%) receiving a variety of food sources

[13–21]. However, iron absorption in both humans and rats depends on iron status; low absorption occurring in normal subjects and animals and high absorption occurring in iron-deficient ones. This is evident from the slightly higher values obtained at the 20 ppm iron level compared with the 75 ppm iron level which would have somewhat higher iron status as the experiment progressed.

The nonheme iron absorption from plant diets varied between 20–23% at the 20 ppm iron level and 16–19% at the 75 ppm iron level and was consistent with the observations of Monsen [22] and Buchowski et al. [23]. The absorption of ^{59}Fe in rats fed 20 ppm dietary iron ranged between 20–23% and 16–19% at 75 ppm iron level which is consistent with the 15% radioiron absorption in normal rats fed plant diet containing 75 ppm dietary iron reported by Buchowski et al. [23]. The radioiron absorption values in this study are similar to those reported by Cook et al. [10] in human subjects (2.3–31.3%) on regular diet receiving extrinsically tagged rolls. We used plant diets as the nonheme iron source because these plant sources represent a variety of products differing in their mechanism of iron binding which influences the exchange of extrinsic ^{59}Fe with the native food nonheme iron.

In several studies [23–25], radioiron was administered by gavage in water after a meal; however, this methodology was not validated against iron balance an accepted reference methodology. We evaluated three methods of dosing the extrinsic ^{59}Fe by comparing apparent absorption against ^{59}Fe absorption using diets containing only nonheme iron. Absorption of ^{59}Fe and total dietary iron were not significantly different ($P \leqslant 0.05$) for any of the treatments. Absorption of ^{59}Fe from a single test meal was similar to the apparent iron absorption of the same nonheme iron source, regardless of dietary iron level. The high correlation between ^{59}Fe absorption and apparent iron absorption ($r = 0.88$ for data in Table 2) indicates that the extrinsic tagging methods used in this study are valid for estimating nonheme iron bioavailability. Nonheme iron absorption values obtained by testing the plant diet in a single meal in animals normally consuming a ferrous sulfate supplemented diet compared well with the values obtained from the rats which were regularly consuming these plant diets (Table 2, treatments 7 *vs* 1; 8 *vs* 3; 9 *vs* 4; and 10 *vs* 6). Cook et al. [21] and Björn-Rasmussen et al. [26] observed in humans that soluble forms of inorganic iron, when added extrinsically to a meal, exchange completely with other dietary nonheme iron being released into a common pool of available iron in the acidic environment of the stomach; and this is reflected in the iron absorption. We have found that the radioiron and apparent iron absorption were similar between plant and reference diets. This is important considering the complexity of the plant mixture, which contained soy flour, wheat bran, potato

flour, spinach and parsley. The extrinsic labelling, as evaluated in this study, is proposed as a method for screening bioavailability of iron in plant and vegetable foods. The high correlation obtained between radioiron absorption and apparent iron absorption from the same meal for the three methods of dosing radioiron indicates that all of them are valid for estimating absorption of nonheme food iron. However, owing to the convenience of administration, we suggest that gavaging the isotope in water 1 h after consuming a single meal is the preferred method. In this way iron bioavailability of different foods can be studied in rats receiving a standardized diet. If experimental protocols, nutritional status and iron intake relative to need as followed in human studies are employed, we conclude that the rat can be a suitable model to predict iron bioavailability for humans.

Acknowledgements

Paper 3549 of the Utah Agricultural Experiment Station. Partially supported by the National Live Stock and Meat Board and CSRS Project W-143.

