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Equivalent Effects on Fecal Reactive Oxygen Species Generation with Oral Supplementation of Three Iron Compounds: Ferrous Sulfate, Sodium Iron EDTA and Iron Polymaltose

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Key Words
Fecal iron · Ferrous sulfate · Free radical damage · Iron polymaltose · Na Fe-EDTA · Oral iron supplementation

Abstract
Background: In any context of iron supplementation in the prenatal prophylaxis or therapeutic dosage range, a large amount will remain unabsorbed and pass through the intestinal tract into the colonic digesta possibly causing increased oxidation. ***Aim:*** To compare the generation of fecal reactive oxygen species (ROS) in situ after daily consumption of 100 mg of elemental iron in three frequently used forms of iron supplements. ***Methods:*** Ten healthy, iron-repleted adult males were investigated before and during supplementation with three oral iron compounds: 100 mg of oral iron were given as ferrous sulfate, Na Fe-EDTA and iron polymaltose for 6 days to each subject in an individually stratified sequence. Stool samples were collected and analyzed for iron content and the in situ generation of fecal ROS. ***Results:*** Significant increases in fecal ROS generation were observed during oral iron supplementation. No statistical differences were seen in either residual concentrations of non-heme iron in stool or the level of fecal ROS generation between the three Fe compounds. There was, however, a significant association between the iron concentration in the stool and ROS generation. ***Conclusion:*** In spite of the differences in their chemical characteristics, none of the three distinct iron complexes reduced oxidative stress in the intestinal lumen.
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Introduction

The criteria for the administration of a drug are that it be safe and effective. When it comes to the therapeutic role of nutritional supplements, scientific evaluation of their benefit, i.e. their efficacy in improving the nutritional status, is often overemphasized in comparison to related safety issues. Iron supplements are a case in point. High hepatic iron stores increase hepcidin expression which, in turn, down-regulates intestinal iron absorption [1, 2]. This mechanism helps to prevent excess iron accumulation in the body and the ensuing toxic consequences [3]. However, when highly bioavailable iron supplements are ingested in excessive dosages, this protection is overwhelmed [4]. Moreover, the state of iron repletion is more frequently encountered in elderly people consuming diets with high iron bioavailability or iron

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supplementation [5], and iron-repleted infants showed impaired growth [6–9] and more severe courses of malaria after oral iron supplementation at recommended daily allowance levels [10]. Therefore, down-regulation of intestinal iron absorption does apparently not protect against systemic iron overload in all cases, since highly bioavailable oral iron preparations, for example, can abrogate this effect.

Independent of the discussion to what extent homeostatic regulation of iron absorption can or cannot reduce detrimental effects of iron in tissues and in the circulation [11], oral iron exerts a detrimental effect on the small intestines from the luminal side in a dose-dependent manner [12]. The upper tolerable level of iron, which was established by the Food and Nutrition Board of the US Institute of Medicine [13], is based on the gastric and small intestinal irritation produced by daily doses of iron, ranging from 45 to 65 mg Fe depending on age.

Adverse effects of luminal iron on the lower intestinal tract have been reported repeatedly [14–16]. Only a minor fraction of an iron compound is absorbed with the residual metal passing through the gastrointestinal lumen to be excreted in the feces. Epidemiological and clinical observations have shown that toxic oral iron doses damage the gastrointestinal tract, resulting in bleeding and subsequent scarring of the lumen that may require surgical intervention [4, 17]. Due to its oxidative capacity, iron can also induce pathological alterations in the lower gastrointestinal tract at lower dose levels [15, 16]. In laboratory animals and humans, it has been demonstrated that oral iron supplementation induces free radical formation in the intestinal lumen. As a consequence of this process documented by Lund et al. [16], the exposure of the intestinal tract to continuous supplementation with oral iron depletes the capacity of the fecal material to resist oxidative reactions, as shown earlier by us [18]. Such iron-mediated oxidative stress aggravates the course of inflammatory bowel diseases [19, 20]. Moreover, it was suspected to participate in the pathogenesis of colon cancer [21, 22], though this is not unequivocal [23].

