Both Iron Deficiency and Daily Iron Supplements Increase Lipid Peroxidation in Rats[1,2]

Mitchell D. Knutson,*[3] Patrick B. Walter,† Bruce N. Ames† and Fernando E. Viteri*

*Departments of *Nutritional Sciences and †Molecular and Cell Biology, University of California, Berkeley, CA 94720*

ABSTRACT Numerous studies have shown that iron-loaded diets increase markers of lipid peroxidation in rats, but few have addressed the effects of oral iron supplements on these markers. We investigated the effects of daily and intermittent iron supplements on iron and vitamin E status, and lipid peroxidation. Iron supplements were administered in doses equivalent to those often given to pregnant women in the developing world. In Study 1, iron-deficient (D) and iron-normal (N) rats were fed either 0 or 8000 μg of supplemental iron daily for 21 d. In Study 2, D rats were fed either the same supplements daily or once every 3 d (8 supplements total). Lipid peroxidation was assessed by breath ethane and pentane and by malondialdehyde (MDA) (using GC-MS). In Study 1, daily supplemented N and D rats had liver nonheme iron concentrations that were 1.8- and 2.7-fold higher, respectively, than those in unsupplemented N rats. Breath ethane levels were also higher in supplemented rats ($P < 0.05$), but MDA (in plasma, liver, kidney) and liver vitamin E did not differ. Unexpectedly, severely D, anemic rats had significant elevations in the levels of breath ethane, liver MDA and kidney MDA. In Study 2, liver iron and breath ethane decreased progressively ($P < 0.05$) from 1 d to 3 d after the last iron dose in intermittently supplemented rats. We conclude that iron deficiency results in lipid peroxidation, but that its correction with daily iron supplements results in abnormal iron accumulation and increased lipid peroxidation in rats. These effects are mitigated by intermittent iron supplementation. J. Nutr. 130: 621–628, 2000.

KEY WORDS: • *iron supplements* • *lipid peroxidation* • *breath ethane* • *malondialdehyde* • *iron deficiency* • *rats*

Lipid peroxidation can result in reversible and irreversible cell and tissue damage (Amdur et al. 1991, Dargel 1992). The process of lipid peroxidation is initiated by reactive oxygen species, such as hydroxyl radicals, and is terminated by antioxidants such as vitamin E. Lipid peroxidation is also stimulated by iron ions (Braughler et al. 1986, Minotti and Aust 1987), which can catalyze the formation of the hydroxyl radical and accelerate the decomposition of lipid hydroperoxides (Davies and Slater 1987, Donovan and Menzel 1978). In biological systems, the steady-state level of lipid peroxidation is often assessed by the measurement of lipid peroxidation breakdown products such as malondialdehyde (MDA)[4] (Janero 1990) or breath hydrocarbon gases ethane and pentane (Kneepkens et al. 1994). The degradation of lipid peroxides into hydrocarbon gases appears to be dependent on the presence of transition metal ions (Halliwell and Chirico 1993), and a number of animal studies have shown that excess iron, when given either intraperitoneally (Dillard and Tappel 1979, Dougherty et al. 1981, Harvey and Klaassen 1983) or in the diet (Dresow et al. 1995, Younes et al. 1989), resulted in increased exhalation of ethane and/or pentane. Increases in tissue MDA in rat models of dietary iron overload have also been reported (Brown et al. 1997, Houglum et al. 1990, Lee et al. 1981). In some of these studies, supplementation with vitamin E mitigated the increased lipid peroxidation (Brown et al. 1997, Dillard and Tappel 1979, Dougherty et al. 1981, Dresow et al. 1995, Lee et al. 1981).

Despite extensive literature on iron and lipid peroxidation, few studies have investigated the effects of oral iron supplements on lipid peroxidation. Iron supplements are almost universally prescribed for pregnant women at doses ranging from 30 mg/d in the United States (IOM 1993) to as high as

[1] Portions of this study were presented at the 5th Annual Meeting of the Oxygen Society, Washington D.C., 1998 [Walter, P. B., Knutson, Paler-Martinez, A., Yu, X., Viteri, F. E. & Ames, B. N. (1998) Iron deficiency and supplementation: Damage to DNA, mitochondria and lipids. Free Radic. Biol. Med. 25 (Suppl. 1): p. S80 (abs.).]; at the 4th Annual Meeting of the Oxygen Society, San Francisco, CA, 1997 [Knutson, Walter, P. B., Ames, B. N. & Viteri, F. E. Iron supplements increase lipid peroxidation in normal and iron-deficient rats. p. 101 (abs.)]; and at the 16th International Congress of Nutrition, Montreal, Canada, 1997 [Knutson, Walter, P. B., Ames, B. N. & Viteri, F. E. Daily oral iron supplementation causes sustained iron accumulation and oxidative damage which are mitigated by intermittent iron supplementation. p. 63 (abs.)].

[2] Supported by Bristol Myers Squibb-Mead Johnson, International Nutrition Foundation and Agricultural Research Station Grant CA-B-*-NTS-5854-H (M. D. K. and F. E. V.), National Institute of Environmental Health Sciences Grant ES07075 (P. B. W.), the National Cancer Institute Outstanding Investigator Grant CA39910, and the National Institute of Environmental Health Sciences Center Grant ES01896 (B. N. A).

[3] To whom correspondence should be addressed.

[4] Abbreviations used: D, iron-deficient; ds, daily iron supplements; Dds, iron-deficient + daily iron supplements; Dis, iron-deficient + intermittent iron supplements; is, intermittent iron supplements; MDA, malondialdehyde; N, iron-normal; Nds, iron-normal + daily iron supplements; TBARS, thiobarbituric acid reactive substances; TBHQ, *tert*-butylhydroquinone; TIBC, total iron-binding capacity

Manuscript received 4 August 1999. Initial review completed 7 September 1999. Revision accepted 5 November 1999.

240 mg/d where prevalence of anemia is high (Baker and DeMaeyer 1979, Charoenlarp et al. 1988) For iron supplementation programs where anemia prevalence is high, the International Nutritional Anemia Consultative Group (INACG) has recently changed its recommendation from 120 to 60 mg/d (Stoltzfus and Dreyfus 1999). However, if duration of iron supplementation during pregnancy is short, or if anemia is present, 120 mg/d is still recommended.

