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Abstract

Prophylactic doses of 120 mg of iron (Fe) are commonly used to prevent Fe-deficiency anemia in vulnerable populations, especially in developing countries. Evidence shows that residual Fe in the large bowel may alter the normal antioxidant capacity of the fecal stream. Our objective was to evaluate the effect of dietary antioxidants from the Carotino Tocotrienol-Carotene Mixed Concentrate (CTCMC) on the depletion of fecal antioxidant capacity by oral Fe supplementation. In total, 17 healthy male adults participated in the 2 phases of the study, 5 in the pilot study and 12 in the definitive intervention trial. Participants received different treatments, separated by washout periods. These included: 120 mg Fe; 120 mg Fe and refined palm oil (FeOil); and 120 mg Fe in refined palm oil combined with 1 of 2 dosages (0.4 g and 0.8 g) of CTCMC/5 mL of refined palm oil (CTCB and CTCA treatments, respectively). Fecal samples were collected and analyzed to quantify the products of hydroxyl radical attack on salicylic acid (2,5 dihydroxybenzoic acid, 2,3-dihydrobenzoic acid, and catechol) at baseline and after active supplementation. Fe supplementation in either form (Fe or FeOil treatments) increased the concentrations of hydroxylated compounds in fecal samples. The production of hydroxylated compounds was significantly lower in treatments CTCB and CTCA than in the FeOil reference. Baseline antioxidant capacity state was virtually restored with dietary carotenoids and tocotrienols from the CTCMC. In conclusion, dietary antioxidants can reverse the depletion of fecal antioxidant capacity induced by oral Fe supplements. J. Nutr. 140: 1105–1110, 2010.

Introduction

Free radicals are atoms, ions, or molecules with reactive, unpaired electrons (1). We have an emerging, but still incomplete, understanding of free radical function in biological systems and their role in health. Free radicals have been implicated in the origins of aging (2) and degenerative diseases (3,4). In biological systems, iron (Fe) plays a role in cellular and tissue oxidation via Fenton chemistry (4,5). We have an emerging, but still incomplete, understanding of free radical function in biological systems and their role in health. Free radicals have been implicated in the origins of aging (2) and degenerative diseases (3,4). In biological systems, iron (Fe) plays a role in cellular and tissue oxidation via Fenton chemistry (4,5).

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Fe deficiency is an endemic, worldwide public health problem (9), provoking the need for prophylactic or therapeutic administration of Fe. Although the official recommendation for daily prophylactic Fe of the United Nations agencies and the International Nutritional Anemia Consultative Group (10) is 60 mg, doses of up to 240 mg are used in populations where the prevalence of Fe deficiency anemia is high (11). Lund et al. in Norwich, UK conducted a series of studies in rodents (12,13) and humans (14) exploring oxidation in the intestinal lumen and its consequences. In the latter study (14), they showed that daily supplementation with 19 mg of ferrous Fe over a 2-wk period increased the in vitro production of free radicals by 40% in sporadic stool samples collected during the intervention. Owen et al. (15) developed a fast and convenient HPLC-based method that can be used to detect fecal oxidation, because it precisely quantifies the appearance of oxidized metabolites derived from free-radical attack on salicylic acid in stool samples.

Diseases, including Crohn’s disease (6) and ulcerative colitis (7). Organic Fe from the heme moiety of hemoglobin has been related to colonic carcinogenesis (8).

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of Owen et al. (15) in a metabolic study performed in healthy, nonanemic Guatemalan men. We further refined the principles of Owen et al. (15) into a practical, quantitative assay for the generation of reactive oxygen species (ROS) in fecal material as an index of in situ antioxidant capacity of stools. This was applied to the daily administration of oral Fe over 7-d periods to determine whether Fe affects the antioxidant buffer capacity of the fecal matrix. To these design elements, we added oral supplementation with 2 dosages of dietary antioxidants derived from the palm fruit (Elaeis guineensis). The following report focuses on the development of these novel methods as well as the results from the pilot study and definitive intervention trial.

