

Daily Supplementation with Iron Increases Lipid Peroxidation in Young Women with Low Iron Stores

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The aim of this study was to determine whether women with low iron stores (plasma ferritin ≤ 20 $\mu\text{g/L}$) receiving a daily iron supplement for 8 wks at a level commonly used to treat poor iron status develop increased lipid peroxidation as measured by ethane exhalation rates and plasma malondialdehyde. The women served as their own control as pre- and post-supplementation periods were compared. Twelve women participated in the study for a 70-day period and consumed daily iron supplements (98 mg of iron as ferrous sulfate) from day 14 to day 70. Baseline blood and expired air samples were obtained on days 1 and 14; measurements during supplementation were performed on days 56 and 70, that is at 6 and 8 weeks of supplementation. Iron status improved during the iron supplementation period; biochemical indicators of lipid peroxidation also increased. After 6 wks of iron supplementation, serum ferritin almost doubled and body iron more than doubled.

Hemoglobin levels increased slightly and other indicators of iron status became normal. However, plasma malondialdehyde (MDA) and breath ethane exhalation rates (BEER) increased by more than 40% between baseline and 6 wks of supplementation; these increases correlated significantly with plasma iron and ferritin levels. MDA was positively correlated with BEER. BEER increased further after 8 wks of iron supplementation. The increased indicators of lipid peroxidation with duration of supplementation and as iron status improved suggest that providing daily nearly 100 mg iron may not be a totally innocuous regimen for correcting iron depletion in women. *Exp Biol Med* 233:701–707, 2008

Key words: iron; supplements; malondialdehyde; breath ethane; lipid peroxidation; oxidative stress; iron status; women

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Introduction

Iron supplements are almost universally recommended to pregnant women in most countries at doses ranging from 30–120 mg/day (1). The International Anemia Consultative Group (2) recommends 60 to 120 mg supplemental iron per day for pregnancy; the higher dose is recommended for areas where the prevalence of anemia is high among women of childbearing age. These recommendations exceed the iron RDA for pregnant women by 2- to 3-fold (3). It is recognized that excess body iron and “free iron” stimulate lipid peroxidation that leads to cell and tissue damage (4–6). Yet, only a few studies have been conducted to investigate the effect of iron supplementation on oxidative stress in humans and results are inconclusive (7–11).

Animal studies have demonstrated that excess iron increases lipid peroxidation (12–14). Lipid peroxidation is often assessed by measuring endproducts of peroxidation such as plasma malondialdehyde (MDA) and breath ethane and pentane (15). In a study by Knutson et al. (16) healthy women given 120 mg oral iron supplements daily for 28

days had increased plasma MDA. Several of the women also had increased breath ethane exhalation rates (BEER). It is not known, however, if supplemental iron causes an increase in endproducts of lipid peroxidation when used by non-anemic women with marginal iron status.

The aim of this study was to determine whether iron supplementation of women with low iron stores at a level commonly used to treat poor iron status (~ 100 mg Fe/d) for 8 weeks increases lipid peroxidation, as measured by BEER and plasma MDA, and to explore if measures of lipid peroxidation are related to indicators of iron status and to duration of supplementation (amount of iron intake). The women served as their own controls as pre-supplementation (baseline) results were compared with values measured during and at the end of supplementation.

Methods

Subjects. Twelve non-pregnant women, aged 18–30, participated in the study. They were non-smokers, apparently healthy, users of oral contraceptives, with acceptable weights for height, with blood hemoglobin >10.0 g/dL, and plasma ferritin ≤ 20 μ g/L at screening. Women who recently used mineral and/or vitamin supplements or who exercised heavily were excluded.

The study was approved by the Committee for Protection of Human Subjects of the University of California, Berkeley, and by the Radiation Safety Committee of the University of California, Berkeley. Written informed consent was obtained from each subject.

