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Is a 40 % Absorption of Iron from a Ferrous Ascorbate Reference Dose Appropriate to Assess Iron Absorption Independent of Iron Status?

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Abstract Although a 40 % absorption of a standard reference dose corresponds to iron (Fe) absorption in borderline Fe-deficient subjects, this percentage is currently applied to all subjects independent of Fe status: (a) to assess the use of the 40 % of Fe absorption of the reference dose (FeRD%) for subjects with iron-depleted stores (IDS), normal Fe status (NIS), Fe deficiency without anemia (IDWA), and Fe deficiency anemia (IDA) and (b) to explore relationships between Fe status biomarkers and FeRD%. Six hundred forty-six participants (582 women and 64 men) were selected from multiple Fe bioavailability studies and classified into four groups based on Fe status: NIS, IDS, IDWA, and IDA. All men were classified as normal. The absorption from FeRD% was calculated in each group and correlated with Fe status biomarkers. (a) Women with IDS absorbed 40 (18.9–84.7)% of the reference dose; (b) for male subjects with NIS, the absorption of the reference dose was 19 (9.8–36.1)%, while for females, absorption was observed as to be 34 (16.7–68.6)%. In the case of subjects with IDWA, a 43 (19.7–92.5)% absorption was observed, while subjects with IDA demonstrated 67 (45.2–98.6)% absorption. Serum ferritin (SF) had the strongest inverse correlation with FeRD% ($r=-0.41$, $p<0.001$). A transferrin saturation (TS) <15 % increases the probability that the FeRD% will be highly elevated (OR, 5.05; 95 % CI, 2.73, 9.31; $p<0.001$). A 40 % absorption as reference dose is only appropriate to assess Fe absorption in subjects with IDS and IDWA. SF had an inverse correlation with FeRD%, and TS increases the probability that the FeRD% will be elevated by over fivefold.

Keywords Iron absorption · Reference dose · Ferrous ascorbate · Ferritin · Iron deficiency · Micronutrients

Introduction

The diets in developing countries typically do not contain enough iron (Fe) to meet nutritional requirements. The population subgroups especially at risk included infants, young children, and women of childbearing age [1]. Inadequate dietary intake increases the risk of Fe deficiency anemia (IDA), which affects more than one third of the world's population [2]. Fe deficiency (ID) affects these subgroups in different ways. For example, in infants and children, ID impairs cognitive development and growth and increases risk of mortality. ID in adulthood lowers physical work capacity and increases risk of mortality in childbirth [3]. Consequently, efforts to improve Fe status are a priority in the realm of public health. Interventions such as Fe supplementation [3], diversification of the diet, and Fe fortification might mitigate the high prevalence of ID. Iron fortification is considered to be the most practical long-term solution for the prevention and control of ID [4]. In order to evaluate the effectiveness of these interventions, assessment of Fe absorption is used as a proxy for bioavailability [3]. Layrisse et al. first established an estimate of 40 % Fe absorption in fasting men [5]. In 1981, Magnusson et al. confirmed the use of this percentage for the absorption of a standard reference dose of 3 mg of Fe as ferrous ascorbate at a 2:1 M ascorbic acid/Fe ratio [6]. Although this percentage corresponds to Fe absorption in

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borderline Fe-deficient subjects, it is currently applied to all subjects independent of Fe status. Thus, we attempted (a) to assess the use of the 40 % of Fe absorption of the reference dose (FeRD%) for subjects with iron-depleted stores (IDS), normal Fe status (NIS), Fe deficiency without anemia (IDWA), and Fe deficiency anemia (IDA) and (b) to explore relationships between Fe status biomarkers and FeRD%.