In healthy Swedish women, iron absorption from supplements of 100 mg/d was found by Svanberg (1975) to average 6–7% in early pregnancy to 8.6% (range from 5.5 to 15%) in the third trimester. These data indicate that, on average, total absorbed iron was close to 1,400 mg in 28 wk (about twice the average iron requirement during pregnancy) and that the gastrointestinal tract was loaded with over 90 mg of unabsorbed iron each d (18,200 mg in 28 wk). With these iron doses, rates of undesirable gastrointestinal side effects are high, suggesting some "toxic effects," possibly involving iron-related oxidative stress (Hollan and Johansen 1993) This has motivated a few experimental studies searching for efficient and efficacious iron supplementation schemes that minimize undesirable side effects. In a rat model, administration of iron supplements in synchrony with gut mucosal turnover rates (every 3 d) was equally effective at correcting iron deficiency and anemia as was daily supplementation. What is more, intermittent supplementation reduced the constant gut mucosal iron load accompanying daily supplementation and improved the efficiency of iron absorption 2.6-fold (Viteri et al. 1995). Based on these results, the administration of iron supplements weekly instead of daily in humans (in which gut mucosal turnover occurs every 5–6 d) has been proposed and is being actively investigated as a viable means of controlling iron deficiency in populations, including pregnant women (Viteri 1997, Viteri 1998).

We report here the results of two studies. In Study 1, we investigated the effects of daily iron supplements on iron status, vitamin E status and lipid peroxidation in iron-normal (N) and iron-deficient (D) rats. In Study 2, we compared the effects of daily and intermittent iron supplements on these measurements in D rats. In both studies, we used the same rat model that was developed by Viteri et al. (1995) in which iron supplements were administered daily or intermittently (i.e., once every 3 d) in doses equivalent to 120 mg in humans.

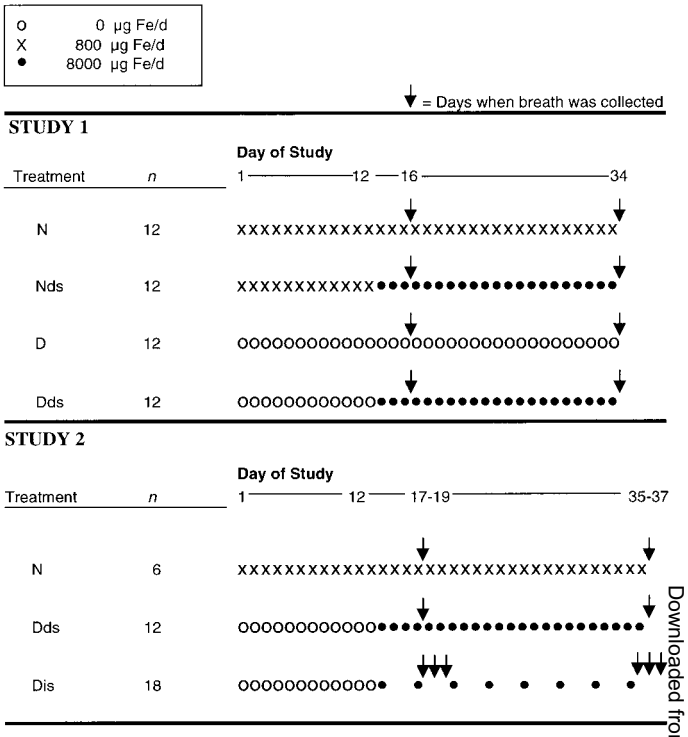

FIGURE 1 Experimental designs. In Study 1, 48 rats were divided into four treatment groups that consumed different amounts of iron during a 34-d period. N = iron normal; D = iron deficient; ds = + daily iron supplements. Between d 1–12, Nds rats consumed normal amounts of iron (800 μg Fe/d) while Dds rats consumed essentially no iron (to induce iron deficiency). Starting on d 13, Nds and Dds rats were fed daily iron supplements (8000 μg Fe/d). In Study 2, 36 rats were divided into three treatment groups that consumed different amounts of iron during a 37-d period. The two iron-supplemented groups received essentially no iron during the first 12 d of the study, but starting on d 13 received iron supplements (8000 μg Fe) either every d (Dds) or intermittently (Dis) (once every 3 d). For the 18 Dis rats, breath samples were collected from groups of 6 rats/d on d 17–19 and on d 35–37 corresponding to 1, 2 and 3 d after receiving an iron dose, respectively. In both studies, rats were fed the appropriate amount of iron in the form of a premeal and consumed a modified AIN-93G diet prepared without iron. Rats were killed after the last breath collection, and blood and tissues were collected.

MATERIALS AND METHODS

Animals. Weanling male Sprague-Dawley rats (Bantin and Kingman, Fremont, CA) were weighed and assigned to treatment groups to ensure similar mean initial weights (ca. 50 g). Rats were housed individually in wire-bottomed cages in a temperature-controlled (25°C ± 2°C) and humidity-controlled room that was lighted between 0700 and 1900 h. The experimental protocol was approved by the Animal Care and Use Committee of the University of California at Berkeley.

Study 1. The effects of daily iron supplements (ds) on iron status, vitamin E status, and lipid peroxidation in N and D rats were investigated (**Fig. 1**). A 2 X 2 factorial design was used with an N, a D, and two similar groups that received daily iron supplements (Nds) and (Dds). Supplementation began on d 13 of the study.

Study 2. The effects of daily iron supplements (ds) and intermittent iron supplements (is) on iron status, vitamin E status and lipid peroxidation in D rats were investigated (Fig. 1). Study 2 included three treatments: an N group and two D groups that received either daily iron supplements (Dds) or intermittent supplements (Dis), beginning on d 13 of the study. To investigate the possibility that iron status and lipid peroxidation could vary considerably in the days following intermittent iron dosing, the 18 Dis rats were killed in groups of six rats 1, 2, and 3 d after receiving the last iron dose.

Diet and premeal preparations. Purified diet was prepared according to the AIN-93G formulation (Reeves et al. 1993) with several modifications. The diet was prepared without iron in the mineral mix, and tocopherol-stripped soybean oil (stabilized with *tert*-butylhydroquinone, TBHQ) (Dyets, Bethlehem, PA) was used. Vitamin E (107 mg *d*-α-tocopherol acetate) was added per kg of tocopherol-stripped soybean oil to account for the amount (estimated from food composition tables) that was removed during the tocopherol-stripping process. Oil and vitamin E were included in the diet in this fashion to administer the desired amount of vitamin E because commercial oil sources vary in vitamin E content. Other modifications were: BHT was used as the antioxidant instead of TBHQ in the diet; menadione was used instead of phylloquinone; and retinol acetate was used instead of retinol palmitate.

Three bulk batches of "premeals" were prepared by mixing diet and powdered sucrose (1:1). Iron (crystalline ferrous sulfate, $FeSO_4 \cdot 7H_2O$) was finely ground by mortar and pestle and then added to the bulk batches of premeals to achieve either 0, 400 or 4000 μg of elemental iron per 0.7 g of premeal.

Animal feeding. Rats were fed twice daily, once at 0700 h and once at 1700 h. Each feeding consisted of premeal feeding (10–20

min), followed by meal feeding (2 h ± 15 min). In the premeal feeding, rats were given 0.7 g of premeal in a small glass dish. Rats were given the food bowl (meal feeding) only after completely consuming the premeal; this ensured the ingestion of the proper amount of iron per day. Between feedings, food bowls and bulk batches of premeal were stored at 4°C. All rats had free access to distilled water throughout the studies.

