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Limited effects of combined dietary copper deficiency/iron overload on oxidative stress parameters in rat liver and plasma☆

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Abstract

Copper (Cu) deficiency decreases the activity of Cu-dependent antioxidant enzymes such as Cu,zinc-superoxide dismutase (Cu,Zn-SOD) and may be associated with increased susceptibility to oxidative stress. Iron (Fe) overload represents a dietary oxidative stress relevant to overuse of Fe-containing supplements and to hereditary hemochromatosis. In a study to investigate oxidative stress interactions of dietary Cu deficiency with Fe overload, weanling male Long–Evans rats were fed one of four sucrose-based modified AIN-93G diets formulated to differ in Cu (adequate 6 mg/kg diet vs. deficient 0.5 mg/kg) and Fe (adequate 35 mg/kg vs. overloaded 1500 mg/kg) in a 2×2 factorial design for 4 weeks prior to necropsy. Care was taken to minimize oxidation of the diets prior to feeding to the rats. Liver and plasma Cu content and liver Cu,Zn-SOD activity declined with Cu deficiency and liver Fe increased with Fe overload, confirming the experimental dietary model. Liver thiobarbituric acid reactive substances were significantly elevated with Fe overload (pooled across Cu treatments, 0.80±0.14 vs. 0.54±0.08 nmol/mg protein; $P<.0001$) and not affected by Cu deficiency. Liver cytosolic protein carbonyl content and the concentrations of several oxidized cholesterol species in liver tissue did not change with these dietary treatments. Plasma protein carbonyl content decreased in Cu-deficient rats and was not influenced by dietary Fe overload. The various substrates (lipid, protein and cholesterol) appeared to differ in their susceptibility to the in vivo oxidative stress induced by dietary Fe overload, but these differences were not exacerbated by Cu deficiency.

Keywords: Copper deficiency; Iron overload; Oxidative stress

1. Introduction

Copper (Cu) deficiency leads to reduced activity of the antioxidant cuproenzymes such as Cu,zinc-superoxide dismutase (Cu,Zn-SOD), which is present in many tissues including the liver, and ceruloplasmin in plasma, thereby increasing susceptibility to oxidative stress [1–3]. Thus, Cu-deficient individuals who are exposed to an oxidative stress would be expected to show greater signs of oxidative damage.

Free iron (Fe) in solution can readily cause the oxidation of biologic molecules such as lipids and proteins, so Fe is normally sequestered in vivo by specific binding proteins. If the capacity of these binding proteins is exceeded, oxidative complications can occur [4]. Elevated Fe status, whether acute through excessive supplement intake or chronic due to genetic predisposition such as hereditary hemochromatosis, has been associated with oxidative stress and adverse health outcomes [5]. Severe dietary Fe overload (2–3% carbonyl Fe in the diet of rats) has been shown to increase liver F2 isoprostane levels [6] or 4-hydroxynonenal levels and to decrease Cu,Zn-SOD activity [7], but it may be more nutritionally relevant to examine the effect of milder dietary Fe loading. In the present study, the effects of moderate

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dietary Fe overload in combination with dietary Cu deficiency on oxidative changes in multiple tissue substrates were investigated.

Cu is necessary for proper mobilization and metabolism of Fe [8], so Cu deficiency may be expected to exacerbate the effects of Fe overload. In Cu deficiency, Fe tends to accumulate in the liver, suggesting that this would be a target organ for oxidative changes. Plasma is a readily accessible biologic fluid, suitable for routine monitoring of health status, so the potential impact of combined Cu deficiency with Fe overload was investigated in this tissue as well. Previous work has suggested that macromolecules can differ in their susceptibility to oxidative stress induced by Fe-overloaded diets [9]. Hence, indices of protein, lipid and cholesterol oxidation were examined in the present study.