Subjects and Methods

Database

Six hundred forty-six participants (582 women and 64 men) were selected from 40 Fe bioavailability studies and classified into four groups based on Fe status: NIS, IDS, IDWA, and IDA. A standard dose of 3 mg of ferrous ascorbate was used in all studies. The absorption from FeRD% was calculated in each group and correlated with Fe status biomarkers. All subjects were between 18 and 56 years of age and female subjects tested negative on a study-administered pregnancy test, and used contraceptives for the duration of the study. All studies in the database were conducted at the Institute of Nutrition and Food Technology (INTA), University of Chile between 1977 and 2005. Fe absorption protocols were approved by the Ethics Committee at INTA in accordance with the Helsinki Declaration and the Nuremberg code before the execution of the studies. Moreover, radioactive Fe doses were approved by the Chilean Nuclear Energy Commission. Participation was voluntary, and all participants signed an informed consent.

Iron Absorption

All subjects received one dose of 0.05 mmol (3 mg) of elemental Fe as ferrous ascorbate (molar ratio, 2:1 ascorbic acid to Fe). The standard solutions were diluted in deionized water (MilliQ Plus, Millipore Iberica, Madrid, Spain). A solution of 1 g Fe/L was prepared for calibration of the equipment. Fe isotopes (^{55}Fe and ^{59}Fe) of high-specific activity (New England Nuclear, Boston, MA, USA) were used as tracers of Fe bioavailability. Both isotopes were Fe (III) chlorides. The isotopes were mixed with the ferrous ascorbate before administration. The reference dose solution was labeled with 111 kBq ^{55}Fe or with 37 kBq ^{59}Fe (New England Nuclear, Boston, MA, USA). Bloodstream radioactivity was calculated using a liquid scintillation counter (Beckman LS 5000 TD, Beckman Instruments, Fullerton, CA, USA and TRICarb 2000, Canberra Packard, Downers Grove, IN, USA) in accordance with the double isotope technique described by Eakins and Brown [7]. Fe absorption values were calculated under the assumption that 80 % of the radioactivity absorbed was incorporated into the hemoglobin (Hb) of circulating erythrocytes [8]. Tulane's tables were used to estimate blood volume as a function of gender, weight, and height [9]. Single absorptions were calculated based on the ratio between the total circulating radioactivity and ingested radioactivity.

Iron Status Biomarkers

Blood samples used to determine Fe status were obtained after a 12-h overnight fast. Three milliliters of blood were obtained by venipuncture from the antecubital vein through vacutainers containing ethylenediaminetetraacetic acid (EDTA). The following parameters were determined: Hb and mean corpuscular volume (MCV) (Coulter Model ZBI, Hialeah, Fla. and CELL-DYN 1700 Diagnostic, Abbott Park, IL, USA), free erythrocyte protoporphyrin (FEP) (Hematofluorimeter model 206D, AVIV, Lakewood, NJ, USA), serum Fe (SFe) and total Fe-binding capacity (TIBC) [10], and serum ferritin (SF) (Gamma Dab ^{125}I ferritin Radioimmunoassay, Travenol, Cambridge, MA, USA) by ELISA [11]. The percentage of transferrin saturation (TS) was calculated from the formula TS=SFe/TIBC×100.

Classification of Iron Status

The classification of Fe status was defined in accordance with the cutoffs established by the Centers for Disease Control and Prevention (1998) for each of the above mentioned Fe status biomarkers [12]. The lower-normal limits used are as follows: (1) Hb, 120 g/L for men and 130 g/L for women; (2) MCV, 80 fL; (3) SFe, 60 μmol/L; (4) TS, 15 %; and (5) SF, 12 μg/L. The upper-normal limits used are as follows: (1) FEP, 70 μg/dL RBC and (2) TIBC, 450 μmol/L. The biomarkers that were inferior or superior to these cutoff points were considered altered (Table 1). NIS was defined as normal Hb and the presence of no more than one altered value of the following biomarkers: MCV, FEP, SFe, TIBC, or TS (Table 1). IDS was defined as both normal Hb and SF <12 μg/L. IDWA was defined as normal Hb and two or more of any of the previously mentioned altered biomarkers (Table 1). IDA was defined as low Hb and two or more of any of the previously mentioned altered biomarkers (Table 1).

