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Studies on variation in fecal reactive oxidative species generation in free-living populations in Guatemala*

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Abstract: Among the factors associated with the risk of colorectal cancer and other large bowel diseases are gender, with women having lower incidence than men, and free-radical mediated oxidation. Dietary fiber has been attributed a protective role in human gastrointestinal health. The main aim of this study was to determine the degree of association between dietary fiber consumption and fecal free-radical production in healthy rural and urban Guatemalan women, moreover, to look for associations between gender and fecal reactive oxidative species (ROS) basal production, a marker of in situ colonic free-radical-based oxidation. For this purpose, we assessed the dietary fiber consumption, using two 24-h recalls, in urban and rural females, and compared the baseline data, i.e., of iron-supplement-free periods, in three previous studies. Two of these trials quantified the fecal ROS generation as total hydroxylated products resulting from free-radical attack on salicylic acid along with residual non-heme iron content in stool samples from 27 Fe-replete men. The third study assessed the same variables in 20 rural and 20 urban women, all consuming their respective habitual diets. The average fiber consumption for females was more than double in the rural group than in the urban population. As for the average ROS responses, a 2.5-fold difference was observed between men and women, with men having the higher concentrations of total hydroxylated products. This difference was sex-linked, unaffected by statistically significant differences in dietary fiber intake, nor by different concentrations of residual fecal non-heme iron between rural and urban women. The difference in background ROS production between men and women suggests a gender-related influence on intraintestinal oxidation that may protect women from harmful effects of dietary oxidants, such as iron.

Keywords: dietary fiber; dietary iron; free radicals; gender; Guatemala; oxidation.

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INTRODUCTION

The human gastrointestinal tract is among the organs most prone to degenerative and neoplastic diseases. It has been hypothesized that carcinogenesis is promoted by chronic inflammation, through the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are produced by inflammatory cells and may alter gene regulation mechanisms [1]. Model experimental systems were developed to demonstrate and quantify in situ susceptibility of the fecal milieu to oral iron supplementation [2–5]. The equilibrium between intraluminal free-radical buffering capacity and in situ generation of ROS is likely to have a significant impact on intestinal pathophysiology. Accordingly, there are effects of dietary oxidants, such as iron [2–5] and of antioxidative substances [4] imposing the free-radical abundance. With these concepts in mind, it is surprising that ambient background oxidation in the intestinal lumen at dietary iron intake levels has heretofore received little investigative attention. There are no data or estimates on the intra-individual variance in intrinsic free-radical quenching and buffering capacity in the intestinal lumen or on its stability or lability within a given individual, nor on whether sex has an impact.

A prospective intervention study and a series of secondary analyses of a previous data set [4,5] were drawn together to address the issue of fecal oxidation buffering capacity in the absence of provocation by supplemental oral iron. We examined various influencing variables with a potential to modulate the basal status of resistance to fecal oxidation [4]. As a convenient hypothesis to examine variance in fecal oxidation buffering on a population level, we hypothesized that differences in habitual dietary fiber intakes, or associated dietary or life-style features that contrast between urban and rural conditions in contemporary Guatemala, might influence fecal oxidation in a discernable manner. This hypothesis was addressed in a cross-sectional fecal sampling survey associated with dietary intake interviews. In combination, we mobilized and reanalyzed the data from an earlier study in young urban men, focusing on those stool samples collected under basal conditions, i.e., when they were consuming nothing but their habitual diet. This permits to explore a rank assortment among individuals for their intrinsic capacity to quench fecal oxidation. As non-heme iron concentration can easily be assessed in human feces, we were able to examine any associations between residual fecal iron and concomitant ROS generation in the ambient state, to see if they were similar to the direct relationship observed during oral supplementation in the same Guatemalan men [4]. We present here findings on the association between different diets consumed in rural and urban environments leading to different residual fecal iron and fiber content, and the stability of intra-individual capacity of the stools to buffer free-radical formation in situ.

SUBJECTS AND METHODS

Prospective-study and archival subjects

Subjects of the present study were 40 new female subjects, as well as 27 male volunteers who were enrolled in the two prior iron supplementation fecal ROS metabolic studies previously presented [4,5]. From the 40 women participating in the prospective study, 20 were recruited in the rural town of Santo Domingo Xenacoj, Chimaltenango, and the remainder in the urban area among the students attending Guatemala's national university, "Universidad de San Carlos de Guatemala". Among women living at the rural area, no exclusion criteria were applied; for the women recruited in the urban area, only those individuals who had a diet rich in refined carbohydrates, as established by previous interview screening, were included. The data in urban males used in this analysis come from 27 nonsmokers, recruited at two universities in Guatemala City. We used fecal samples collected during three consecutive days prior to beginning the iron supplementation trial as our "basal" samples, assumed to be reflecting the subjects' "habitual" diet.