2. Methods

2.1. Animals and diet formulations

Weanling male Long–Evans rats were obtained from Charles River Canada (St. Constant, QC, Canada). Upon arrival, rats were randomly assigned to four groups of eight rats each and fed sucrose-based modified AIN-93G diets [10] formulated to differ in Cu (adequate 6 mg/kg vs. deficient 0.5 mg/kg) and Fe (adequate 35 mg/kg vs. overload 1500 mg/kg) (Table 1) in a 2×2 factorial design for 4 weeks. Sucrose-based diets were used to take advantage of the known interaction between dietary carbohydrate source and Cu deficiency in male rats, which exacerbates the signs of Cu deficiency and results in higher susceptibility of tissues to ex vivo oxidative stress [11]. Diets were stored frozen (−80°C) in batches, each sufficient for 1 week of feeding. Each week, one bag of each diet was removed to a refrigerator (4°C) adjacent to the animal room. Feed in animal cages was replaced daily. These procedures were intended to minimize oxidative changes in diets prior to feeding to the rats. The experimental protocol was approved by the institutional animal care committee of the Health Products and Food Branch of Health Canada.

Table 1
Composition of modified AIN-93G diets fed to rats for 4 weeks

Ingredient	g/kg diet
Casein	200.00
Cornstarch	132.00
Sucrose	477.50
Soybean oil[a]	70.00
Fiber	50.00
Fe premix[b]	20.00
Mineral premix (AIN-93G-MX) with (Diets 1 and 3) or without (Diets 2 and 4) Cu	35.00
Vitamin premix (AIN-93G-VX)	10.00
L-Cystine	3.00
Choline bitartrate (41.1% choline)	2.50

[a] Containing *t*-butylhydroquinone at 0.2 mg/g oil (0.014 g/kg diet).
[b] Diets 1 and 2, 20.00 g of sucrose; Diets 3 and 4, 9.091 g of ferric citrate (16.5% Fe)+10.909 g of sucrose.

2.2. Tissue sampling

After 4 weeks, the rats were killed by isoflurane anesthesia and exsanguination. Blood was collected by syringe from the abdominal aorta and immediately transferred into tubes containing EDTA (1.8 mg/ml blood), tubes containing heparin (15 USP units/ml blood) and serum separator tubes (Vacutainer, Fisher Scientific, Nepean, Canada). EDTA-treated blood samples were held briefly on ice and centrifuged (1500×*g*, 10 min, 4°C) to separate plasma. Blood samples in heparin tubes were used for analyses of Vitamins C and E. Blood in serum separator tubes was allowed to clot for 30 min at room temperature before centrifugation. Serum and plasma samples were stored at −80°C until analyzed. Livers were removed at necropsy, rinsed in 0.25 mol/L sucrose [12], blotted dry, weighed, divided into separate lobes and promptly stored at −80°C until analyzed.

2.3. Mineral analyses

Diet (~3 g), liver (~5 g) and plasma (500 μl) samples for mineral analyses were dry ashed in a programmable furnace (Model 497, Fisher Scientific) at 450°C using concentrated nitric acid as an oxidizing agent [12]. The ash was dissolved in 2.9 mol/L of hydrochloric acid and analyzed for Cu and Fe by flame atomic absorption spectrophotometry (Perkin Elmer 5100PC, Perkin-Elmer, Norwalk, CT, USA). Analytic standards were prepared from certified single-element stock solutions (SPEX Chemical, Metuchen, NJ, USA). We have previously shown the content of Cu and Fe in any one lobe to be representative of the entire liver [12].

2.4. Liver enzymes

Liver tissue (~200 mg) for determination of SOD and glutathione peroxidase (GSH-Px) activities was homogenized in 39 volumes of ice-cold 0.2% Triton X-100 (vol/vol in distilled water) in a ground glass homogenizer and centrifuged briefly to remove cell debris. Cu,Zn-SOD activity was determined in ethanol/chloroform extracts of liver homogenates according to the method of L'Abbé and Fischer [13] based on xanthine/xanthine oxidase and cytochrome *c*, modified for use on a microplate spectrophotometer (SpectraMax Plus, Molecular Devices, Sunnyvale, CA, USA). Total SOD activity was determined using the unextracted homogenates and manganese SOD (Mn-SOD) was calculated by the difference. One unit of SOD activity is defined as the amount of the enzyme that causes a 50% inhibition of the rate of cytochrome *c* reduction under the conditions of the assay. Selenium-dependent GSH-Px (Se-GSH-Px) activity was determined using the automated coupled assay method of L'Abbé et al. [14], adapted for use on a microplate spectrophotometer, and using 0.3 nmol/L *t*-butylhydroperoxide as substrate. One unit of Se-GSH-Px activity is defined as the amount of the enzyme that results in