Statistical Analysis

The values were log-transformed because the distributions of FeRD% and SF were skewed. The results were then retransformed into antilogarithms to recover the original units and were expressed as geometric means with a range of ±1 SD. FeRD% values were compared using ANOVA, and post hoc Fischer's tests were performed through Statgraphics Plus 5 (Statistical Graphics Corp, Rockville, MA, USA). Pearson correlation coefficients and univariate logistic regression

Table 1 Iron status biomarkers

Parameters	*n*	Mean±SD	Cutoff value[a]	%[b]
Hb (g/L)	646	149±15	< 130 (m) or<120 (f)	6.8
MCV (fL)	341	87.6±6.0	<80	7.6
FEP (μg/dL RBC)	341	70.7±40.3	>70	37.5
SFe (μmol/L)	563	85.9±32.4	<60	20.2
TIBC (μmol/L)	563	334.7±64.5	>450	6.0
TS (%)	646	27.5±11.5	<15	13.8
SF (μg/L)[c]	646	26 (10–69)	<12	16.9

Hb Hemoglobin, *MCV* median corpuscular volume, *FEP* free erythrocyte protoporphyrin, *SFe* serum iron, *TIBC* total iron-binding capacity, *TS* transferrin saturation, *SF* serum ferritin, *m* males, *f* females

[a] Cutoff points to define iron status were based on CDC values (1998) [24]

[b] Percent of subjects below or upper cutoff value

[c] Values are expressed as geometric means and ranges±1 SD

models were conducted using the Infostat® statistical package (Grupo InfoStat, 2002). The statistical significance level was set at $p < 0.05$.

Results

Fe status biomarkers and the prevalence of altered values are shown in Table 1. A 6.8 % of subjects were anemic (<130 and <120 g/L for men and women, respectively). Prevalence of altered biomarkers values ranged from 6 to 37.5 %. The biomarkers with the lowest and highest prevalence of abnormality were TIBC and FEP, respectively. Of the entire population, 74 % presented with NIS and all men were classified as normal. The prevalence of IDS, IDWA, and IDA were 5, 15, and 6 %, respectively. Table 2 shows iron status biomarkers according to Fe status classification.

Figure 1 demonstrated that subjects with IDS absorbed 40 % of the reference dose. The figure included the geometric mean and range±1 SD (18.9–84.7). It is important to highlight that this group was comprised solely by women. For male subjects with NIS, the absorption of the reference dose was 19 (9.8–36.1)% while for females, absorption was observed as to be 34 (16.7–68.6)%. In the case of subjects with IDWA, a 43 (19.7–92.5)% absorption was observed, while subjects with IDA demonstrated 67 (45.2–98.6)% absorption (Fig. 1). This figure also expressed that the FeRD% for each Fe status classification was significantly different, with the exception of IDS as compared with IDWA.

There were significant inverse correlations between FeRD% and Hb (−0.33), MCV (−0.35), SFe (−0.29), TS (−0.37), SF (−0.41), and significant positive correlations between FeRD% and both FEP (0.29) and TIBC (0.29). SF had the strongest inverse correlation with FeRD% ($r = -0.41$, $p < 0.001$). Univariate logistic regression models indicated significantly positive associations between the dependent variable, FeRD%, and each Fe status biomarker. The presence of a TS <15 % increases the probability that the FeRD% will be elevated by over fivefold (OR, 5.05; 95 % CI, 2.73, 9.31; $p < 0.001$) as compared with a normal TS (>15 %). Similarly, the presence of an altered Hb, TIBC, or SF biomarker increases the probability that the FeRD% will be elevated by over fourfold (Table 3).

Discussion

The present article confirmed that a 40 % Fe absorption of the reference dose in subjects with IDS and IDWA is the most appropriate estimate to use as a reference in women of childbearing age. Moreover, this study provides the FeRD% for subjects with NIS and IDA. The FeRD% for NIS men is 19 %, NIS women is 34 %, and IDA women is 67 %.