Study Design. Subjects kept a 4-day record of all food and beverage intake prior to the start of the study. Nutrient intake was estimated using The Food Processor (ESHA Research, OR, USA). The subjects were instructed not to change their dietary or activity habits during the study. Each subject participated in the study for a 70-day period. The women were given iron supplements (98 mg Fe/d as ferrous sulfate, United Research Laboratories, Inc., Philadelphia, PA) to take from day 14 to day 70 daily (8 weeks), in the evening 2 hours after dinner. Venous blood and expired air were obtained on days 1 and 14, prior to supplementation (basal values) and repeated on days 56 and 70, that is, after 6 and 8 weeks of supplementation. All collections were done in the Metabolic Unit, Department of Nutritional Sciences and Toxicology, UC Berkeley.

Sample Collection. Fasting blood samples were drawn into trace metal-free polypropylene syringes (Sarstedt Monovette, NH_4 -heparin, Sarstedt Inc., Newton, NC, USA) and kept on ice for no more than 1 hour before processing. Whole blood aliquots were used to determine packed cell volume, hemoglobin, and zinc protoporphyrins. Plasma was obtained from the remaining blood by centrifugation, transferred into polyethylene tubes and stored at -70°C until analysis. Plasma measurements included iron, total iron binding capacity, ferritin, transferrin receptors (TfR),

and malondialdehyde (MDA). The latter two measurements were done only on days 1 and 56.

For the expired-air collections, an air leak-free system was used and tested. Subjects sat upright in a chair and breathed hydrocarbon-free air (HCFA) through a vinyl 109T mouthpiece (Vacu-Med, Ventura, CA) connected to a two-way non-rebreathing valve. A disposable nose-clip was used to ensure only mouth breathing. Samples of expired air were collected 2–4 hours after a standardized breakfast meal was fed. The collection setup and materials were those described by Knutson and Viteri (17). Briefly, the subjects breathed HCFA for 6 minutes (USP, zero-grade air; Puritan Bennett, Lenexa, KS) from a 150 L Tedlar “reservoir” bag in order to wash out any alkanes in their lungs from breathing ambient air. A 5 L collection bag previously flushed with HCFA was filled twice at 6 and 10 minutes following that “wash-out” period. Three liters of expired air from this bag were transferred to sampling bags with an airtight 1.5 L syringe. The remaining air was recorded and discarded. The syringe and bags had been flushed previously 5 times with nitrogen (UCB Storehouse). Three 3.0-L samples were taken directly from the “reservoir” bag for background analysis. The samples were then stored at room temperature until they were analyzed later that same day for ethane and pentane concentrations. Recovery of alkanes from stored bags used in this study was previously determined: mean $97\% \pm 4\%$ SD.

Analytical Methods. Blood packed cell volume in capillary tubes was determined by centrifugation, blood hemoglobin with a HemoCue System (AB Leo Diagnostics, Medical Equipment Design Inc, Helsingborg, Sweden), and blood zinc protoporphyrin by hematofluorometry (Helena ProtoFluor Reagent System, Helena Laboratories, Beaumont, TX, USA).

Iron and total iron-binding capacity in plasma were measured by a colorimetric assay using bathophenanthroline sulfonate and magnesium carbonate (18). Plasma ferritin and plasma transferrin receptors were determined by enzyme-linked immunosorbent assay kits (Spectro Ferritin, S-22 and TfR, TF-94, Ramco Laboratories Inc., Houston, TX, USA). Plasma MDA was measured by gas chromatography-mass spectrometry using the method of Yeo et al. (19) including the modifications in Knutson et al. (14).

Breath samples (3 L) were analyzed for ethane concentration by cryofocusing (final volume, 50 ml) in a column containing activated alumina (80–100 mesh) and Porasil C (80–100 mesh) (Alltech Associates Inc.), followed by gas chromatography, as described by Knutson & Viteri (20) including the modifications by Knutson et al. (15). A Varian 3700 gas chromatograph (Varian Instruments, Palo Alto, CA, USA) fitted with a flame ionization detector and a $15\text{ m} \times 3.18\text{ mm OD}$ column filled with 80–100 mesh Porosil (Alltech) was used.