Tissue collections. After the collection of air-breath samples on d 34 (Study 1) or d 35–37 (Study 2), rats were anesthetized with Nembutal® and killed by exsanguination by aortic puncture. Blood was collected into heparinized syringes and stored on ice until centrifuged to separate plasma. Livers and kidneys were quickly removed, weighed (±0.002 g), immediately frozen in liquid nitrogen and then stored at −80°C until analysis.

Iron status measurements. Hemoglobin was determined by the azidemethemoglobin method using the HemoCue blood Hemoglobin system (HemoCue,, Mission Viejo, CA). Liver and kidney nonheme iron was measured colorimetrically after acid digestion of tissues (Torrance and Bothwell 1968). Plasma iron and total iron-binding capacity (TIBC) were determined colorimetrically (INACG 1985). For standards, iron reference solution (1 g Fe/L) was used (Fisher Scientific, Santa Clara, CA). A control serum (Iron/UIBC Control, Level 1; Sigma Chemical, St. Louis, MO, Catalog #1389) sample was included in the analysis of the plasma iron and TIBC determinations to verify accuracy of measurement.

Liver vitamin E measurement. Liver tocopherol was measured by the method of Taylor et al. (1976). Briefly, tissue homogenates were incubated for 30 min at 70°C in ethanolic KOH in the presence of excess ascorbate. After saponification, the nonsaponifiable lipids (which includes tocopherols) were extracted into hexane, and the tocopherol was measured fluorometrically.

Plasma triglyceride measurement. Triglycerides in plasma were measured by absorbance of quinoneimine dye at 540 nm using a kit (triglycerides, GPO-Trinder; procedure No. 339, Sigma Chemical).

Assessment of lipid peroxidation. Breath ethane and pentane and tissue and plasma MDA were used as markers of in vivo lipid peroxidation. Air-breath samples were collected for the subsequent determination of ethane and pentane by cryofocusing and gas chromatography (Knutson and Viteri 1996). In Study 1 breath was collected on d 16 and before the rats were anesthetized on d 34. In Study 2, breath from the same six rats of the Dis group was collected on d 17, 18 and 19—i.e., 1, 2 and 3 d after their second intermittent iron dose. These rats had breath collected again, together with four other rats before killing either 1, 2 or 3 d after the last supplementation day. All breath collections were performed before the morning feeding. Liver, kidney and plasma MDA were measured by GC-MS according to Yeo et al. (1994), but with two modifications: desferrioxamine was added to the homogenization buffer (final concentration of 3.5 mmol/L) and the amount of BHT was increased from 10 μmol/L to 3.85 mmol/L (concentration in assay). These modifications were made to prevent iron-catalyzed, ex vivo MDA formation that might occur during sample processing and analysis.

Statistical analyses. All values are expressed as means ± SEM for the number of rats in parentheses. Statistical analyses were performed using SPSS software, 1997 version (SPSS, Chicago, IL). For Study 1, ethane and pentane data were analyzed by three-factor, repeated measures ANOVA, with two grouping factors (iron status, iron supplementation) and one trial factor (time). All other data in Study 1 were analyzed by two-way ANOVA with two grouping factors (iron status, iron supplementation). If interactions were found between grouping factors, data were reanalyzed by one-way ANOVA, followed up by Tukey's studentized range test at a type I error of 0.05. For Study 2, the data for the Dis rats were analyzed as one combined group (18 rats) and as three subgroups (six rats/group), grouped by date of killing. Data were analyzed by one-way ANOVA, with accepted level of significance of 0.05. When differences were statistically significant, we used Bonferroni-Dunn techniques to follow up the 10 pairwise comparisons for the 5 means (N, Dds and 3 Dis groups). In addition, two more pairwise comparisons were made: the combined Dis group vs. N and the combined Dis group vs. Dds. Each of these 12 total follow-up comparisons were performed at $\alpha = 0.01$ for a maximum procedure-wise error rate of 0.12. In both studies, data were log-transformed when the standard deviation was proportional to the mean. Statistical outliers, as determined by Dixon's test (Dixon and Massey 1969), were omitted from statistical analyses.

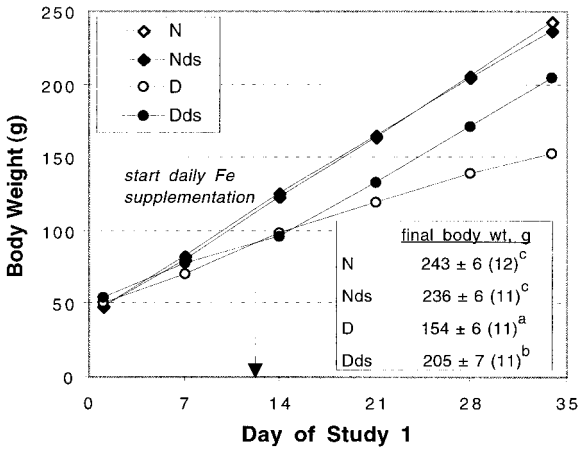

FIGURE 2 Effect of daily iron supplements on body weights of iron-normal and iron-deficient rats (Study 1). N = iron normal; D = iron deficient; ds = + daily iron supplements (8000 μg Fe/d for 21 d). Results at each day are group means. Final body weights are mean ± SEM, with the number of rats in parentheses. Values not sharing a superscript are significantly different ($P < 0.05$).

RESULTS

Study 1

Morphometry. D rats grew poorly (**Fig. 2**); the mean body weight of these rats at the end of the study was 63% of normal rats. Weight gain in the Dds rats increased after the consumption of ds, and by 22 d, mean body weight was 85% of that of N rats. The ds did not affect body weights of Nds rats. The data from three "abnormal" rats were omitted from all analyses: one D rat which had a kidney tumor and a final body weight that was 53 g below the group mean; one Dds rat which had frequent diarrhea and a final body weight that was 69 g below the group mean; and one Nds rat which frequently refused to consume the iron containing premeal and which had a final body weight that was 92 g below the group mean.

Iron status. As indicated by hemoglobin concentration, D rats were severely anemic after 35 d (**Table 1**). The markedly reduced liver nonheme iron concentrations and percentage of transferrin saturations in these rats confirmed the presence of iron deficiency. Nds and Dds rats had liver nonheme iron levels that were 1.8- and 2.7-fold higher, respectively, than liver nonheme iron levels in N rats.

Liver vitamin E and plasma triglycerides. D rats had significantly elevated liver vitamin E concentrations and markedly elevated plasma triglyceride concentrations relative to N, Nds and Dds rats (**Table 2**).