For several decades, Fe absorption studies in vivo have been considered the most appropriate method of determining Fe bioavailability from foods [13–15]. In vivo studies are advantageous in that radioisotopes can be incorporated into Hb and used as direct tracers of Fe bioavailability. Layrisse et al. developed a reference dose based on the observation that Fe absorption from varied plant and animal sourced foods highly related with the absorption of a single dose of inorganic Fe as ferrous sulfate [5]. In 1981, Magnusson et al. confirmed the use of a 40 % absorption of a standard reference dose of 3 mg of Fe as ferrous ascorbate as a reference [6]. However, this percentage was calculated from data on borderline Fe-deficient subjects and therefore may not be applicable for people with normal or more severely deficient Fe status. The present article validated the use of this reference in subjects with IDS (FeRD%=40 %). Moreover, this percentage did not differ significantly from FeRD% in IDWA ($p < 0.05$). Therefore, we hypothesized that this absorption factor can be appropriately applied to both groups. On the other hand, we reported that for subjects classified with NIS and IDA, the absorption of the dose varied significantly. Subjects with NIS demonstrated decreased absorption of the reference dose and significant gender differences, with 19 % FeRD% for men and 34 % FeRD% for women. Women demonstrated higher absorption of Fe than men. This is consistent with our findings that women are more prone to developing ID and that poor Fe status is associated with higher absorption [16]. The absorption of the dose was highest (67 %) in subjects with IDA, as compared with all the other classification groups. The information provided by the present article regarding the FeRD% for subjects with NIS and IDA can be applied to estimate the absorption of the reference dose of Fe in future studies.

Table 2 Iron status biomarkers according to iron status classification

Groups					
Biomarkers	NIS		IDS	IDWA	IDA
	Male (n=64)	Female (n=415)	(n=34)	(n=94)	(n=39)
Hb (g/L)	159±9	141±10	136±7	135±10	104±13
MCV (fL)	–	88±4	89±3	85±5	74±11
FEP (μg/dL RBC)	–	71.2±20.8	49.5±13.1	87.8±23.6	170.3±73.2
SFe (μmol/L)	111.0±36.4	90.6±26.0	84.3±18.1	58.6±28.0	35.3±16.3
TIBC (μmol/L)	315±38	321±50	339±49	381±95	396±76
TS (%)	35.3±11.1	29.3±9.5	25.5±7.0	15.8±7.3	8.8±5.4
[a]SF (μg/L)	85 (46–158)	32 (17–60)	6 (4–11)	14 (6–35)	7 (3–16)

Values are expressed as mean±SD for Hb, MCV, FEP, SFe, TIBC, and TS

NIS Normal iron status, *IDS* iron-depleted stores, *IDWA* iron deficiency without anemia, and *IDA* iron deficiency anemia, *Hb* hemoglobin, *MCV* median corpuscular volume, *FEP* free erythrocyte protoporphyrin, *SFe* serum iron, *TIBC* total iron-binding capacity, *TS* transferrin saturation

[a] Values are expressed as geometric mean and range±1 SD for serum ferritin (SF)

Currently, the mean Fe absorption from foods is corrected using 40 % FeRD% in accordance with the following formula (Eq. 1) [17]:

$$\text{Adjusted to } 40\,\% \text{ absorption of the reference dose} = \frac{(\%Femeal \times 40)}{\%FeRD} \quad (1)$$

where:

$\%Fe_{meal}$ percentage of Fe absorption of the tested meal

40 percentage of Fe absorption of the reference dose estimated in borderline Fe-deficient populations