The gas chromatograph was calibrated each study day using a hydrocarbon standard gas mixture (0.493 ppm ethane/0.516 ppm pentane in nitrogen; Praxair, Los

Table 1. Biochemical Indices of Iron Status in the Women Before (Baseline) and After 6 and 8 Weeks of Daily Iron Supplementation^{1,2,3}

Index	Baseline		Iron supplementation	
	Day 1	Day 14	Day 56, 6 wk	Day 70, 8 wk
Packed blood cell volume, %	39.0 ± 1.6	39.8 ± 2.7	40.3 ± 3.5	41.3 ± 2.7
Blood hemoglobin, g/dL	12.4 ± 0.6a	12.7 ± 1.0a	12.9 ± 1.0ab	13.3 ± 0.8b
Blood zinc protoporphyrin, µmol/mol Hb	44.3 ± 10.4ab	43.1 ± 9.8ab	45.3 ± 10.1a	39.3 ± 7.7b
Plasma iron, µg/dL	77.9 ± 25.5a	65.6 ± 25.1a	105.5 ± 43.1b	97.1 ± 34.2ab
Plasma total iron-binding capacity, µg/dL	388 ± 62	409 ± 65	369 ± 47	398 ± 69
Plasma transferrin saturation, %	19.9 ± 5.3a	16.6 ± 6.6a	28.4 ± 10.3b	24.5 ± 8.3ab
Plasma ferritin, µg/L	10.5a (3.1–20.3)	11.0a (3.1–24.8)	20.6b (10.2–32.7)	18.9b (10.9–33.9)
Plasma transferrin receptors, µg/ml	3.90 ± 1.13	–	3.51 ± 0.84	–

¹ Iron supplements (98 mg Fe as ferrous sulfate) were consumed daily from days 14 to 70 of the study. Days 56 and 70 correspond to 6 and 8 wks of iron supplementation, respectively.

² Values are mean ± SD, *n* = 12. For plasma ferritin, values are given as geometric mean and range in parentheses.

³ Values in the same row with different superscripts differ significantly, *P* < 0.05.

Angeles, CA). The calibration standard, 4 ml of the standard gas mixture diluted to 50 ml with nitrogen in a gas-tight glass syringe, was injected directly into the gas chromatograph to determine concentrations. The coefficient of variation for the calibration standard over the course of the study was 4.5%. A standard sample (4 ml of standard gas mixture injected into a 3 L bag of HCFA) was subjected to the cryofocusing procedure and analyzed before samples were run to ensure proper recovery of ethane. Recovery was 98.0% ± 1.2% for ethane. Background concentrations of ethane and pentane in HCFA were also determined for appropriate correction. Results were expressed as pmol of hydrocarbon exhaled per kg body weight per min.

Statistical Analysis. Normality of distribution was tested with the Kolmogorov-Smirnov test. Where distribution was not normal, data were log-transformed prior to analysis. Differences in means were analyzed using one-way ANOVA with repeated measures. When differences were significant, the Tukey-Kramer Multiple Comparisons Test was performed for further analysis. The changes (deltas) in biochemical indices of iron status and lipid peroxidation status between the periods of the study were compared by paired Student's *t* tests. Changes were compared between the following periods: Period 1: Change between day 0 and day 14 (baseline); Period 2: Change between days 14 to day 56; Period 3: Change between days 56 to day 70; Period 2 + 3: Change between days 14 to day 70.

Correlations between measurements of oxidative stress and iron status were computed by the Pearson correlation coefficients. A probability ≤0.05 was considered to be significant. Non-parametric methods were used to test for differences in proportions. Statistical analyses were performed using SAS software, Version 8.