Lipid peroxidation. Ethane evolution rates showed significant main effects for iron status, iron supplement intake and time (**Table 3**). At d 34, ethane evolution rates were higher in both Nds and Dds rats (1.59, $n = 22$) than in N and D rats (1.30, $n = 23$). Ethane and pentane evolution rates decreased between d 16 and 34, and were elevated in D rats. MDA levels were significantly elevated in kidney and liver of the D rats (**Table 4**).

TABLE 1

Effect of daily iron supplements on iron status in iron-normal and iron-deficient rats[1] (Study 1)

Treatment[2]	Hemoglobin	Liver nonheme Fe[3]	TIBC saturation[4]
	g/L	*μmol/g wet liver*	*%*
N	133 ± 1.9 (12)[b]	2.3 ± 0.24 (12)[b]	40.1 ± 2.5 (12)[b]
Nds	135 ± 2.2 (11)[b]	6.4 ± 0.73 (11)[c]	38.3 ± 4.2 (11)[b]
D	38 ± 1.9 (11)[a]	0.4 ± 0.02 (11)[a]	5.7 ± 0.3 (10)[a]
Dds	131 ± 2.0 (10)[b]	8.6 ± 0.62 (11)[c]	35.2 ± 2.6 (8)[b]

[1] Values are means ± SEM; values in parentheses indicate number of samples analyzed. Within a column, values not sharing a superscript are significantly different ($P < 0.05$).
[2] N = iron-normal; D = iron-deficient; ds = + daily iron supplements (8000 μg Fe/d for 21 d).
[3] Data were log-transformed prior to statistical analysis. Untransformed means ± SEM are presented.
[4] TIBC = total iron-binding capacity of plasma. TIBC saturation (%) = (plasma Fe/TIBC) × 100.

Study 2

Morphometry. Throughout the study, body weights of the D rats did not differ whether they received daily or intermittent iron supplements (is) (**Fig. 3**).

Iron status and liver vitamin E. Hemoglobin and liver vitamin E levels did not significantly differ among groups at the end of the study (**Table 5**). In both Dds and Dis rats, liver nonheme iron levels were higher than those of N rats. However, Dds rats had higher liver nonheme iron levels than did the Dis rats.

Lipid peroxidation. For the first set of breath collections (d 17–19) (**Table 6**), the 1, 2 and 3 d post-Fe dose in the Dis rats represents the means for the same six rats that were measured on three successive days. This is in contrast to the second set of breath collections (d 34–36), in which the successive day in the Dis group represents the means for six different rats at each time point (18 rats total). It would have been ideal at the second collection set to have collected from the same rats for three consecutive days as was done in the first collection set, but this was not possible, because the rats were killed immediately after their breath was collected. No significant differences in ethane or pentane evolution rates were found between treatment groups at either collection time point. However, among the Dis rats at d 34–36, rats killed 3 d after receiving the last iron supplement had significantly lower ethane evolution rates when compared with those killed 1 d after.

TABLE 2

Effect of daily iron supplements on liver vitamin E and plasma triglycerides in iron-normal and iron-deficient rats[1] (Study 1)

Treatment[2]	Liver vitamin E	Plasma triglycerides
	nmol/g wet tissue	*mmol/L*
N	114 ± 7.0 (12)[a]	0.32 ± 0.03 (12)[a]
Nds	119 ± 4.9 (11)[a]	0.34 ± 0.04 (11)[a]
D	171 ± 20 (11)[b]	1.89 ± 0.41 (8)[b]
Dds	103 ± 6.0 (11)[a]	0.33 ± 0.02 (11)[a]

[1] Values are means ± SEM; values in parentheses indicate number of samples analyzed. Within a column, values not sharing a superscript are significantly different ($P < 0.05$).
[2] N = iron-normal; D = iron-deficient; ds = + daily iron supplements (8000 μg Fe/d for 21 d).

TABLE 3

Effects of daily iron supplements on breath ethane and pentane in iron-normal and iron-deficient rats at d 16 and d 34[1,2] (Study 1)

Treatment[3]	Day[4]	Ethane	Pentane
		pmol · 100 g body^{-1} · min^{-1}	
N	16	1.39 ± 0.11 (12)	1.00 ± 0.07 (11)
N	34	1.12 ± 0.05 (12)	0.79 ± 0.10 (12)
Nds	16	1.69 ± 0.14 (11)	1.16 ± 0.09 (11)
Nds	34	1.36 ± 0.12 (11)	0.75 ± 0.09 (12)
D	16	1.83 ± 0.17 (11)	1.55 ± 0.17 (9)
D	34	1.50 ± 0.13 (11)	0.97 ± 0.14 (11)
Dds	16	2.02 ± 0.37 (11)	1.49 ± 0.12 (9)
Dds	34	1.82 ± 0.13 (11)	0.85 ± 0.11 (11)
ANOVA (*P* value)[5]			
Fe status (N vs. D)		0.002	0.004
Fe supplements (ds vs. no ds)		0.038	0.692
Fe status × Fe supplements		0.961	0.500
Days (d 16 vs. d 34)		0.028	0.000
Fe status × days		0.875	0.014
Fe supplements × days		0.883	0.431
Fe status × Fe supplements × days		0.727	0.758

[1] Values are means ± SEM; values in parentheses indicate number of samples analyzed.
[2] Data were log-transformed prior to statistical analysis. Untransformed means ± SEM are presented.
[3] N = iron-normal; D = iron-deficient; ds = + daily iron supplements (8000 μg Fe/d for 21 d).
[4] At d 16, Fe-supplemented rats had consumed Fe supplements for 3 d; at d 34, rats had consumed Fe supplements for 21 d.
[5] Data were analyzed by three-factor, repeated measures ANOVA with two grouping factors (iron status, iron supplements) and one trial factor (days). Differences of $P < 0.05$ were considered significant for main treatment effects and their interaction.

TABLE 4

Effect of daily iron supplements of malondialdehyde (MDA) levels in iron-normal and iron-deficient rats[1,2] (Study 1)

Treatment[3]	Liver MDA	Kidney MDA	Plasma MDA
	nmol/g wet tissue		*nmol/L*
N	0.66 ± 0.08 (12)[a]	1.02 ± 0.01 (12)[a,b]	61.2 ± 4.5 (10)
Nds	0.52 ± 0.05 (11)[a]	0.96 ± 0.10 (11)[a]	64.5 ± 6.1 (11)
D	2.24 ± 0.24 (11)[b]	2.56 ± 0.19 (11)[c]	57.1 ± 6.4 (10)
Dds	0.78 ± 0.13 (11)[a]	1.51 ± 0.16 (11)[b]	62.2 ± 5.9 (8)

[1] Values are means ± SEM; values in parentheses indicate number of samples analyzed. Within a column, values not sharing a superscript are significantly different ($P < 0.05$).
[2] Data were log-transformed prior to statistical analysis. Untransformed means ± SEM are presented.
[3] N = iron-normal; D = iron-deficient; ds = + daily iron supplements (8000 μg Fe/d for 21 d).