Materials and Methods

Study design
This was a longitudinal, metabolic study with repeated measures for which each participant served as his own control; no separate control group was included. The experimental design consisted of 2 phases: a pilot study and a definitive intervention trial.

The study included a convenience sample of 17 healthy male adults recruited at the local national university in Guatemala City, Guatemala. Five men participated in the first phase (pilot study) and 12 in the second phase (intervention trial). A preliminary meeting was held to inform interested participants about the objectives and procedures of the study. Potential participants were given a short, prescreening questionnaire about health status and smoking habits. Individuals who smoked; presented anemia or a hematological disorder; had a history of gastrointestinal disorders or an intolerance to Fe supplements; consumed other nutritional supplements, including Fe supplements; or had inflammatory or infectious conditions during the study were excluded from the study. Inflammatory or infectious conditions were assessed systematically by direct oral reports from the participants.

The Bannatyne Campus Research Ethics Board at the University of Manitoba and Center for the Studies of Sensory Impairment, Aging, and Metabolism’s Human Subjects Committee in Guatemala City granted ethical approval of the study protocol. Eligible participants who signed the form for informed consent were asked to give a blood sample for initial routine hematological screening to detect the presence of anemia. Those who were not anemic were able to continue participating in the study.

Dietary intake diaries
Because foods rich in Fe, fiber, dietary antioxidants, or any of the aforementioned in the diet could be confounders of the responses in the study, we asked participants to complete a food diary to monitor their eating habits, recording their food consumption in common household measures. Men in both phases were required to record their total consumption of food and beverages, including drinking water, during 2 separate 7-d periods (including weekends). In total, we collected 224 d [of a maximum of 238 d (14 d/participant × 17 participants)] of dietary data.

Reported food portions were converted from household measures to grams. The total Fe, fiber, carotenoid, vitamin E, and vitamin C contents of each food were estimated using several food composition references, including the USDA online food composition database (16), the Food Composition Table for the Central American area (17), and nutritional labels on food products.

Oral supplementation regimens and sample collection
The pilot study, involving 5 healthy male volunteers, allowed for the testing and implementation of the laboratory methods used in the definitive intervention trial. It also aided in determining whether the proposed supplementation schedules and doses were adequate to produce detectable responses in the free-radical biomarker of the fecal matrix. The strong flavor and thick consistency of the lipid-based antioxidant mixture made it difficult to consume without further dissolution in an appropriate solvent, for which we chose refined palm oil. Therefore, it was necessary to assess whether the addition of this component to the treatment mixture would have any effect on the studied fecal biomarker.

Two treatments were used within the pilot study: 1) the Fe treatment, which consisted of 120 mg of elemental Fe in the form of commercial FeSO₄ syrup; and 2) the FeOil treatment, which included 120 mg of Fe as FeSO₄ from the syrup plus 5 mL refined palm oil. After 3 d of baseline stool collections, treatments were administered during 2 7-d cycles of supplementation, separated by a 14-d washout period (see Fig. 1A for summarized supplementation scheme). The last supplementation period was followed by a final 12-d washout period. Participants were asked to provide a fecal sample in the morning during baseline and supplementation and during each washout period. During 7-d active supplementation, samples were collected on the last 3 d, whereas during the washout phases, samples were collected on nonconsecutive days. All men produced and provided all the fecal samples requested.

Mixtures containing all the elements of the treatments were prepared 1 d prior to administration. A commercial ferrous sulfate preparation, 125 g/L (Fer-In-Sol, Mead Johnson Nutricionales, Bristol-Myers Squibb) containing 25 mg elemental Fe/mL was used as the supplement source. The participants ingested the supplement during the fasting state. Participants were allowed to drink water ad libitum to help swallow the Fe-containing mixture. Approximately 60 min after ingesting the supplement, the men were given a breakfast meal that terminated their overnight fast.