Results

At baseline, subjects were 24.0 ± 2.3 years of age, weighed 60.0 ± 7.3 kg, were 1.6 ± 0.1 m tall, and had an

appropriate body mass index (22.4 ± 2.2 kg/m²). These characteristics remained stable throughout the study. All subjects were taking oral contraceptive pills that contained 0.3–0.4 mg ethinyl estradiol. Mean habitual nutrient dietary intakes of Energy (*kcal/d*), Protein (*g/d*), Vitamin A (*RE/d*), Vitamin C (*mg/d*), Folate (*µg/d*), Copper (*µg/d*), Zinc (*mg/d*), Selenium (*µg/d*) and Calcium (*mg/d*) were adequate compared to reference values (3) except for vitamin E and iron (geometric mean) whose average intakes were 51 and 78% of the reference values.

At baseline (day 1), iron status values were normal except for plasma transferrin saturation and plasma ferritin, which were low (Table 1). This happened in spite of the fact that all the women were taking oral contraceptives that protect from iron deficiency because they result in smaller menses and lower menstrual iron losses (21). There were no significant changes in the indicators of iron status at the end of the baseline period (day 14). No woman was anemic at baseline; their hemoglobin and packed blood cell volume values were within normal limits (1). At day 56, after 6 wks of supplementation, Hb, packed cell volume and indices of iron status improved, serum iron, plasma Transferrin Saturation and serum ferritin reaching statistical significance. By day 70 (8 weeks of supplement intake) the biochemical indicators either continued to improve slightly or became stable (Table 1). The comparisons of changes between periods of study are presented in Table 2. These results essentially confirm those presented in Table 1.

Initially, the mean iron stores of the women were low, about 28 mg, based on plasma ferritin values above 12 µg/L, assuming a value of 8 mg iron stores per µg/L ferritin (22). Considering the mean changes in hemoglobin and ferritin concentrations, and assuming a mean blood volume of 63 ml/kg body weight (23), increments in Hb iron (total circulating Hb × 3.34 mg/g Hb) and in iron reserves (8 µg/L ferritin above 12 µg/L) body iron after 6 weeks of supplementation averaged 118 mg. This amounts to

Table 2. Comparison of Changes (Deltas), by Paired Student's *t* Tests, in Biochemical Indicators of Iron Status and Lipid Peroxidation Between the Periods of the Study^{a,b}

Biochemical index	Comparison of changes		
	Period 2 vs period 1	Period 3 vs period 1	Period 2 + 3 vs period 1
Hemoglobin	NS	NS	0.049
Zn protoporphyrin	NS	0.033	NS
Plasma iron	0.043	NS	0.031
Plasma transferrin saturation	0.024	NS	0.011
Plasma ferritin	0.005	NS	0.028
Breath ethane	NS	0.042	0.002

^a OBS: Changes in plasma Malondialdehyde were not compared because measurements were done only on days 0 and 56 (please see Table 3).

^b Changes were compared between the following periods:

Period 1: Change between days 0 and 14 (baseline);

Period 2: Change between days 14 and 56;

Period 3: Change between days 56 and 70.

approximately 3% of the ingested supplementary iron, without considering any retention from food iron.

By the TfR/ferritin ratio approach (24), the initial mean body iron reserves amounted to 48 mg. With the intake of iron supplements for 6 weeks, plasma TfR decreased 10% (Table 1). The mean changes in the TfR/ferritin ratio between basal values and those after 6 weeks supplementation translate to 159 mg of retained iron and to approximately 3.9% of the supplementary iron intake without considering any additional retention from food iron.

Two women after 6 wks and one woman after 8 wks of supplementation remained slightly iron depleted (serum ferritin 10.2 µg/L but not iron deficient by TfR/ferritin ratio). After 6 weeks of supplementation only one woman had <16% transferrin saturation, indicative of poor iron supply to the bone marrow, and another had TfR/ferritin ratio indicative of iron deficiency.