The Center for the Studies of Sensory Impairment, Aging, and Metabolism (CeSSIAM) Human Subjects Committee in Guatemala City granted ethical approval to the study protocols. The participants

were informed about the objectives and procedures of the study during a preliminary meeting. Subjects signed the informed consent forms assuring that they understood the nature, purposes, inconvenience, risks, and benefits of the study. Subjects were compensated for their participation.

Estimated intake of total energy and dietary fiber

A retrospective history of food and beverage intake on the previous calendar day was obtained from each of the 40 participants by a team of three experienced research nutritionists. The amount of items consumed was reported in common household units. These were converted into grams for formal portion-size tabulations for each subject day. Food composition table values for energy values and dietary fiber were obtained from two reference sources [6,7]. The two consecutive days' intakes of energy (in kcal) and dietary fiber (in g) were averaged as a daily mean for each subject, and then combined across individuals to calculate subgroup averages.

Stool collections

All subjects participating in the study were asked to provide two stool samples during a period of one week. Equal numbers of rural and urban samples were collected in the same weeks. Every time the subjects delivered a fresh sample, a trained nutritionist carried out a 24-h dietary recall, which assessed the food intake on the day prior to producing the stool sample.

Quantification of total iron concentration in stool

A commercial spectrophotometric assay (Feren-B-Method kit, Bioanalytic, Umrich, Freiburg, Germany) was used to quantify non-heme iron in the fecal samples. This allowed examining the relationship between free-radical production and residual non-heme iron content in feces. Readings were made in a spectrophotometer (Thermo Scientific Genesys 10uv, Thermo Fisher Scientific, Waltham, MA, USA). Non-heme iron was expressed as μg/g 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 a high-performance liquid chromatography (HPLC)-based method adapted from Owen et al. [8], also used in a previous study to evaluate the effects of supplemental iron and antioxidants on the production of ROS in human stools [4]. The basis of the method is the generation and detection of hydroxylated products resulting from the hydroxyl radical attack on salicylic acid. The amount of 2,5-dihydroxybenzoic acid (2,5-DHBA) and 2,3-dihydroxybenzoic acid (2,3-DHBA) serves as a measure of ROS production as described elsewhere [4]. Results are given as the sum of the concentrations of hydroxylated products, 2,5-DHBA, and 2,3-DHBA, expressed in mmol/L.

Nutritional data analysis

Data on dietary characteristics, in particular on fiber content, were obtained using the 24-h recall method. Descriptive statistics were used to analyze this data. The differences between the two groups were analyzed using two independent samples *t*-test. The study sought to compare the effects of the diet rich in fiber on the fecal generation of ROS.

Data handling and statistical analysis

Data were entered into an electronic spreadsheet (Excel, 2003, Microsoft, Redwood, WA, USA) and analyzed with statistical software (SPSS 12.0.1 for Windows, Statistical Package for the Social Science Inc., Chicago, IL, USA). Values for the stool iron concentration and fecal ROS generation, assayed for consecutive-day fecal samples, were treated as repeated measures and analyzed with a repeated measures linear model (MANOVA), with the least statistical difference test to assess intertreatment differences. For comparisons between subgroups of the same gender, or between the combined gender-specific samples, the two-tailed *t*-test was used. Spearman's rank-order correlation coefficient (nonparametric measure of association, which does not require normal distribution) was used to measure the strength of the correspondence between fecal non-heme iron and ROS production. A probability of <0.05 was accepted as the level for statistical significance.

RESULTS

Demographic characteristics of the subjects

The age distribution in groups and subgroups are given in Table 1. There was no significant difference in the age of the 20 women in the rural and urban subgroups ($p = 0.22$), respectively. Their combined age was 29 ± 9 y, which did not differ from the 27 ± 9 y of the 27 urban men in the combined male group ($p = 0.083$).

Table 1 Age distributions by sex and study series.