The second phase (intervention trial) consisted of 12 volunteer participants. This phase was designed to test the effectiveness of the palm fruit-derived antioxidant supplement, [Carotino Tocotrienol-Carotene Mixed Concentrate (CTCMC); Carotino SDN BHD] in reducing the potential oxidative effects of the FeOil treatment. The supplementation

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**FIGURE 1** Schematic summarized supplementation scheme and sample collection design used in the pilot study (A) and definitive intervention trial (B).
schedule was similar to that of the pilot study. CTCMC is a thick, deep-red, fatty paste, with a concentration of 7500 mg of mixed carotenoids [35% β-carotene and 59% α-carotene with the remaining 5% of other carotenoids (18)] and 9200 mg of total vitamin/100 g CTCMC (approximately, as reported by the manufacturer). However, this trial used 2 concentrations of CTCMC: 0.4 g or 0.8 g/5 mL of refined palm oil, designated as CTCB or CTCA treatment, respectively, as well as the FeOil treatment used in the pilot phase. A locally produced commercial refined palm oil (Olmeca) was used to dilute the CTCMC and make it easier for consumption. This design effectively determined the dose-dependent effect of these antioxidants.

In the intervention trial, men were divided into 2 groups, each of which received the CTCA and CTCA treatments in a randomized order (Fig. 1B). As in the pilot phase, the mixtures were prepared 1 d in advance, were given during the fasting state, and a breakfast meal was provided within 60 min of ingestion.

At collection for both phases, participants were asked to collect a fecal sample in a scaled 50-mL plastic container (Amsino International) according to their normal bowel habits and transport the sample to the study site in an icebox with a chemical coolant gel. Samples were transferred to properly labeled plastic bags and frozen at -50°C until they were ready to be analyzed for total hydroxylated products and nonheme Fe.

**Assay procedures**

**Generation and detection of hydroxylated products in fecal matter.** Our interest was in an assay that assessed the buffer capacity of fecal material to retard free-radical generation and, as a result, in situ luminal oxidation. We adapted an HPLC-based method, which was originally developed and validated by Owen et al. (15). This method is based on the generation and detection of products resulting from the hydroxyl radical attack on salicylic acid, which serves as a measure of ROS production: 2,5 dihydroxybenzoic acid (2,5 DHBA), 2,3-dihydroxybenzoic (2,3 DHBA) acid, and catechol. (Chemical formulas are illustrated in Supplemental Fig. 1).

Standards of catechol (Sigma Aldrich), salicylic acid, 2,3 DHBA, and 2,5 DHBA (both from Sigma Aldrich, Merck) were used to construct the calibration curve to calculate the concentrations of hydroxylated products and residual salicylic acid that did not react in the fecal samples. Dilutions of a stock solution containing the 4 compounds were prepared to obtain the following concentrations: 0.2 mmol/L, 0.4 mmol/L, 0.6 mmol/L, 0.8 mmol/L, 1 mmol/L, and 2 mmol/L.

For assessing the generation of total hydroxylated products, fecal samples were prepared as indicated in Owen et al. (15) with some modifications, which included an extended incubation period of the sample at 37°C from 18 to 21 h (PREMLAB incubator) and changes in the HPLC run and the detector used. Briefly, 20 μL of the sample were injected directly into the HPLC (UV/VIS detector, Hewlett Packard Series 1100 HPLC equipment, Agilent Technologies) with a Hypersil ODS column (200 × 2.1 mm, 5 μm, Thermo Fisher Scientific). The mobile phase consisted of 2% acetic acid glacial (Merck) in water (Lichrosolv water for chromatography, Merck); this constituted solvent A, and methanol HPLC-grade for chromatography (Merck), which comprised solvent B. The following gradient was used throughout the HPLC run: 95% A/5% B for 2 min, 75% A/25% B for 8 min, 60% A/40% B for 10 min, 30% A/50% B for 10 min, and 0% A/100% B for 10 min. The UV/VIS detector was set at 278 nm for 5.5 min and then changed to 301 nm until the end of the analysis. Flow rate was set at 0.5 mL/min. Instrument control and data handling was conducted with HP Chemstation analysis software (Agilent Technologies). (A sample chromatogram is available in Supplemental Fig. 1).