The fact that oral iron supplements are available in various chemical forms, some of which may persist during their passage through the gut, led us to investigate if different iron compounds might produce more or less oxidative stress in the fecal milieu. We selected three commercially available candidate iron compounds of proven bioavailability and distinct complex chemistry: ferrous sulfate ($FeSO_4$), iron polymaltose (IPM) and sodium sodium iron EDTA (Na Fe-EDTA). All three compounds were administered for 6 days in sequential order and at the same dose level to the same healthy individuals with a washout period of 10 days in between. Fecal iron content and antioxidant capacity were determined during the last 3 days of each iron supplementation period and during the iron-free interval [14, 18] in order to determine intra-individual differences in the oxidative effect of the three iron compounds in the gut lumen.

Subjects and Methods

Subjects

This study comprised 10 apparently healthy males, who were recruited among the students and employees of the Universidad del Valle de Guatemala. Interested participants were informed about the objectives and procedures of the study during a preliminary meeting and were given a short, pre-screening questionnaire assessing health status and smoking habits. Exclusion criteria were smoking, anemia or a hematological disorder, a history of gastrointestinal disorders or intolerance to iron supplements, recent consumption of nutritional supplements including iron, or inflammatory or infectious conditions interfering with the study.

The CeSSIAM (Center for the Studies of Sensory Impairment, Aging and Metabolism, Guatemala) Human Subjects Committee granted ethical approval of the study protocol. Subjects signed informed consent forms assuring that they understood the nature, purposes, inconvenience, risks and benefits of the study. Subjects were compensated for their participation.

Screening Measurements

An initial blood sample to detect possible anemia was obtained from eligible subjects. Those who were not anemic were able to continue their participation in the trial. Serum ferritin concentrations were analyzed in blood drawn at the end of the study, using an automated immunoassay performed by the Abbott AxSYM™ analyzer (Abbott Laboratories) at the clinical laboratory of Sanatorio Nuestra Señora El Pilar (Guatemala City, Guatemala). Any subject with a ferritin level $\leq$40 ng/ml was not considered sufficiently iron repleted to be included in the final analysis. Baseline and final C-reactive protein (CRP) concentrations were analyzed at the same laboratory in Guatemala City, using a quantitative immunoturbidimetric method (Turbiquant). A CRP cutoff point of 5 mg/l was used as a criterion for a normal state of systemic inflammation.

Oral Iron Supplementation Regimen and Fecal Collection

The study tested the effects of three iron supplements – $FeSO_4$, Na Fe-EDTA and IPM – on the fecal generation of reactive oxygen species (ROS). An initial baseline period of 3 days, during which no iron treatment was administered, was followed by three 6-day cycles of supplementation in which all of the subjects ingested the three 100-mg iron supplements in an individually stratified order to assure a balanced sequence of presentation. Each supplementation cycle was separated by a 10-day washout period. Fecal samples were collected consecutively over the last 3 days of the baseline, active iron supplementation, and washout periods.

Table 1. Characteristics of the subjects

Variable	Baseline	Final	p value
Age, years	30 ± 13 (18.0–56.0)	–	NA
Weight, kg	73.4 ± 11.1 (54.5–85.4)	–	NA
Height, m	1.7 ± 0.1 (1.6–1.8)	–	NA
Body mass index	25.2 ± 4.6 (18.6–30.6)	–	NA
Hemoglobin, g/dl	16.0 ± 0.7 (14.7–17.0)	16.2 ± 0.8 (15.0–17.7)	0.288
Hematocrit, %	48.3 ± 2.5 (43.4–52.0)	48.6 ± 2.9 (45.0–53.9)	0.465
Ferritin, μg/l	–	105.4 ± 42.9 (44.3–169.0)	NA
CRP <5 mg/l	10 of 10 subjects	8 of 10 subjects	NA

Two-tailed t test for paired samples. NA = Not applicable.

Treatments were prepared 1 day prior to administration. Individual aliquots of $FeSO_4$ were prepared by mixing 4 ml of a commercially available $FeSO_4$ syrup (Fer-In-Sol®; Mead Johnson Nutricionales, Bristol-Myers Squibb, Quito, Ecuador; 125 mg/ml), containing 25 mg Fe/ml (i.e. a total of 100 mg Fe) with 25 ml of water. Individual solutions of Na Fe-EDTA were prepared by dissolving 0.77 g of powdered Ferrazone® (Akzo-Nobel, Amersfoort, The Netherlands), containing 100 mg of elemental iron in 25 ml of drinking water. The tablets of IPM (Maltofer®; Vifor, Glattbrugg, Switzerland) were administered to the subjects without any further preparation concomitant with 25 ml of drinking water. The participants ingested the supplement during the fasting state. Approximately 60 min after ingesting the supplements, the subjects were given a breakfast meal of identical composition that terminated their overnight fast.