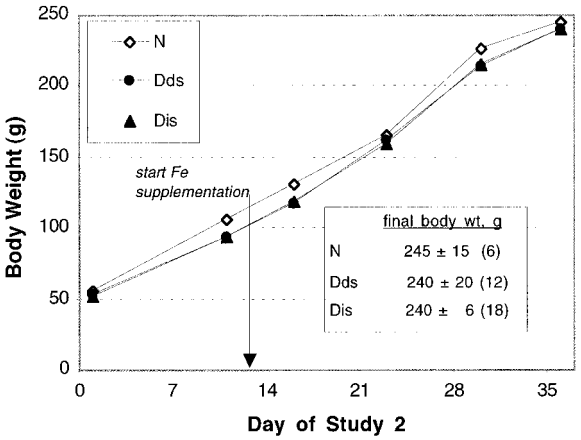

FIGURE 3 Effect of daily and intermittent iron supplements on body weights of iron-deficient rats (Study 2). N = iron normal; Dds = iron deficient + daily iron supplements (8000 μg Fe/d for 24 d); Dis = iron deficient + intermittent iron supplements (8000 μg Fe/every 3 d, 8 total doses). Results at each day are group means. Final body weights are mean ± SEM, with the number of rats in parentheses.

DISCUSSION

The consumption of ds resulted in an accumulation of liver nonheme iron and increased lipid peroxidation, as measured by breath ethane (Study 1). The finding of significantly elevated ethane exhalation rates in daily iron-supplemented rats is consistent with previous studies of animals with minor iron overload. Younes et al. (1989) found that rats with 2.5-fold higher liver nonheme iron levels (subsequent to the feeding of a 3.5% ferrous fumarate diet) had 60% higher ethane exhalation rates when compared with iron-normal controls. Similarly in carbonyl-iron-fed mice, Kawabata et al. (1989) found that six-fold higher liver nonheme iron levels were associated with 2.8-fold higher ethane exhalation rates. A positive correlation between ethane exhalation rates and liver iron concentrations has also been described in ferrocene-iron-loaded rats (Dresow et al. 1995).

In contrast to ethane, breath pentane levels did not increase in the iron-supplemented rats. Different sensitivities of ethane and pentane as markers in vivo lipid peroxidation may account for this. In previous studies in which both ethane and pentane have been measured, ethane has been shown to be more sensitive than pentane in response to intraperitoneal injections of iron (Dillard and Tappel 1979, Filser et al. 1983). These reports, in addition to the numerous difficulties encountered in the measurement and interpretation of pentane measurements (Kohlmuller and Kochen 1993, Springfield and Levitt 1994), have led to ethane as being the marker of choice in studies of dietary iron overload—studies more relevant to the present study in which iron was administered orally. We are aware of no studies of dietary iron overload and pentane measurements. Another factor that may contribute to the different responses in the hydrocarbons may be that pentane is metabolized extensively in vivo whereas ethane is not (Filser et al. 1983).

Ethane production can be modulated by dietary vitamin E. Dougherty et al. (1981) found that supplementation of rats with 200 mg of vitamin E/kg diet could prevent increases in ethane production caused by injection of iron dextran. Similarly, Dresow et al. (1995) noted that increases in ethane exhalation rates in iron-loaded rats were delayed in rats fed 164 mg of vitamin E/kg diet when compared to rats fed 1 mg of vitamin E/kg diet. In the present study, exhalation rates were elevated in daily iron-supplemented rats despite their consumption of diet that contained high levels of vitamin E (150 mg/kg diet).

Although ethane appears to be a sensitive and reliable index of lipid peroxidation (Filser et al. 1983, Kneepkens et al. 1994), certain factors complicate the interpretation of ethane measurements in studies of iron overload. For example, the breakdown of lipid hydroperoxides into hydrocarbon gases in vivo appears to be dependent upon the presence of transition metal ions (Halliwell and Chirico 1993), and thus an increase in the formation of ethane from already peroxidized lipids in conditions of iron overload may reflect an increase in the availability of reactive iron ions and not necessarily an increase in overall lipid peroxidation. It is also conceivable that the elevated ethane levels in the iron-supplemented rats was not due to an increase in in vivo peroxidation of cell membrane lipids, but to an increase in ethane generated by the iron-catalyzed peroxidation of contents in the gut lumen (e.g., undigested foodstuffs or unexcreted fecal material), for the gut lumen of animals receiving iron supplements was nearly constantly loaded with iron. Alterations in gut flora in the iron-loaded animals might also have altered breath ethane levels. The influence of these factors on breath ethane exhalation requires further investigation.

That MDA levels in liver, kidney and plasma were not elevated in daily iron-supplemented rats may indicate that there was no increase in lipid peroxidation in these tissues. Conversely, there may have been an increase in MDA formation that was accompanied by a commensurate increase in the metabolism of MDA. Like pentane, MDA is extensively metabolized in vivo (Draper and Hadley 1990). Several studies have reported elevated MDA levels in homogenates of iron-loaded livers (Brown et al. 1997, Houglum et al. 1990, Lee et al. 1981), but thiobarbituric acid reactive substances (TBARS) were measured instead of MDA specifically. The TBA-test measures MDA present in the sample, plus MDA that is purposefully generated by the breakdown of lipid hy-

TABLE 5

Effects of daily and intermittent iron supplements on hemoglobin, liver nonheme iron, and vitamin E levels in iron-deficient rats[1] (Study 2)

Treatment[2]	Hemoglobin	Liver nonheme Fe[3]	Liver vitamin E
	g/L	*μmol/g wet liver*	*nmol/g wet liver*
N	140 ± 3.0 (6)	1.3 ± 0.29 (6)[a]	100 ± 4.2 (6)
Dds	131 ± 3.0 (12)	7.3 ± 0.71 (12)[c]	101 ± 3.5 (12)
Dis			
1-d post[4]	136 ± 7.0 (6)	5.1 ± 0.80 (6)[b,c]	91 ± 6.5 (6)
2-d post	129 ± 1.0 (6)	3.9 ± 0.67 (6)[b]	102 ± 6.7 (6)
3-d post	134 ± 2.0 (6)	3.1 ± 0.44 (6)[b]	102 ± 4.2 (6)
Dis (combined)[5]	133 ± 2.0 (18)	4.0 ± 0.41 (18)[b]	101 ± 3.7 (18)

[1] Values are means ± SEM; values in parentheses indicate number of samples analyzed. Within a column, values not sharing a superscript are significantly different ($P < 0.05$).

[2] N = iron-normal; Dds = iron-deficient + daily iron supplements (8000 μg Fe/d for 24 d); Dis = iron-deficient + intermittent iron supplements (8000 μg Fe/every 3 d, eight total doses).