The limit of quantification for one of the hydroxylated products, catechol, was too high to ensure analytical quality, so the quantification of this compound was eliminated from the method. The quantitative index for the generation of ROS was established as the sum of the concentrations of hydroxylated products, 2,5 DHBA and 2,3 DHBA, which were expressed in mmol/L.

**Nonheme Fe quantification in fecal matter.** Nonheme Fe was quantified using the commercial spectrophotometrically based assay Feren-B-Method kit (Bioanalytic, Ulmrich) to examine the relationship between free radical production in feces and the presence of residual Fe in the intestinal lumen. Readings were made in an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies). Nonheme Fe was expressed as μg/g wet weight of native stool (Fe molecular weight = 55.84).

**Statistical analysis**

Data were entered into an electronic spreadsheet (Excel, 2003, Microsoft) and analyzed with statistical software (SPSS 12.0.1 for Windows). Values for the fecal free radical biomarker were treated as repeated measures and analyzed with a repeated-measures linear model (MANOVA), with the least statistical difference test for treatment differences. Spearman rank correlation coefficient (nonparametric measure of correlation, which does not assume normality) was used to measure the strength of the association between fecal nonheme Fe and ROS production. Values in the text are means ± SD unless otherwise noted.

**Results**

**Demographic, body composition, and hematological characteristics of the participants.** The demographic and hematological characteristics of the participants are summarized in Table 1 by both study phase and cumulatively. Volunteers ranged in age from 18 to 35 y. Nine volunteers had a normal BMI, whereas 7 were classified as overweight, with a BMI >25 kg/m², and 1 as underweight, with a BMI <18.5 kg/m². None of the men was anemic at either the beginning or end of the study, according to a cutoff criterion of hemoglobin <137 g/L adapted from the WHO criterion for adult men (19), adding an altitude adjustment according to Dirren et al. (20).

**Dietary pattern and estimated intra-study intake of Fe and antioxidant nutrients.** The selection of food by the study group was modern and Westernized, with generous consumption of meat and fast-food/take-away restaurant food and minimal consumption of the traditional Guatemalan maize and bean fare. An analysis of the foods and beverages with respect to days with and without Fe supplements showed no obvious trends in food selection or intake of specific nutrients.

There were no apparent excesses in the participants’ intakes of dietary constituents that might influence antioxidant status. The consumption of dietary supplements, of course, was an exclusion criterion. The mean daily fiber intake, at 19.2 ± 10.9 g/d, was below the Adequate Intake standard of 38 g recommended by the U.S. Food and Nutrition Board (21). The global mean vitamin C intake was 74.6 ± 78.8 mg/d, which is twice the Estimated Average Requirement (EAR), 37 mg/d (22), derived from the WHO/FAO standards (23). The mean vitamin E intake of 6.8 ± 5.1 mg/d was slightly below the 8 mg WHO/FAO EAR (23). There is no accepted intake recommendation for carotene as an antioxidant (22).

Nevertheless, individual mean intakes of total carotenoids were well below the ~1260 μg, which would contribute 25% to the vitamin A EAR (23), if present in provitamin A form.

On the oxidant side, the mean daily intake of dietary Fe, combining both heme and nonheme Fe, was 15.9 ± 7.3 mg. This

| TABLE 1 Demographic and hematological status of the 17 participants1 |
|------------------|--------------|--------------|---|
| Characteristic   | Phase 1 (pilot study) | Phase 2 (intervention trial) | Total |
| n                | 5            | 12           | 17    |
| Age, y           | 30.0 (21–35) | 24.1 (18–34) | 25.8 (18–35) |
| Weight, kg       | 79.8 ± 11.5  | 64.4 ± 11.2  | 69.0 ± 13.1 |
| BMI, kg/m²       | 26.7 ± 3.9   | 22.2 ± 3.4   | 23.5 ± 4.0  |
| Hemoglobin, g/L  | 164.6 ± 6.1  | 157.6 ± 5.4  | 159.6 ± 6.3 |

1 Values are means ± SD or means (range).
is 74% of the WHO/FAO EAR (24) of 21.6 mg/d for men, with the most conservative assumption of the lowest dietary bioavailability for Fe (5%) (24). Given the Westernized consumption pattern observed with these men, dietary Fe bioavailability would be expected to be moderate (10%) if not high (15%).