Rural women ($n = 20$)	Urban women ($n = 20$)	Women combined ($n = 40$)	Men combined ($n = 27$)
31 ± 10 (19–56)	28 ± 8 (19–52)	29 ± 9 (19–56)	27 ± 9 (18–56)

Effects of geography and dietary fiber intake on ROS generation in women

Our design aimed to test the hypothesis that rural and urban woman may show marked differences in dietary fiber intake that can easily be detected in two days of stool sampling per individual. Daily fiber consumption by the rural subjects was more than twice as high as in urban ones (Table 2) as was fiber density expressed per 1000 kcal of estimated energy intake in the diet (data not shown). Meanwhile, the two groups showed no difference in average daily dietary energy intake (Table 2).

Table 2 Comparison of dietary fiber, energy, dietary heme and non-heme iron in rural and urban Guatemalan women.

Area of study	Fiber (g/day)	Energy (kcal/day)	Dietary heme iron (mg/day)	Dietary non-heme iron (mg/day)
Rural	40 ± 21	2528 ± 624	2.5 ± 2.5	15.8 ± 8.7
Urban	17 ± 8	2360 ± 1121	3.3 ± 1.4	15.5 ± 7.2
p-values*	0.0001	0.56	0.19	0.89

*Two-tailed *t*-test.

Total dietary iron intake was approximately 19 mg Fe/d, which was distinguished and displayed as to the heme and non-heme components (Table 2). There were no significant differences between groups regarding intake of either variety of dietary iron.

To test the hypothesis of a geographical distinction in fecal ROS generation, we compared the average in vitro ROS responses across the subgroups in the female sample (Fig. 1). We found no difference in ROS production between rural and urban samples from the stools collected across the one-week interval. Hence, we also pooled all values collected at both points in time into a global average (Fig. 1). There was no significant difference in the mean ROS production (p = 0.86) between both groups for rural (0.077 ± 0.020 mg/ml) and urban (0.078 ± 0.023 mg/ml) women, which was unexpected in view of the different dietary fiber consumption, which was 135 % higher in the rural area. The lack of a fiber effect on basal ROS production is also demonstrated by the absence of any association between grams of fiber ingested and fecal ROS generation (r^2 = 0.093, p = 0.57) (Fig. 2).

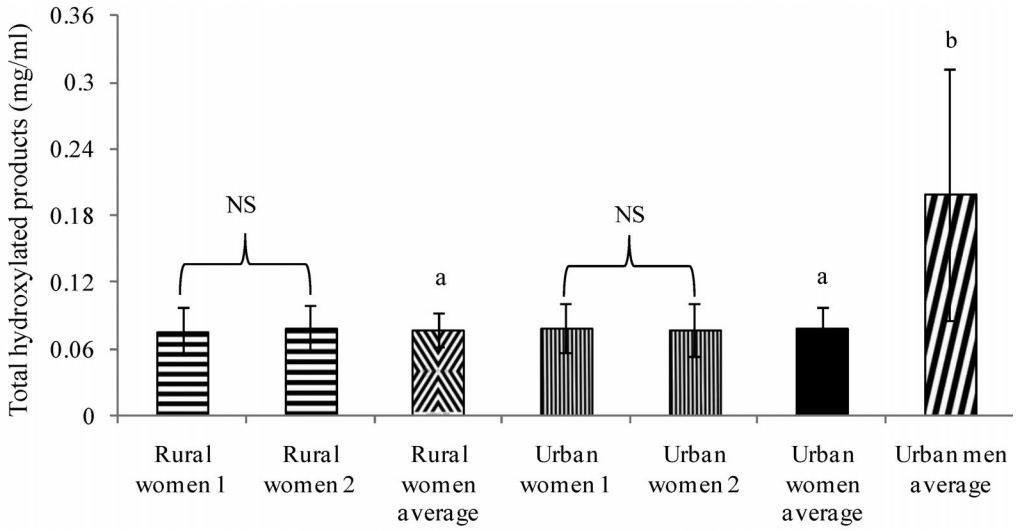

Fig. 1 Production of total hydroxylated products per day in stool sampled for rural women, urban women, and urban men. (The values "women 1" and "women 2" were determined in samples collected in the same individuals at one-week interval.)