The indicators of lipid peroxidation increased with iron supplementation (Table 3). On study day 56, after 6 wks of supplementation, both MDA and BEER were more than 40% higher than baseline values; BEER increased further after 2 more weeks of iron supplementation. After 8 wks of

iron supplementation (day 70), BEER was 157% higher than baseline values. Pentane breath exhalation rates did not change significantly during the study (results not shown). Based on a 2.5 pmol/kg/min cut off level of ethane exhalation rates, the percent of women with values above this norm at baseline and after 6 or 8 wks supplementation increased from 17 to 58 and 92%, respectively. Only about 17% of a population of normal, non-smoking, healthy adults not taking supplemental iron have values above this level (Viteri FE and Knutson MD, unpublished).

After 6 wks of iron supplementation (day 56), plasma MDA correlated positively with plasma iron ($r = 0.578$, $P < 0.05$) (Fig. 1), with plasma ferritin ($r = 0.647$, $P < 0.04$) (Fig. 2), and with BEER ($r = 0.548$, $P < 0.04$) (Fig. 3). BEER correlated with plasma MDA at all study time points ($r = 0.419$, $P < 0.05$), with plasma iron ($r = 0.362$, $P < 0.02$), with transferrin saturation ($r = 0.352$, $P < 0.02$), and with plasma ferritin ($r = 0.506$, $P = 0.016$). Similarly, plasma MDA correlated with plasma ferritin ($r = 0.453$, $P = 0.034$). No significant correlations were observed between dietary intake of micronutrients and indicators of iron status and of lipid peroxidation.

Discussion

At baseline, all women were chosen to be iron deficient in spite of taking oral contraceptives. As a group, these women exhibited "normal" levels of MDA and BEER and lower intakes than recommended of vitamin E and iron. Consumption of 98 mg of daily iron as ferrous sulfate for 8 wks improved the iron status of women with low iron stores. By the sixth week of supplementation, plasma ferritin increased by 96%, plasma transferrin saturation by 55%, and hemoglobin by 7% (9 g/L). All indicators of iron nutritional status reached normal levels.

A substantial increase in markers of lipid peroxidation occurred at both periods of evaluation during iron supplementation suggesting that the women were experiencing oxidative stress. These data are consistent with previous studies in animals and humans. The 44% rise in plasma MDA after 6 wks of 98 mg supplemental iron is similar to the rise observed in women after 4 wks of 120 mg of iron per day (16). BEER more than doubled after 8 wks of iron supplementation, a finding that has been observed

Table 3. Indices of Lipid Peroxidation in the Women Before (Baseline) and After 6–8 Weeks of Daily Iron Supplementation^{a,b,c}

Index	Baseline		Iron supplementation	
	Day 1	Day 14	Day 56, 6 wk	Day 70, 8 wk
Ethane breath exhalation, pmol/kg/min	1.35 ^a (0.58–4.28)	1.69 ^a (0.67–4.28)	2.33 ^b (0.92–5.15)	3.90 ^c (2.16–9.03)
Plasma malondialdehyde, pmol/ml	103 ^a (24–198)	–	148 ^b (94–225)	–

^a Iron supplements (98 mg Fe as ferrous sulfate) were consumed daily from days 14 to 70 of the study. Days 56 and 70 correspond to 6 and 8 wks of iron supplementation, respectively.

^b Values are given as geometric mean and range in parentheses.

^c Values in the same row with different superscripts differ significantly, $P < 0.05$.

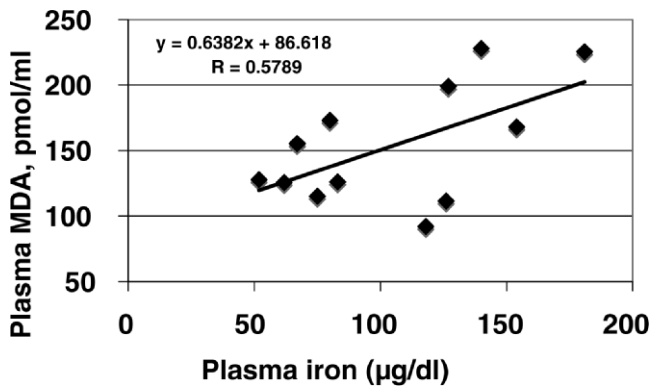


Figure 1. Linear regression between plasma malondialdehyde (MDA) and plasma iron in non-pregnant, non-anemic, iron depleted women after 6 wks of daily 98 mg iron supplementation.