FeRD% Fe absorption of reference dose

This formula needs to be corrected for the difference in absorption of subjects with NIS and IDA. Additionally, we applied the corrected FeRD% reported in the present article to three Fe absorption studies that included subjects with varied classifications of Fe status: (a) the first study published by Olivares et al. [18] compared the absorption of iron bis-glycine chelate in water and milk with the reference dose of ferrous ascorbate in a population with low prevalence of IDA, (b) the second study was performed by Murray-Kolb et al. [19], who reevaluated soybean iron bioavailability in a small sample of women (n=18), which presented with marginal ID, and (c) the third study by Disler et al. was conducted in an anemic population to assess the effect of tea on the absorption of Fe from different foods and Fe solutions [20]. After applying the correction values reported above (34 and 67 % in NIS and IDA, respectively), we found minimal differences in the percentage of Fe absorption in the studies performed by Olivares et al. and Murray-Kolb et al. In the study performed

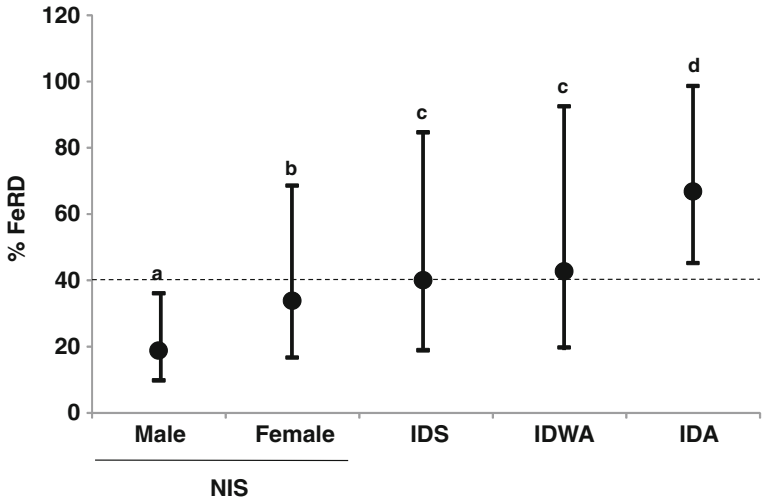

Fig. 1 Percentage of Fe reference dose absorption (FeRD%) by iron status expressed as geometric mean and range±1 SD. Differing *letters* indicated significant difference (p<0.05). *NIS* normal iron stores, *IDS* iron-depleted stores, *IDWA* iron deficiency without anemia, and *IDA* iron deficiency anemia

Table 3 Univariate logistic regression models

Biomarkers	OR	95 % CI		*p* value
Hb	4.50	1.56	13.02	0.0055
SFe	2.79	1.77	4.39	<0.0001
TIBC	4.50	1.82	11.17	0.0012
TS	5.05	2.73	9.31	<0.0001
SF	4.39	2.63	7.32	<0.0001

Hb Hemoglobin, *SFe* serum iron, *TIBC* total iron-binding capacity, *TS* transferrin saturation, *SF* serum ferritin

by Disler et al., however, the Fe absorption mean increased after using these new correction factors. This suggests that the most appropriate percentage to correct Fe absorption in anemic individuals would be 67 % FeRD%, as compared to 40 %.

Fe status biomarkers have also been used to estimate Fe absorption. Several attempts have been made to develop algorithms to estimate the bioavailability of Fe using Fe status biomarkers [21–23]. In our study, SF had the strongest inverse correlation with FeRD%. Magnusson et al. [6] reported that 40 % of the absorption of the reference dose corresponds to 30 μg/L SF, same value described by Reddy et al. [24]. On the other hand, Cook et al. reported 40 μg/L SF to assess dietary absorption of Fe [14], while Hallberg and Hulthén described an SF value of 23 μg/L [25]. In the present study, the corresponding SF value for a 40 % of Fe absorption was 19 μg/L. However, it is important to mention that the participants of the present study came from an environment where the prevalence of infection and inflammation are low.

On the other hand, this study also shown that a TS <15 % increases in more than five times the probability of reducing Fe absorption of the reference dose, followed by Hb and TIBC which represents 4.5 times higher risk of reduced Fe absorption.

Conclusions

A 40 % absorption as reference dose is only appropriate to assess Fe absorption in subjects with IDS and IDWA.

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