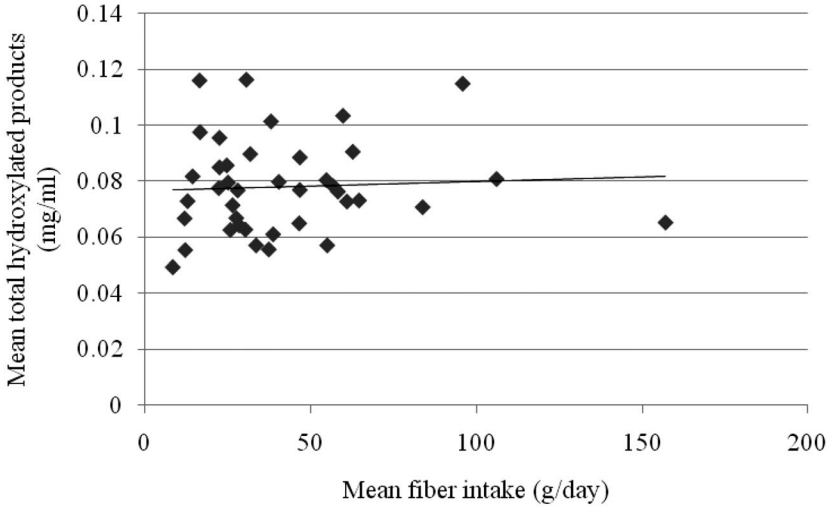

Fig. 2 Association between average individual fecal ROS production and average individual dietary fiber in the female sample (n = 40). (Spearman rank correlation: r^2 = 0.093, p = 0.57.)

Effects of sex on fecal ROS generation

Compared to the archival data on ROS production in 27 urban males on their habitual diet (0.20 ± 0.11 mg/ml) (Fig. 1), the female responses (above) were significantly lower ($p = 7.88 \times 10^{-6}$). This reveals difference in baseline antioxidant buffering capacity based on sex, which was unexpected. There was an 155 % higher average ROS generation response for the 3-day mean of 27 males pooled from two studies, as compared to the 2-day means for 40 females ($p = 7.90 \times 10^{-6}$).

Association of fecal ROS generation with residual fecal iron concentration

In view of the consistently found relationship between fecal oxidation indicators and the residual stool iron after iron supplement in our experience [4] and in Norwich data [3], we assessed the fecal iron content and related it to the oxidation capacity in the stools in a gender-specific way. We compared residual fecal non-heme iron content in the female subgroups to the archival data in men (Fig. 3). Again, no significant differences were seen over the one-week interval of data collection in women. A significantly higher fecal non-heme iron content showed in urban women than in their rural counterparts ($p = 0.0015$). Compared to the pooled male fecal iron content rural women showed no significant difference, but urban women showed significantly higher fecal non-heme iron content ($p = 0.003$). Examining the association between ROS generation and fecal non-heme iron (Fig. 2), we found no significant correlations, neither in male, nor in combined female samples or after combining all samples (Figs. 4a–c).

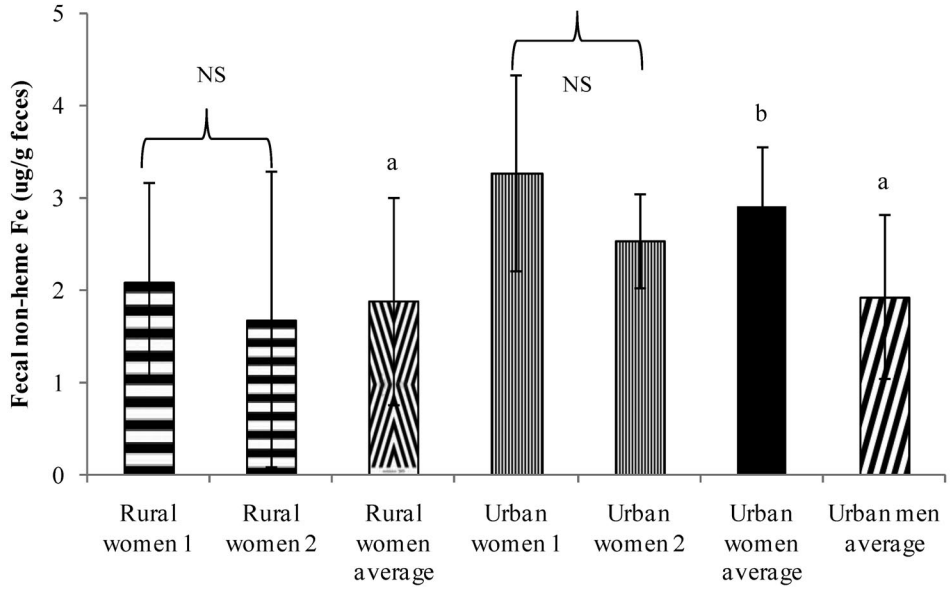

Fig. 3 Residual fecal non-heme iron in the rural women, urban women, and urban men samples in average and by day.