previously in animals (5, 13, 14), but not in humans. Other researchers have measured the effects of supplemental iron, increased dietary iron, and iron injections on the susceptibility to oxidation of very low density (VLDL) and low density (LDL) plasma lipoproteins in humans (9), lipid hydroperoxides in rats (25), and thiobarbituric reactive substances (TBARS) in rats (12). In all cases, lipid peroxidation increased in response to iron supplementation, as seen in the present study.

The results presented here are the first to clearly demonstrate that supplemental oral iron intake at usual recommended doses for correcting iron deficiency are excessive as manifested in elevated lipid peroxidation products. Other investigators have not found a relationship between iron intake or iron status and lipid oxidation in humans (8, 11, 26, 27). A low supplemental iron dose (14 mg/d) with vitamin C (260 mg/d) for 12 wks did not elevate LDL oxidation in a group of healthy men and women (8). Also, 50 mg of iron daily by non-anemic iron deficient college women for 8 wks did not affect plasma lipid hydroperoxide and protein carbonyl concentrations (26). In a retrospective study in men and women, dietary iron intake (17–21 mg/d) was not related to levels of non-transferrin-

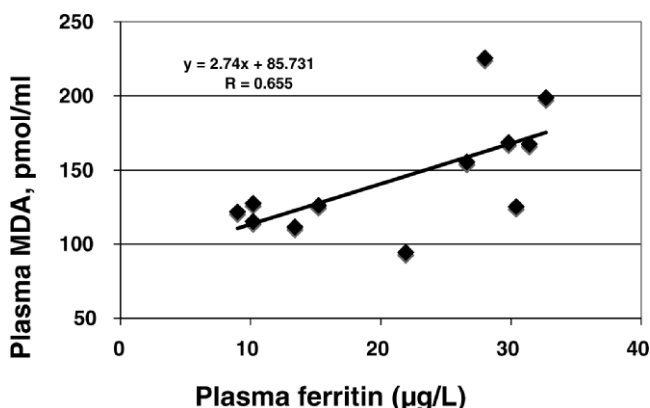


Figure 2. Linear regression between plasma MDA and plasma ferritin in non-pregnant, non-anemic, iron depleted women after 6 wks of daily 98 mg iron supplementation.

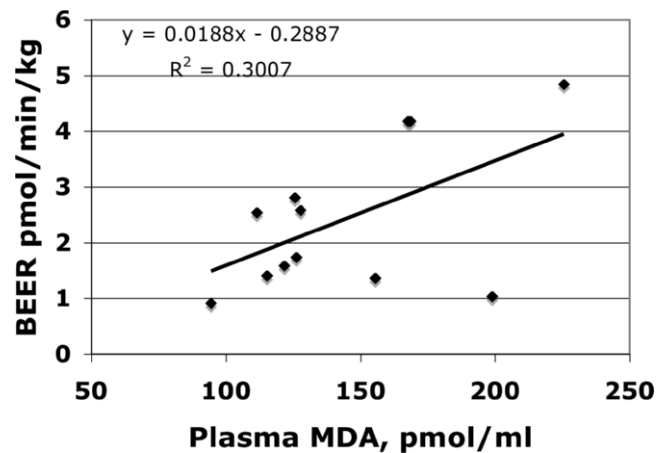


Figure 3. Linear regression between plasma MDA and Breath Ethane Exhalation Rate (BEER) in non-pregnant, non-anemic, iron depleted women after 6 wks of daily 98 mg iron supplementation. Note: Two subjects had the same BEER (4.18 pmol/min/kg) and MDA (168 pmol/ml).