[3] Data were log-transformed prior to statistical analysis. Untransformed means ± SEM are presented.

[4] The 18 Dis rats were killed in groups of six rats 1, 2 and 3 d after having received the last iron dose. The results for these three groups are also presented as a combined mean.

[5] Statistical analyses of the Dis rats, when combined as one group, reflect comparison to only the N and Dds groups.

TABLE 6

Effects of daily and intermittent iron supplements on breath ethane and pentane in iron-deficient rats at d 17–19 and 35–37[1,2,3] (Study 2)

Treatment[4]	Ethane d 17–19	Ethane d 34–36	Pentane d 17–19	Pentane d 34–36
		pmol · 100 g body wt^{-1} · min^{-1}		
N	1.62 ± 0.26 (6)	2.20 ± 0.31 (6)[a,b]	0.93 ± 0.17 (6)	0.76 ± 0.16 6)
Dds	2.33 ± 0.49 (11)	3.17 ± 0.60 (12)[a,b]	1.12 ± 0.16 (12)	0.98 ± 0.12 (11)
Dis				
1-d post[5]	1.99 ± 0.35 (6)	3.39 ± 0.48 (6)[b]	0.96 ± 0.07 (6)	0.96 ± 0.07 (6)
2-d post	2.29 ± 0.38 (6)	2.12 ± 0.36 (6)[a,b]	1.13 ± 0.09 (6)	0.83 ± 0.04 (6)
3-d post	1.44 ± 0.19 (6)	1.60 ± 0.09 (5)[a]	1.00 ± 0.22 (6)	0.81 ± 0.17 (5)
Dis (combined)[6]	1.91 ± 0.19 (18)	2.42 ± 0.28 (17)[a,b]	1.03 ± 0.08 (18)	0.87 ± 0.06 (17)

[1] Values are means ± SEM; values in parentheses indicate number of samples analyzed. Within a column, values not sharing a superscript are significantly different ($P < 0.05$).
[2] Data were log-transformed prior to statistical analysis. Untransformed means ± SEM are presented.
[3] Breath samples for each group were collected on only one of these days.
[4] N = iron-normal; Dds = iron-deficient + daily iron supplements (8000 μg Fe/d for 24 d); Dis = iron-deficient + intermittent iron supplements (8000 μg Fe/every 3 d, eight total doses).
[5] The 18 Dis rats were killed in groups of six rats 1, 2 and 3 d after having received the last iron dose. The results for these three groups are also presented as a combined mean.
[6] Statistical analyses of the Dis rats, when combined as one group, reflect comparison to only the N and Dds groups.

droperoxides during the assay. The TBA-test can also measure various TBARS other than MDA (Janero 1990). What is more, we have found that unless a strong iron chelator is added to the homogenization buffer, a large amount of MDA is formed from iron-loaded liver, probably through iron-catalyzed lipid peroxidation/decomposition that occurs during the homogenization process. Thus, it is possible that in other studies, the increase in lipid peroxidation (as measured by TBARS) in iron-loaded livers may have occurred during homogenization and not in vivo.

In a recent study using a roughly similar study design, Srigiridhar and Madhavan Nair (1998) reported that ds increased lipid peroxidation, as well as protein oxidation, in D rat intestinal mucosa. Weanling female rats were made D by consuming a low-iron diet and were then supplemented with 8000 μg of iron (as ferrous sulfate) for 15 d. Iron-supplemented, D rat intestine had two times more MDA than did unsupplemented D rats and 1.6 times more MDA than did unsupplemented normal control rats. However, because TBARS were measured, it is unclear if the higher levels of MDA resulted from increased in vivo lipid peroxidation or from increased iron-catalyzed, ex vivo lipid peroxidation/decomposition. In this same study, the D, iron-supplemented rats had liver nonheme iron levels that were 2.9 times higher than those of unsupplemented N rats. These levels of liver iron are similar to what we report here. These authors did not measure liver MDA.

Contrary to expectations, we found that D rats had increased lipid peroxidation: ethane, pentane, liver MDA and kidney MDA were all significantly increased in these rats. This is in contrast to most previous reports of iron deficiency. One group has reported significantly decreased liver MDA (as measured by TBARS) in D rats (Rao and Jagadeesan 1996), and other reports provide evidence that iron deficiency is protective against in vivo lipid peroxidation (Chandler et al. 1988) and hydroxyl radical formation (Patt et al. 1990). However, increased lipid peroxidation in iron deficiency has been reported by Uehara et al. (1997), who found that D rats had increased levels of serum and liver phosphatidylcholine hydroperoxide, an indicator of cell membrane lipid peroxidation.

Several factors may contribute to elevated lipid peroxidation in D rats. First, it has been demonstrated repeatedly that D rats rapidly accumulate liver copper (Sherman and Moran 1984, Sherman and Tissue 1981, Sourkes et al. 1968). As with excess iron, excess copper can also catalyze lipid peroxidation (Bremner 1998). The D rats that had increased lipid peroxidation in the study by Uehara et al. (1997) had copper concentrations in liver mitochondrial and nuclear subcellular fractions that were >8-fold higher than those in N rats. Second, D rats have been shown to accumulate triglycerides in liver and plasma (Masini et al. 1994, Uehara et al. 1997). High concentrations of triglyceride provide more lipid substrate for lipid peroxidation, and this may have contributed to the high levels of liver and kidney MDA in our D rats (provided that triglycerides were elevated in these tissues). Third, the increase in lipid peroxidation may be due to increased fragility of mitochondrial membranes of D rat tissue mitochondria, which have been described as being greatly enlarged and swollen (Dallman and Goodman 1970).

The increased ethane and pentane exhalation rates in the D rats may have been exaggerated by the low body weights of these rats. Hydrocarbon exhalation rates were expressed per 100 g of body weight, and thus elevations in hydrocarbon exhalation rates may simply reflect the significantly lower body weights of the D rats. Indeed, a negative correlation between ethane exhalation rate and body weight in rats has been reported (Topp et al. 1995). However, because both liver and kidney MDA levels were also markedly elevated in the D rats, it seems probable that the increased ethane and pentane exhalation rates were due to increased lipid peroxidation and not merely to low body weights.

In studies of rats with chronic iron overload, liver vitamin E levels have been negatively correlated with liver iron levels (Dresow et al. 1995, Ward et al. 1991). In our rats with subacute, minor iron overload, we observed no relationship between concentrations of liver vitamin E and liver iron, but we did find significantly elevated liver vitamin E levels in the D rats. The elevated liver vitamin E may be attendant to the accumulation of liver triglycerides that has been reported to occur in D rats (Masini et al. 1994, Uehara et al. 1997).

Although we did not measure liver triglycerides in these rats, it is likely that they were increased considering the five-fold increase in plasma triglyceride concentrations.