1 μmol of NADPH oxidized per minute. Catalase activity was determined in separate liver homogenates prepared in 0.01 mol/L of sodium phosphate buffer, pH 7.4, and pretreated with ethanol and Triton X-100 [15]. The protein concentration of homogenates was determined using a bicinchoninic acid protein assay kit (Sigma).

2.5. Thiobarbituric acid reactive substances and lipid peroxides

Thiobarbituric acid reactive substances (TBARS) in liver homogenates (prepared in 0.2% Triton X-100) were determined by our modification [3] of the method of Ohkawa et al. [16], using 0.01 N NaOH to prepare the TBA reagent. Lipid peroxide levels in these homogenates were determined using a commercially available kit (LPO-CC Lipid Peroxides, Kamiya Biomedical, Seattle, WA, USA).

2.6. Vitamins C and E

Vitamin C levels [both reduced ascorbic and dehydroascorbic acids (DHAA)] in heparin plasma were determined using reversed-phase HPLC with electrochemical detection following the method of Behrens and Madere [17]. Vitamin E levels in plasma and liver were determined by the HPLC method of Thompson and Hatina [18].

2.7. Protein carbonyls

Liver cytosol was prepared from homogenates (in 50 mM HEPES, 100 mM KCl, 10 mM $MgCl_2$, 1.1 mM EDTA, pH 7.2, 9 volumes of buffer per gram of tissue) by ultracentrifugation at 100,000×*g* for 1 h at room temperature (Beckman XL-80 ultracentrifuge with 42.2 Ti rotor, Beckman Instruments, Palo Alto, CA, USA). Liver cytosolic protein carbonyls and plasma protein carbonyls were determined by slot-blot immunoassay using reagents from an OxyBlot Protein Oxidation Detection Kit (Intergen, NY, USA). Briefly, 2 μg/slot of sample or standard proteins, derivatized with 2,4-dinitrophenylhydrazine, was applied to a nitrocellulose membrane and sequentially reacted with rabbit anti-DNP and HRP-conjugated goat anti-rabbit antibodies. Chemiluminescence detection used ECL detection reagents (Amersham Pharmacia Biotech, Baie D'Urfé, QC, Canada). Oxidized and reduced protein standards were prepared by reaction of bovine serum with hypochlorite and sodium borohydride, respectively [19]. Some standards were prepared from pooled control rat serum or plasma incubated with 1 mmol/L $FeSO_4$ and 1 mmol/L H_2O_2 for 10 min (oxidized standard), after which a portion was reduced with 200 mmol/L $NaBH_4$ [20]. Oxidized or reduced proteins prepared by either method were collected by centrifugal filtration (10 kDa MW cutoff, Microcon YM-10, Millipore) and washed with Tris-EDTA buffer (85.7 mmol/L Tris, 0.857 mmol/L EDTA, 20 mmol/L NaOH, pH 8.5), and the carbonyl content of the standards was determined colorimetrically following reaction with 2,4-dinitrophenylhydrazine [21]. Oxidized and reduced proteins were mixed in varying proportions to generate a standard curve on the slot blot.