bound iron (NTBI) and of LDL oxidation (27). Also, iron supplementation (50 mg of elemental iron, twice daily with meals for two 3-wk periods with a 2-wk break) did not increase the susceptibility of LDL to oxidation in women with low iron status (11). However, in these studies, the amount of iron ingested and/or the duration of iron supplementation were probably insufficient to cause detectable oxidative responses in plasma lipoproteins, and/or the markers of oxidation used might not be as sensitive responders as plasma MDA and BEER to oxidative stress. In contrast, supplementation with only 19 mg of iron per day as ferrous sulfate for 2 wks was sufficient to increase the concentration of weakly bound iron and the production of free-radicals in feces of adult men and women (7), a marker of increased oxidative stress.

In our study, both BEER and plasma MDA were positively correlated with several indicators of iron status (serum iron, transferrin saturation, and plasma ferritin) suggesting that iron status as modified by iron supplementation increased lipid peroxidation. Although we did not measure non-transferrin bound iron (NTBI) or 'free iron' there is evidence that this iron can catalyze the formation of hydroxyl radicals and stimulate lipid peroxidation (4). Clear elevations of plasma NTBI have been demonstrated in anemic women for at least 4 hours after the ingestion of 65 mg of iron as FeSO_4 both in the fasting state or with food (28). NTBI has been found in the plasma of hemochromatotic (29) and thalassemic (30) patients, but the relationship between liver iron, NTBI, and MDA in these last patients is not always clear (31). Although transferrin saturation was less than 30% in our study after iron supplementation, and the subjects were healthy, the repeated ingestion of highly absorbable ferrous sulfate may have temporarily increased NTBI and exhaust the free radical trapping capacity of the plasma, leading to increased lipid peroxidation. Recently, subjects heterozygous for hereditary hemochromatosis, with

no evidence of iron overload, were found to have significantly higher NTBI than controls, although their transferrin saturation and ferritin values did not differ (6). Thus, NTBI occurs in a number of physiological states affecting iron metabolism and following iron supplementation. Oxidative stress can also initiate tissue injury and inflammation promoting a vicious cycle of supplemental iron, lipid peroxidation and inflammation explaining why plasma MDA levels increased about 45% and BEER by nearly 2-fold in our women.

This paper does not go into the mechanisms involved in producing the elevations of indicators of lipid peroxidation or in defining possible adaptation mechanisms to what appears to be an excessive production of free radicals. The fact is that after 6 weeks and even more, after 8 weeks of iron supplementation at the dose administered, breath ethane exhalation rate, a very specific indicator of lipid peroxidation, kept on rising. At week 6 of supplementation MDA was also elevated. These changes suggest that adaptation to the damage being caused by the daily intake of 98 mg of iron was not occurring even after 2 months of supplement intake. Readers interested in exploring mechanisms in the production of free radicals and the defense mechanisms against ROS and RNS and its health consequences, are referred to References 32–34.

The marked increase in plasma ferritin (about 100%) during the study may reflect, in part, an inflammatory response due to free radical generation consequent to repeated loading of the intestine with unabsorbed iron (7) or the oxidative stress may be due to a more generalized nature in the whole organism. The fact that a rise in serum ferritin with daily iron supplementation could be due in part to inflammation is supported by the fact that the decline seen in plasma ferritin 3 months after discontinuation of iron supplementation in non-pregnant women cannot be fully explained by the normal iron loss (35). Further studies of iron supplementation and markers of inflammation are needed to differentiate the effects of iron storage and inflammation on circulating levels of ferritin.

In conclusion, supplementation with 98 mg iron per day increased indicators of lipid peroxidation in non-anemic, iron-depleted women. Therefore, this regimen of supplemental iron may provide excessive amounts of iron and appears to increase the risk of oxidative stress. Larger and longer term studies with different doses and modalities of iron of supplementation on lipid peroxidation and oxidative stress in women of childbearing age, pregnant or not, and in children, need to be undertaken to expand the results of this study.

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