Pilot study on feasibility of in situ ROS modification by oral Fe and effects of aqueous and lipid solvents. The pilot phase served the dual purpose of determining if consecutive-day Fe supplementation had an effect on fecal ROS production and whether the effect was distorted when Fe was given in a lipid emulsion rather than a completely aqueous solution. Results in both a daily format and a 3-d mean show that Fe supplementation with and without refined palm oil (FeOil and Fe, respectively) increased the generation of hydroxylated products in fecal matter by 23% \( (P = 0.024) \) and 36% \( (P = 0.026) \), respectively, compared with baseline (Fig. 2). In fact, the addition of refined palm oil to the ferrous sulfate (FeOil treatment) showed higher levels of ROS production \( (P = 0.014) \) by repeated-measures analysis compared with the Fe treatment.

Definitive intervention trial with oral Fe alone and with 2 doses of carotene-tocotrienol antioxidant concentrate. After observing significant increases in ROS production with consecutive daily doses of 120 mg of Fe with and without refined palm oil, the research study was extended to a definitive, controlled intervention. This extension was designed to determine whether the effect would be attenuated by oral supplementation of a rich source of lipid-soluble antioxidants. The FeOil treatment produced an increase over basal fecal ROS responses \( (P < 0.001) \), as demonstrated in the pilot phase (Fig. 3).

Turning to the hypothesis of a dose-response effect, the addition of both dosages of CTCMC during oral Fe administration returned fecal ROS to a level comparable with basal or washout periods. This was further tested through analyses of the ROS responses for the respective no-Fe periods, either combined and averaged across the study (pooled baseline) or related to the specific collections within the treatment unit (specific baseline). Again, there was no evidence of a CTCMC dose-response effect compared with the CTCA and CTCB responses (Fig. 3).

Association of residual fecal nonheme Fe and in situ ROS production. Because Lund et al. (14) related fecal oxidation to the residual nonheme Fe in the stools of their Fe-supplemented male participants, we examined this association in our participants’ stool samples. The concentration of nonheme Fe ranged from 0 to 10.2 \( \mu g/g \) among the entire 287 stool samples collected and analyzed throughout the entire study. We did not obtain ROS and nonheme Fe for 6 samples, so only 281 were available for association analysis. The fecal nonheme Fe concentration in the samples collected at baseline or during washout periods was 1.8 \( \pm 0.34 \) \( \mu g/g \) (mean \( \pm \) SEM, \( n = 281 \)). At the remaining collections, which occurred during Fe supplementation, the nonheme Fe concentration of 4.4 \( \pm 0.47 \) \( \mu g/g \) was greater \( (n = 281; \ P < 0.00001) \). As could be expected, the fecal Fe concentrations increased by 145% when an additional 120 mg of Fe was ingested daily. The coordinates of the scattergram in Figure 4 illustrate the fecal heme-Fe concentrations and ROS responses for all 281 samples analyzed using both assays. The Spearman correlation coefficient was 0.255 \( (P < 0.001) \), confirming an association between the 2 phenomena.

In Figure 4, the data for treatment with or without CTCMC are indicated with distinct symbols. Additional demonstration of the effect of oral antioxidants is provided through the partial regression analysis. When the regression was rerun, eliminating the 140 pairs related to the baseline and washout periods with CTCMC, the strength of the Fe to ROS association increased to \( r = 0.395 \) \( (P < 0.001) \). Further evidence of an attenuating effect of antioxidant supplementation can be found in a series of partial correlations. The correlation coefficient for collections from the entire set of values for treatment without CTCMC was 0.351 \( (P < 0.001) \) compared with 0.166 \( (P = 0.05) \) when the antioxidant extract was orally administered.