Quantification of the Total Iron Concentration in Stool

The Feren-B-Method kit (Bioanalytic, Umkirch, Germany) was used to quantify non-heme iron in the fecal samples. This allowed examining the relationship between free radical production in feces and the iron content in the intestinal lumen. Spectrophotometric readings were made using a spectrophotometer (Thermo Scientific Genesys 10uv; Thermo Fisher Scientific, Waltham, Mass., USA). Non-heme iron was expressed in micrograms per gram of native stool.

Assessment of in situ ROS Generation with HPLC

The buffering capacity of fecal material to quench free radical generation, an indirect measure of in situ luminal oxidation, was assessed with an HPLC-based method adapted from Owen et al. [14] used previously to evaluate the effects of supplemental iron and antioxidants on ROS production in human stool [18]. The method is based on the generation and detection of two hydroxylated products (2,5-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid) resulting from the hydroxyl radical attack on salicylic acid, which serves as a measure of ROS production.

Data Handling and Statistical Analysis

Data were entered into an electronic spreadsheet (Excel, 2003; Microsoft, Redwood, Wash., USA) and analyzed with statistical software (SPSS 12.0.1 for Windows; SPSS, Chicago, Ill., USA). Values for the iron concentration in the stool and fecal ROS generation were treated as repeated measures and analyzed with a repeated measures linear model (MANOVA), with the least statistical difference test for assessment of intertreatment differences. Spearman's rank-order correlation coefficient (non-parametric measure of association, which does not assume normality of distribution) was used to measure the strength of the correspondence between fecal non-heme iron and ROS production. A probability of $p < 0.05$ was accepted as the level for statistical significance.

Results

Characteristics of the Subjects

The baseline characteristics, age, body composition and hematological status, are provided in table 1. The male subjects ranged in age from 18 to 56 years, with a mean age of 30 ± 13 years. None of the 10 males was classified as underweight according to their body mass index, but 5 were classified as overweight. Using hemoglobin concentration >13.7 g/l as a cutoff criterion (adjusted for the 1,500-m altitude of Guatemala City [24]), none of the subjects were anemic. The subjects' serum ferritin concentrations at the end of the study ranged from 44.3 to 169.0 μg/l (all of them within the normal range). All 10 subjects had CRP values below the threshold criterion of 5 mg/dl before the study. After three rounds of iron supplementation, 8 subjects' CRP values remained <5 mg/l, whereas the remaining 2 had slightly increased levels at 5.36 and 6.83 mg/dl, which, however, cannot be regarded as indication for severe inflammation.

Fecal Iron Concentration

The overall concentration for fecal non-heme iron in the sets of three stool specimens collected prior to iron