An important finding in Study 2 was that liver nonheme iron levels and ethane exhalation rates varied considerably in the 3 d following intermittent iron dosing. When the intermittently supplemented rats were grouped by killing date (either 1, 2 or 3 d post last iron dose), liver nonheme iron levels decreased progressively. The stepwise decrease may reflect the rat's ability to actively excrete iron. Cheney et al. (1967) estimated that 10–15% of plasma iron is normally taken up by the gut mucosa and lost from the body when these cells are sloughed. Iron-supplemented rats may excrete even a higher percentage of plasma iron by this mechanism (National Research Council 1979). Alternatively, the decreasing liver iron concentrations may be partly due to the increasing body weights; the final body weights (mean ± SEM) of the rats killed 1, 2 and 3 d after the last iron dose were 221 ± 6, 246 ± 30, 253 ± 19 g, respectively.

The parallel decreases in liver iron and breath ethane are consistent with the hypothesis that iron levels can modulate lipid peroxidation. Unfortunately, the considerable variability in iron levels and ethane exhalation rates in the 3 d following the last intermittent iron dose complicates comparisons between intermittent and ds. Nevertheless, several generalizations can be made when the 18 intermittently supplemented rats are considered as one group: *i*) Intermittent iron supplements corrected iron deficiency as well as did daily iron supplements. *ii*) Intermittently iron-supplemented rats accumulated nearly 50% less liver iron than did daily iron-supplemented rats. *iii*) Intermittently iron-supplemented rats had 24% lower levels of breath ethane compared with daily iron-supplemented rats, though the differences did not reach statistical significance.

In conclusion, the finding of increased lipid peroxidation in D rats identifies yet another adverse effect of iron deficiency and further emphasizes the need for preventing and correcting it. However, this study also demonstrates that the consumption of relatively high doses of daily iron supplements promotes abnormal iron accumulation and lipid peroxidation. These potential hazards thus call into question the desirability of consuming relatively large doses of daily iron. We are currently conducting studies of the effects of daily and weekly iron supplements on lipid peroxidation in humans.

ACKNOWLEDGMENTS

We thank Phu Truong for his assistance with feeding the rats, Mark Hudes for his help with statistical analyses and Jiankang Liu and Stephan Christen for their help with the GC-MS analysis of MDA.

LITERATURE CITED

Amdur, M. O., Doull, J. & Klaassen, C. D., eds. (1991) Casarett and Doull's Toxicology: The Basic Science of Poisons, 4th Ed. Pergamon Press: Elmsford, NY.

Baker, S. J. & DeMaeyer, E. M. (1979) Nutritional anemia: its understanding and control with special reference to the work of the World Health Organization. Am. J. Clin. Nutr. 32: 368–417.

Braughler, J. M., Duncan, L. A. & Chase, R. L. (1986) The involvement of iron in lipid peroxidation. Importance of ferric to ferrous ratios in initiation. J. Biol. Chem. 261: 10282–10289.

Bremner, I. (1998) Manifestations of copper excess. Am. J. Clin. Nutr. 67: 1069S–1073S.

Brown, K. E., Poulos, J. E., Li, L., Soweid, A. M., Ramm, G. A., O'Neill, R., Britton, R. S. & Bacon, B. R. (1997) Effect of vitamin E supplementation on hepatic fibrogenesis in chronic dietary iron overload. Am. J. Physiol. 272: G116–123.

Chandler, D. B., Barton, J. C., Briggs, D. D. D., Butler, T. W., Kennedy, J. I., Grizzle, W. E. & Fulmer, J. D. (1988) Effect of iron deficiency on bleomycin-induced lung fibrosis in the hamster. Am. Rev. Respir. Dis. 137: 85–89.

Charoenlarp, P., Dhanamitta, S., Kaewvichit, R., Silprasert, A., Suwanaradd, C., Na-Nakorn, S., Prawatmuang, P., Vatanavicharn, S., Nutcharas, U., Pootrakul, P., Tanphaichitr, V., Thanangkul, O., Vaniyapong, T., Thane Toe, Valyasevi, A., Baker, S., Cook, J., DeMaeyer, E. M., Garby, L. & Hallberg, L. (1988) A WHO collaborative study on iron supplementation in Burma and in Thailand. Am. J. Clin. Nutr. 47: 280–297.

Cheney, B.A., Lothe, K., Morgan, E.H., Sood, S. K. & Finch, C.A. (1967) Internal iron exchange in the rat. Am. J. Physiol. 212: 376–380.

Dallman, P. R. & Goodman, J. R. (1970) Enlargement of mitochondrial compartment in iron and copper deficiency. Blood 35: 496–505.

Dargel, R. (1992) Lipid peroxidation—a common pathogenetic mechanism? Exp. Toxicol. Pathol 44: 169–181.

Davies, M. J. & Slater, T. F. (1987) Studies on the metal-ion and lipoxygenase-catalysed breakdown of hydroperoxides using electron-spin-resonance spectroscopy. Biochem. J. 245: 167–173.

Dillard, C. J. & Tappel, A. L. (1979) Volatile hydrocarbon and carbonyl products of lipid peroxidation: a comparison of pentane, ethane, hexanal, and acetone as in vivo indices. Lipids 14: 989–995.

Dixon, W. J. & Massey, F. J. (1969) Introduction to Statistical Analysis, 3rd Ed. McGraw-Hill, Inc.: New York.

Donovan, D. H. & Menzel, D. B. (1978) Mechanisms of lipid peroxidation: iron catalyzed decomposition of fatty acid hydroperoxides as the basis of hydrocarbon evolution in vivo. Experientia 34: 775–776.

Dougherty, J. J., Croft, W. A. & Hoekstra, W. G. (1981) Effects of ferrous chloride and iron dextran on lipid peroxidation in vivo in vitamin E and selenium adequate and deficient rats. J. Nutr. 111: 1784–1796.

Draper, H. H. & Hadley, M. (1990) A review of recent studies on the metabolism of exogenous and endogenous malondialdehyde. Xenobiotica 20: 901–907.

Dresow, B., Albert, C., Zimmermann, I. & Nielsen, P. (1995) Ethane exhalation and vitamin E/ubiquinol status as markers of lipid peroxidation in ferrocene iron-loaded rats. Hepatology 21: 1099–1105.

Filser, J. G., Bolt, H. M., Muliawan, H. & Kappus, H. (1983) Quantitative evaluation of ethane and n-pentane as indicators of lipid peroxidation in vivo. Arch. Toxicol. 52: 135–147.

Halliwell, B. & Chirico, S. (1993) Lipid peroxidation: its mechanism, measurement, and significance. Am. J. Clin. Nutr. 57: 715S–724S; discussion 724S-725S.