Discussion

We aimed to advance understanding of the mechanisms that underlie epidemiological evidence for an oral Fe and intestinal disease association (6,7). By relating oral Fe supplementation to increased oxidation in the stool, we confirm the seminal observation of the Norwich group (14) that supplemental Fe allows for a more oxidation-prone milieu in the fecal stream. We replaced their dimethyl sulfoxide indicator colorimetric method.
and vitamin E. In their study, 5 healthy adults were randomly selected to consume 3 types of spreads: 2 contained increasing amounts of α-tocopherol equivalents and carotenoids and the other one contained RRR-α-tocopherol without carotenoids. Plasma α-tocopherol concentrations and antioxidant capacity ex vivo increased significantly in the men consuming the spreads with both antioxidants, whereas no significant changes were observed with RRR-α-tocopherol only. In a study of in vivo effects of antioxidant supplementation among 64 elderly Malaysians (28), palm oil extracts were also the source of antioxidants. However, in this case, a carotene-free formula with 160 mg of tocotrienols was supplemented daily for 6 mo. The results from this study demonstrated a significant reduction in systemic DNA damage after 3 mo of supplementation, which remained stable until the end of the study. The locus of interest for the aforementioned studies, however, is at the level of systemic, in vivo oxidation. In situ intestinal-fecal oxidation was the novel focus of the present study.

Important distinctions in the respective designs of the Norwich experiment (14) and the present study are evident and can be combined to provide insight into the possible in situ mechanisms that underlie the findings. In the former study, the cumulative oral Fe dose was 266 mg over 14 d. The association of free-radical generation and fecal Fe, incubated with EDTA and dimethyl sulfoxide, is consistent with the notion that residual Fe initiates the oxidation. A similar process in situ in the intestinal lumen is predicted to occur with oral Fe supplementation. In the present study, we provided 840 mg of oral Fe over 7 d and analyzed the stools using an assay in which additional Fe (ferric chloride) combined with EDTA was added to the reaction flask prior to incubation with salicylic acid. This increased the Fe concentration of the reaction flask by 20–60 times that produced by the background residuals in the fecal samples. The mechanistic synthesis across the complementary experiences is best interpreted as most compatible with a scenario in which oral Fe in the intestinal tract produces a steady low-grade oxidation in situ; the consequence is a cumulative decrease in antioxidant...

Our study design was somewhat analogous to that of Upritchard et al. (27), as it is based on a blend of carotenoids...
capacity of the fecal milieu to buffer extraordinary oxidant challenges. The observed disruption of the association between fecal Fe and ROS generation by an external source of antioxidants, even during oral Fe supplementation, indirectly supports fecal Fe and ROS generation by an external source of antioxidants to prevent potential adverse effects on the ROS responses in fecal matter. Further research is needed to determine the minimum oral dose of the antioxidant extract required to observe effects and the potential of practical doses of antioxidants to prevent potential adverse effects on intestinal health.

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5. Siemianowicz K, Gmínski J, Francuz T, Wójcik A, Posielezna B. Activity of the oxidative process involving ROS in an in vitro, ambient setting is related to the rate and extent of Fe-associated oxidation that would occur under the highly anaerobic condition of the human colon. The combination of at least 3 antioxidants in the CTCMC mixture makes it intrinsically impossible to determine the relative role of α-carotene, β-carotene, and tocotrienols in the suppression of in situ oxidation. As the two antioxidants, carotenes and tocotrienols, are naturally combined within the extract in a fixed chemical composition, further differentiating the relative action of one or the other is not relevant at this time.

In conclusion, we confirm the finding that daily oral Fe supplementation increases the formation of free radicals in the fecal stream. The Fe-induced ROS response is mitigated by simultaneous supplementation with 2 large doses of a carotene-tocotrienol extract, a by-product of refining of crude red palm oil (29), without any dose-response effects. The experimental model could easily be extended to explore the influences of other dietary supplements/nutrients that might have beneficial or adverse effects on the ROS responses in fecal matter. Further research is needed to determine the minimum oral dose of the antioxidant extract required to observe effects and the potential of practical doses of antioxidants to prevent potential adverse effects on intestinal health.

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