References

1. Cook JD (1983) Determination of nonheme iron absorption in man. Food Technol 37: 124–126
2. Consaul JR, Lee K (1983) Extrinsic tagging in iron bioavailability research: A critical review. J Agric Food Chem 31: 684–689
3. Van Campen D (1983) Iron bioavailability techniques: An overview. Food Technol 10: 127–132
4. Cook JD, Layrisse M, Martínez-Torres C, Walker R, Monsen E, Finch CA (1972) Food Iron Absorption measured by extrinsic tag. J Clin Invest 151: 805–815
5. Timm KI (1979) Orbital venous anatomy of the rat. Lab Animal Sci 29: 636–638
6. National Research Council (1978) Nutrient Requirements of Domestic Animals Nutrient Requirements of Laboratory Animals, 3rd ed. Washington, DC: National Academy of Sciences. P 16
7. Crosby W, Munn HS, Furth FW (1954) Standardized method for hemoglobinometry. US Armed Forces Med J 5: 693–703
8. Buchowski MS, Mahoney AW, Carpenter CE, Cornforth DP (1988) Heating and the distribution of total and heme iron between meat and broth. J Food Sci 53: 43–45
9. Steel RGD, Torrie JH (1960) Principles and Procedures of Statistics. New York, NY: McGraw-Hill Book Co
10. Cook JD, Minnich V, Moore CV (1973) Absorption of fortification iron in bread. Am J Clin Nutr 26: 861–872
11. Forbes AL, Adams CE, Arnaud MJ, Chichester CO, Cook JD, Harrison BN, Hurrell RF, Kahn SG, Morris ER, Tanner JT, Whittaker P (1989) Comparison of in vitro, animal, and clinical determinations of iron bioavailability: International nutritional anemia consultative group task force report of iron bioavailability. Am J Clin Nutr 49: 225–238

12. Mahoney AW, Hendricks DG (1984) Potential of the rat as a model for predicting iron bioavailability for humans. Nutr Res 4: 913–922
13. Cook JD, Monsen ER (1976) Food iron absorption in human subjects. III. Comparison of the effect of animal proteins on nonheme iron absorption. Am J Clin Nutr 29: 859–867
14. Olszon E, Iaksson B, Norrby A, Sölvell L (1978) Food iron absorption in iron deficiency. Am J Clin Nutr 31: 106–111
15. Hallberg L, Rossander L (1982) Absorption of iron from Western-type lunch and dinner meals. Am J Clin Nutr 35: 502–509
16. Hallberg L, Rossander L (1982) Effect of soy protein on nonheme absorption in man. Am J Clin Nutr 36: 514–520
17. Lynch SR, Beard JL, Dassenko SA, Cook JD (1984) Iron absorption from legumes in humans. Am J Clin Nutr 40: 42–47
18. Morck TA, Lynch SR, Cook JD (1982) Reduction of the soy-induced inhibition of nonheme iron absorption. Am J Clin Nutr 36: 219–228
19. Gillooly M, Bothwell TH, Charlton RW, Torrance JD, Bezwoda WR, MacPhail AP, Derman DP (1984) Factors affecting the absorption of iron from cereals. Br J Nutr 51: 37–46
20. Hallberg L, Rossander L (1984) Improvement of iron nutrition in developing countries: Comparison of adding meat, soy porotein, ascorbic acid, citric acid, and ferrous sulfate on iron absorption from a simple Latin American-type of meal. Am J Clin Nutr 39: 577–583
21. Lynch SR, Dassenko SA, Morck TA, Beard JL, Cook JD (1985) Soy protein products and home iron absorption in humans. Am J Clin Nutr 41: 13–20
22. Monsen ER (1974) Validation of an extrinsic iron label in monitoring absorption of nonheme food iron in normal and iron-deficient rats. J Nutr 104: 1490–1495
23. Buchowski MS, Mahoney AW, Kalpalathika PVM (1989) Nonheme iron absorption, apparent iron absorption and hemoglobin regeneration efficiency in anemic and normal rats fed with dietary heme and nonheme iron at various levels. Nutr Res 9: 773–778
24. Thannoun AM, Mahoney AW, Buchowski MS, Hendricks DG (1988) Heme and nonheme iron absorption from meat and meat loaf by anemic and healthy rats. Nutr Rpts Intl 37: 487–497
25. Zhang D, Hendricks DG, Mahoney AW (1989) Bioavailability of total iron from meat, spinach (*Spinacea Oleracea* L.) and meat/spinach mixtures by anemic and nonanemic rats. Br J Nutr 61: 331–343
26. Björn-Rasmussen E, Hallberg L, Isaksson B, Arvidsson B (1974) Food iron absorption in man. Application of the two pool extrinsic tag method to measure haem and nonhaem iron absorption from the whole diet. J Clin Invest 53: 247–255