Harvey, M. J. & Klaassen, C. D. (1983) Interaction of metals and carbon tetrachloride on lipid peroxidation and hepatotoxicity. Toxicol. Appl. Pharmacol. 71: 316–322.

Hollan, S. & Johansen, K. S. (1993) Adequate iron stores and the 'Nil nocere' principle. Haematologia 25: 69–84.

Houglum, K., Filip, M., Witztum, J. L. & Chojkier, M. (1990) Malondialdehyde and 4-hydroxynonenal protein adducts in plasma and liver of rats with iron overload. J. Clin. Invest. 86: 1991–1998.

INACG (International Anemia Consultative Group) (1985) Measurements of Iron Status (Laboratory Manual). The Nutrition Foundation: Washington, D.C.

IOM (Institute of Medicine) (1993) Iron-Deficiency Anemia: Recommended Guidelines for the Prevention, Detection and Management Among U.S. Children and Women of Childbearing Age. National Academy Press: Washington, D.C.

Janero, D. R. (1990) Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic. Biol. Med. 9: 515–540.

Kawabata, T., Ogino, T. & Awai, M. (1989) Protective effects of glutathione against lipid peroxidation in chronically iron-loaded mice. Biochim. Biophys. Acta 1004: 89–94.

Kneepkens, C. M., Lepage, G. & Roy, C. C. (1994) The potential of the hydrocarbon breath test as a measure of lipid peroxidation [published erratum appears in Free Radic. Biol. Med. (1994) 17(6): 609]. Free Radic. Biol. Med. 17: 127–160.

Knutson, M. D. & Viteri, F. E. (1996) Concentrating breath samples using liquid nitrogen: a reliable method for the simultaneous determination of ethane and pentane [published erratum appears in Anal. Biochem. (1999) 270: 186]. Anal. Biochem. 242: 129–135.

Kohlmuller, D. & Kochen, W. (1993) Is n-pentane really an index of lipid peroxidation in humans and animals? A methodological reevaluation. Anal. Biochem 210: 268–276.

Lee, Y. H., Layman, D. K., Bell, R. R. & Norton, H. W. (1981) Response of glutathione peroxidase and catalase to excess dietary iron in rats. J. Nutr. 111: 2195–2202.

Masini, A., Trenti, T., Caramazza, I., Predieri, G., Gallesi, D. & Ceccarelli, D. (1994) Dietary iron deficiency in the rat. II. Recovery from energy metabolism derangement of the hepatic tissue by iron therapy. Biochim. Biophys. Acta 1188: 53–57.

Minotti, G. & Aust, S. D. (1987) The role of iron in the initiation of lipid peroxidation. Chem. Phys. Lipids 44: 191–208.

National Research Council (1979). Iron. University Park Press: Baltimore.

Patt, A., Horesh, I. R., Berger, E. M., Harken, A. H. & Repine, J. E. (1990) Iron depletion or chelation reduces ischemia/reperfusion-induced edema in gerbil brains. J. Pediatr. Surg. 25: 224–227; discussion 227–228.

Rao, J. & Jagadeesan, V. (1996) Lipid peroxidation and activities of antioxidant enzymes in iron deficiency and effect of carcinogen feeding. Free Radic. Biol. Med. 21: 103–108.

Reeves, P. G., Nielsen, F. H. & Fahey, G. C., Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 123: 1939–1951.
Sherman, A. R. & Moran, P. E. (1984) Copper metabolism in iron-deficient maternal and neonatal rats. J. Nutr. 114: 298–306.
Sherman, A. R. & Tissue, N. T. (1981) Tissue iron, copper and zinc levels in offspring of iron-sufficient and iron-deficient rats. J. Nutr. 111: 266–275.
Sourkes, T. L., Lloyd, K. & Birnbaum, H. (1968) Inverse relationship of heptic copper and iron concentrations in rats fed deficient diets. Can. J. Biochem. 46: 267–271.
Springfield, J. R. & Levitt, M. D. (1994) Pitfalls in the use of breath pentane measurements to assess lipid peroxidation. J. Lipid. Res. 35: 1497–1504.
Srigiridhar, K. & Madhavan Nair, K. (1998) Iron-deficient intestine is more susceptible to peroxidative damage during iron supplementation in rats. Free Radic. Biol. Med. 25: 660–665.
Stoltzfus, R. J. & Dreyfus, M. L. (1999) Guidelines for the use of iron supplements to prevent and treat iron deficiency anemia: a report of the International Nutritional Anemia Consultative Group (INACG) (Washington, D.C.: The Nutrition Foundation).
Svanberg, B. (1975) Absorption of iron during pregnancy. Acta Obstet. Gynecol. Scand. (Suppl.).
Taylor, S. L., Lamden, M. P. & Tappel, A. L. (1976) Sensitive fluorometric method for tissue tocopherol analysis. Lipids 11: 530–538.
Topp, H., Vangala, M., Kritzler, K. & Schoch, G. (1995) Assessment of lipid peroxidation in rats of different body weight by determining expired ethane. Biol. Chem. Hoppe Seyler 376: 691–694.
Torrance, J. D. & Bothwell, T. H. (1968) A simple technique for measuring storage iron concentrations in formalinised liver samples. S. Afr. J. Med. Sci. 33: 9–11.
Uehara, M., Chiba, H., Mogi, H., Suzuki, K. & Goto, S. (1997) Induction of increased phosphatidylcholine hydroperoxide by an iron-deficient diet in rats. J. Nutr. Biochem. 8: 385–391.
Viteri, F. E. (1997) Iron supplementation for the control of iron deficiency in populations at risk. Nutr. Rev. 55: 195–209.
Viteri, F. E. (1998) A new concept in the control of iron deficiency: community-based preventive supplementation of at-risk groups by the weekly intake of iron supplements. Biomed. Environ. Sci. 11: 46–60.
Viteri, F. E., Liu, X., Tolomei, K. & Martin, A. (1995) True absorption and retention of supplemental iron is more efficient when iron is administered every three days rather than daily to iron-normal and iron-deficient rats. J. Nutr. 125: 82–91.
Ward, R. J., Florence, A. L., Baldwin, D., Abiaka, C., Roland, F., Ramsey, M. H., Dickson, D. P., Peters, T. J. & Crichton, R. R. (1991) Biochemical and biophysical investigations of the ferrocene-iron-loaded rat. An animal model of primary haemochromatosis. Eur. J. Biochem. 202: 405–410.
Yeo, H. C., Helbock, H. J., Chyu, D. W. & Ames, B. N. (1994) Assay of malondialdehyde in biological fluids by gas chromatography-mass spectrometry. Anal. Biochem. 220: 391–396.
Younes, M., Eberhardt, I. & Lemoine, R. (1989) Effect of iron overload on spontaneous and xenobiotic-induced lipid peroxidation in vivo. J. Appl. Toxicol. 9: 103–108.

JN THE JOURNAL OF NUTRITION