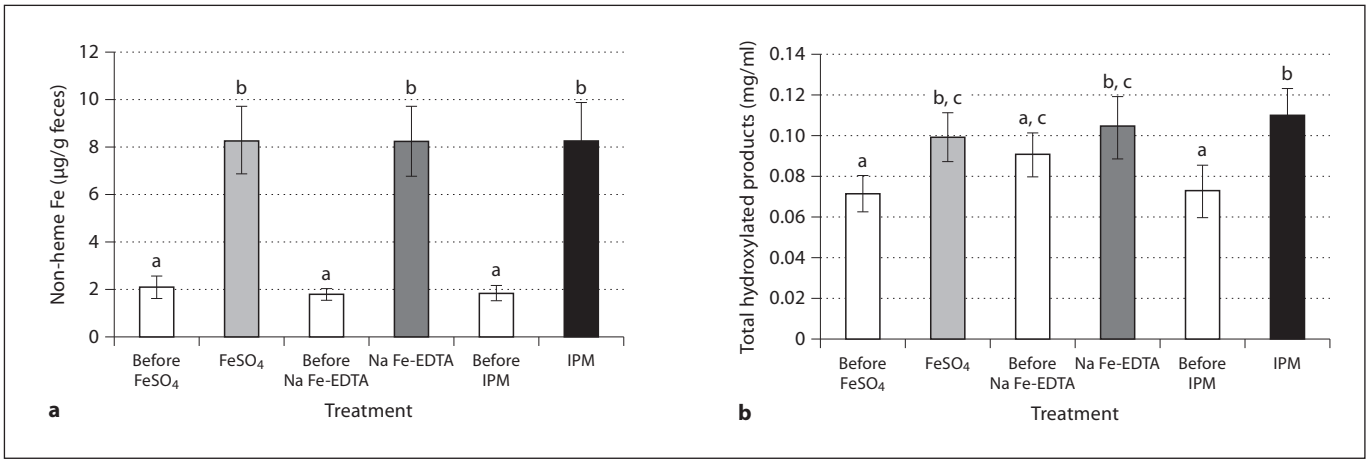

Fig. 1. a Fecal concentration of non-heme iron before supplementation of the respective oral iron (open bars) and during oral supplementation with each iron compound (shaded bars). **b** Fecal in situ ROS responses before supplementation of the respective oral iron (open bars) and during oral supplementation with each iron compound (shaded bars). Bars not sharing common superscript letters were significantly different ($p < 0.05$) by MANOVA.

supplementation was 1.7 ± 1.3 µg/g feces. As shown in figure 1a, an identical concentration of residual fecal non-heme iron was found with each of the three iron compounds supplemented at the same daily dose of 100 mg Fe each. These values were significantly greater than non-supplemented specimens (MANOVA: $p < 0.001$).

Fecal ROS and in situ Antioxidant Capacity of Stool

The ROS responses in fecal samples are shown in figure 1b. There were no differences among the ROS responses after intake of the three iron compounds. ROS generated in stools in the absence of iron supplementation were higher in the Na Fe-EDTA part of the trial and, thus, ROS production during the supplementation period with Na Fe-EDTA showed no significant differences compared to the control period. ROS responses during supplementation with $FeSO_4$ and IPM were significantly greater than those during the corresponding period before iron intake (MANOVA, $p < 0.003$).

Association of Residual Fecal Iron and ROS Responses

Figure 2 illustrates the correlation between fecal iron concentrations (x-axis) and corresponding ROS responses (y-axis) in fecal samples in a scattergram. Both the Pearson product-moment coefficient correlation ($r = 0.35$, $p = 2.0 \times 10^{-6}$) and the Spearman rank-order coefficient correlation ($r = 0.32$, $p = 1.3 \times 10^{-5}$) for the association were statistically significant.

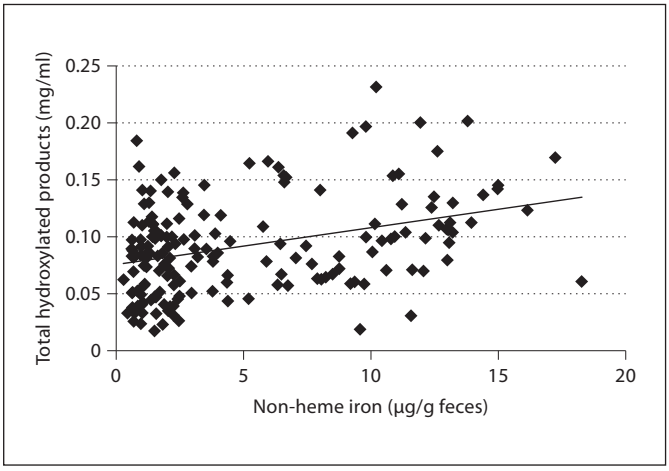

Fig. 2. Association of non-heme iron concentration (x-axis) with the in situ ROS response in stools (y-axis), with the regression line, for 172 paired observations. Pearson's correlation coefficient, r = 0.35; Spearman correlation coefficient, r = 0.32.

Discussion

Iron is an essential micronutrient, which is indispensable for oxygen transport and capture, and instrumental in metabolic oxidation-reduction reactions [25]. Besides its essential functions, the role of iron as an oxidant carries intrinsic and unavoidable risks of oxidative damage

[26]. Iron as a supplement is a double-edged sword, and sufficient investigative attention must be devoted to the side that can induce adverse metabolic pathways. This study focused on the oxidative capacity of residual, unabsorbed iron that passes through the lower intestinal lumen after oral iron supplementation in the upper range of recommended supplementation levels [27].

Iron-induced oxidative stress in the intestinal lumen showed far-reaching metabolic consequences in a murine model of Crohn's disease [28, 29]. It induces endoplasmic reticulum stress, i.e. impaired protein folding in the intestinal mucosa, reduced expression of proteins engaged in cellular energy generation and altered composition of the intestinal flora. These negative health consequences on the intestinal mucosa were avoided with iron-deficient feeding balanced by corresponding parenteral iron supplementation [29]. Therefore, the research hypothesis tested here was that oral iron compounds which lead to slower and less marked increments in post-absorptive plasma iron concentrations may do so by retarding iron release due to their complex structures in the gut lumen. If so, they might cause less oxidative damage at this location as well.