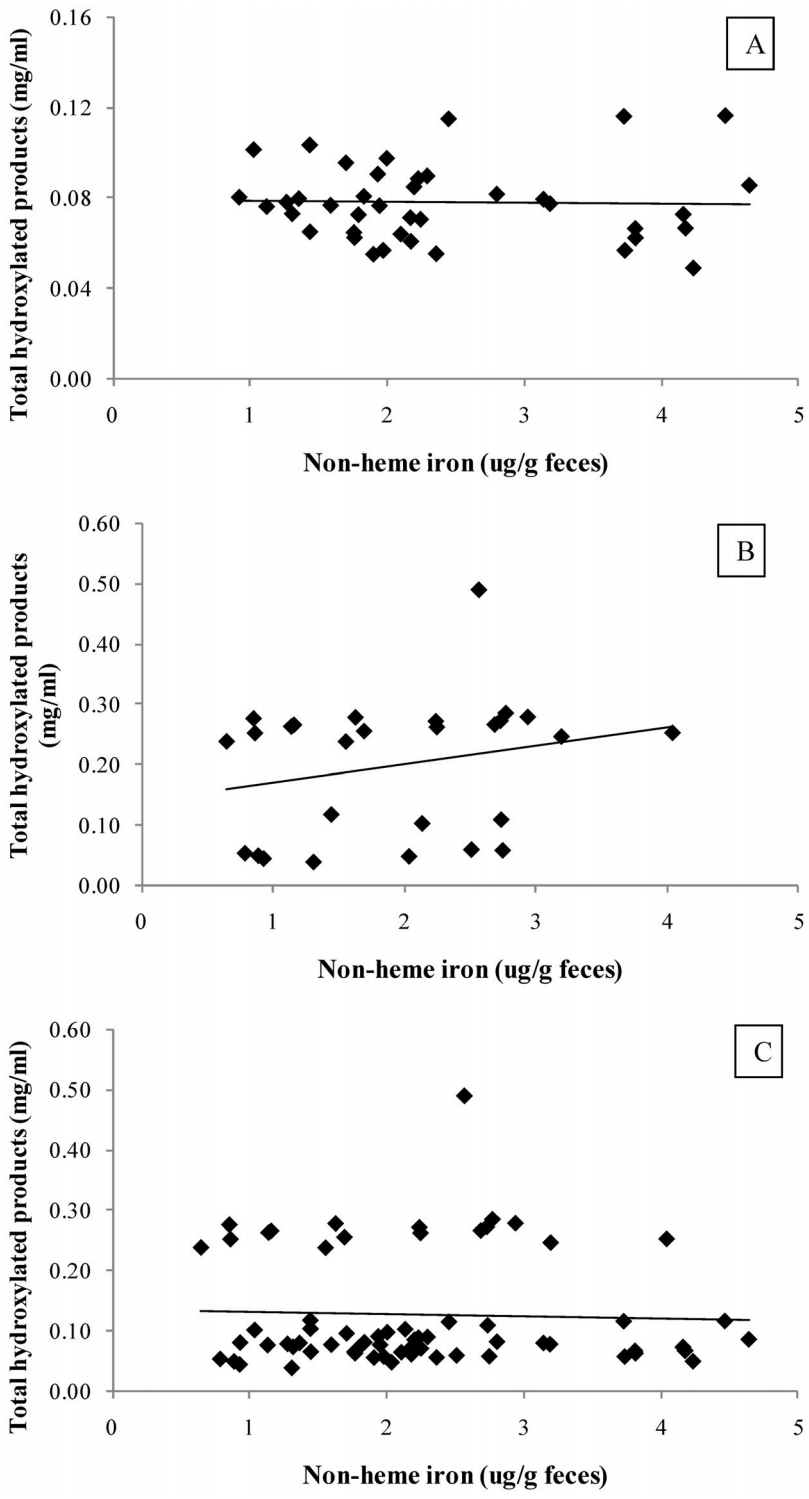

Fig. 4 Association between baseline ROS production and residual non-heme iron concentration in: (A) combined female sample (n = 80, r = –0.093, p = 0.56); (B) baseline values in combined male sample (n = 27, r = 0.31, p = 0.12) and (C), in combined all basal samples from both sexes (n = 67, r = 0.025, p = 0.84).