Consistent with the significant association between fecal non-heme iron content and the extent of fecal free radical formation demonstrated by Lund et al. [16], the amount of residual iron in the feces was a correlate of free radical formation. The Spearman correlation coefficient for the rank-order association of iron concentration with ROS responses in the 172 fecal samples tested here had a value of $r = 0.32$ ($p < 0.00001$), which supports our previous results for 281 comparable samples evaluated in our earlier study ($r = 0.26$; $p < 0.00001$) [18]. This finding suggests that the different formats of the iron ingested lost their differences during their passage through the gastrointestinal tract. Iron offered in three different chemical forms seems equally available to catalyze oxidative stress in the feces and to react with the chromogens used for chemical non-heme iron determination.

All three compounds compared here have proven efficacious to alleviate anemia in earlier reports [30, 31]. Due to its low cost and high bioavailability, $FeSO_4$ is regarded as the reference compound for oral iron supplementation [32]. Similar evidence was established for Na Fe-EDTA in supplemental dosing [30], and for IPM [31]. Each of the three chosen iron compounds, therefore, can be expected to show adequate efficacy in the treatment of anemia or prenatal prophylaxis of iron deficiency. Consistent with our prior experience [18], a comparable daily dose of iron as $FeSO_4$ (100 vs. 120 mg) also produced a significant elevation in fecal ROS generation in situ compared to stool collected in the absence of supplementation. Iron was also given as $FeSO_4$ in the patients investigated by Lund et al. [16]. However, there is a dearth of literature providing a comparative focus on the oxidizing potential after intake of distinct chemical forms of iron.

The present study shows that IPM produced a significant increase in the oxidative potential of the fecal milieu at an equivalent magnitude to that observed with $FeSO_4$ and Na Fe-EDTA. Thus, no differences in the iron-induced oxidative potential were found between the three compounds under investigation. The lack of significance between the periods before and during supplementation with Na Fe-EDTA was due to a higher generation of total hydroxylated products during the corresponding period before supplementation, for which we have no ready explanation. The order of administration of the three compounds was randomly assigned and differed between the 10 individuals; all samples were analyzed at random in order to avoid systemic mistakes influencing the results. The standard deviation is at the same order of magnitude for all three compounds so that outlying results are not a reasonable explanation either. Moreover, the reproducibility of the method is high, as demonstrated by the comparability of corresponding results, and the iron content in the stool was the same. However, in any case, the lack of differences in total hydroxylation products after intake of the three supplements shows that this effect is not due to less oxidative performance of Na Fe-EDTA.

A number of limitations in the design and execution of the study have to be mentioned. The study included a limited number ($n = 10$) of subjects, but our previous experience with 12 subjects in an almost identical format [18] provided easy resolution of effects on ROS generation, using the same repeated measures (MANOVA) analysis as applied here, which corrects for individual variation. The daily 100-mg dose was purposely chosen at the high end of the range of the 60–120 mg commonly used in prenatal supplements in developing countries [27]. Although iron-deficient or pregnant women are the usual beneficiaries of iron supplement prescription, we used iron-repleted males. This was done in order to reduce intersubject variation in iron absorption, which is higher in women due to their individual propensity to become iron deficient. The underlying question revolves around the residual iron quantities that are *not* absorbed. In terms of the actual health consequences, differences in iron absorption could have influenced the results. The results of this study suggest further studies in a prospec-

tive design aiming to reduce iron-induced fecal oxidation are warranted, e.g. by simultaneous intake of antioxidant nutrients.

Conclusion

Daily consumption of 100 mg of elemental iron offered as $FeSO_4$, IPM or Na Fe-EDTA produced an identical content of iron in the stool and identical degrees of oxidative stress, as indicated by in situ generation of ROS. There may be other oral iron supplements with a chemical structure that remains intact and reduces the oxidative effects of non-absorbed iron while successfully alleviating iron deficiency anemia. However, none of the three candidate compounds tested here showed beneficial characteristics in this regard.

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Disclosure Statement

M.N.O., C.A., N.W.S. and K.S. have no potential conflicts of interest.

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