DISCUSSION

Earlier work on fecal ROS production has been linked to the provocation of intra-luminal oxidation by oral iron supplements, e.g., [3–5]. However, even the lower free-radical responses in the non-iron supplemented state are easily quantified and were shown to be stable and uniform within groups of subjects. The study in women aimed to explore the hypothesis that significant differences in dietary fiber intakes, which by itself is a potential antioxidative substance, might modulate the antioxidant capacity in corresponding fecal oxidation in a discernable manner. Our findings, however, failed to show any difference in ROS generation in spite of a greater than two-fold difference in habitual dietary fiber consumption.

This finding, however, does not argue against the use of ambient ROS production to explore biological differences. Thus, our own archival data in males generated in the same laboratory by use of the same methods under the same conditions demonstrate a two-fold, higher ROS production rate in our in situ assay than in females. Our first attempt of explanation was related to an effect of dietary iron, as fecal iron had shown to increase fecal oxidation after oral iron supplementation [3,4]. In principle, the lower fecal iron content reached after dietary iron intake could also exert an oxidizing effect. However, we were unable to attribute individual modulation or group differences in ROS production rates to the iron content in the fecal milieu under these conditions. Data on dietary iron intake in our earlier studies in males are unfortunately not available. In our present study, despite different dietary patterns, total iron intake was remarkably similar in rural and urban women. The fecal iron content, however, showed significantly higher content of non-heme iron in the stools of the urban women. We assume that differences in intestinal transit time might explain the concentration differences in fecal non-heme iron. Increase of stool volume and decreased transit time can be attributed to higher dietary fiber intake [9]. Thus, residual dietary iron content in feces can be distributed in larger volumes of fecal mass in the high-fiber-consuming rural females than in the urban subjects on a more refined diet. In parallel, the differences seen between urban men and women, who were probably on an equally refined diet, may be due to the established more rapid transit of fecal content in men [10].

The nature of the specific gender-associated mechanisms providing the apparently greater fecal antioxidant buffering capacity in stools from women on their habitual diets cannot be inferred from the data of this study. Even if our male subjects had consumed much higher dietary non-heme iron quantities than the 15 mg determined for women, the fecal iron content in the nonsupplemented state does not emerge as a powerful determinant of free-radical production (Figs. 4a–c). Despite the lack of clues to a rationale for gender differences, we can point to a certain consistency with the epidemiology of colonic health. Inflammatory bowel disease shows a markedly higher prevalence in males [11]. As intestinal oxidation has been implicated in the pathogenesis of these conditions, a tighter control of fluctuations in fecal free radicals could explain this gender-associated difference in risks.

Our study has, on the one hand, acknowledged limitations, such as the small sample size, the number of days in which the fecal antioxidant capacity was assessed, different analytical runs for the men and women samples, and the disconnect in timing between the fecal bolus related to the food consumed in the 24-h recall report and the fecal specimens collected for ROS and iron analyses. Thus, we rely on the assumption of habitual intake being reflected by the 2 days of the dietary recall. Moreover, 24-h dietary recalls per se have inherent limitations, since estimation of true portion sizes, and omission or distortion of the identity of food items consumed is problematic. On the other hand, the strengths of this study derive from our refined biomarker of in situ free-radical generation and the possibility to analyze the relationship between non-heme iron, fiber, and ROS contents in the same stool sample.

CONCLUSIONS

The in situ assays for fecal oxidation have provided a new tool to follow the physiological effects of intestine supplemental dosages of oral iron [2–5]. The present study uses the same tools to analyze free-

radical production in ambient stools without pharmaceutical dosing of iron, that is, with iron intake being restricted to the amounts in the habitual diet. On the one hand, nutritional iron and dietary fiber passing through the intestines under such conditions do not appear to be major determinants of fecal ROS production. On the other hand, sex stands out as a determining factor, with females clearly showing a higher antioxidant buffering capacity in stools. These findings set forth a number of opportunities for further inquiry into the relationships between dietary constituents, physiological conditions, genetic constitution, and health and may pave the way to greater insights into the sex-differential epidemiology of intestinal diseases.

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