2.8. Oxysterols

Cholesterol oxidation products (COPs or oxysterols) in liver and plasma samples were determined by GC-MS [22]. Briefly, 19-hydroxycholesterol was added to the samples as an internal standard before extraction of the lipids with a modified Folch procedure. Artifactual oxidation of cholesterol was minimized by incorporation of L-ascorbic acid and sodium acetate to scavenge oxygen and acidic species, respectively. The lipid extract was saponified; unsaponified lipids were extracted with diethyl ether; and free fatty acids were removed with KOH. Bulk cholesterol was removed by solid-phase extraction to facilitate the measurement of the oxysterols, which were eluted with 2-propanol in hexane. After removal of the solvent under nitrogen, the samples were converted to trimethylsilyl ethers for GC-MS analysis (Agilent 6890 GC System with 5973 Mass Selective Detector, Agilent Technologies, Wilmington, DE, USA). Total cholesterol was quantified in a portion of the sample, taken before solid-phase extraction, in a separate GC-MS run using 5-α-cholestane as an internal standard.

Table 2
Diet concentrations of Cu, Fe and TBARS and feed intakes and body weights of rats fed modified AIN-93G diets for 4 weeks

	Control	−Cu	+Fe	−Cu/+Fe	Significance		
					−Cu	+Fe	Cu × Fe
Diet Cu (mg/kg)[a]	6.0±0.5	0.5±0.2	6.6±0.7	0.5±0.3	<.001	N.S.	N.S.
Diet Fe (mg/kg)[a]	46±2	41±1	1621±72	1637±57	N.S.	<.001	N.S.
Diet TBARS (nmol/mg protein)[b]							
Stored at −80°C	0.4±0.2	0.3±0.1	2.5±0.4	2.7±0.9	N.S.	<.001	N.S.
Overnight at room temperature	0.5±0.2	0.4±0.1	5.2±0.6	4.5±1.8	N.S.	<.001	N.S.
After 1 week in 4°C fridge	0.4±0.1	0.3±0.2	3.2±0.7	3.6±0.7	N.S.	<.001	N.S.
After 4 weeks in 4°C fridge	0.5±0.1	0.4±0.0	6.1±1.3	18.2±3.0	<.001	<.001	<.001
Feed intake (g/day)[c]	17.8±1.2	16.6±2.5	19.0±1.7	17.5±0.9	<.05	N.S.	N.S.
Final body weight (g)[c]	232±24	219±32	246±29	233±12	N.S.	N.S.	N.S.

Cu × Fe indicates interaction effects (−Cu × +Fe); N.S., not significant.

[a] Mean±SD, $n=5$.

[b] Mean±SD, $n=3$.

[c] Mean±SD, $n=8$.

Table 3
Liver Cu, Fe and Vitamin E concentrations and antioxidant enzyme activities in rats fed modified AIN-93G diets differing in Cu and Fe content for 4 weeks

	Control	−Cu	+Fe	−Cu/+Fe	Significance		
					−Cu	+Fe	Cu × Fe
Cu (μg/g dwt)[a]	16.7±1.4	4.9±1.9	14.9±3.3	5.6±2.0	<.001	N.S.	N.S.
Fe (μg/g dwt)	218±46	372±72	895±376	1096±369	N.S.	<.001	N.S.
Vitamin E (μg αT/g wwt)	57.9±10.6	46.6±14.5	64.9±21.7	57.8±15.7	N.S.	N.S.	N.S.
Total SOD (U/mg protein)	60±7	35±4	65±5	39±6	<.001	<.05	N.S.
Cu,Zn-SOD (U/mg protein)	56±6	30±5	61±5	35±5	<.001	<.05	N.S.
Mn-SOD (U/mg protein)	4.5±1.7	4.5±1.0	4.0±2.6	3.9±2.6	N.S.	N.S.	N.S.
Se-GSH-Px (U/mg protein)	0.14±0.05	0.12±0.03	0.12±0.01	0.11±0.02	N.S.	N.S.	N.S.
Catalase (k/s/mg protein)	0.53±0.12	0.56±0.12	0.59±0.12	0.52±0.10	N.S.	N.S.	N.S.

dwt indicates dry weight of tissue; wwt, wet weight of tissue; αT, α-tocopherol; U, units.
[a] Mean±SD, $n=8$ for all values in this table.

2.9. Statistical analyses

Statistical analyses by two-way ANOVA (Statistica 6.1, StatSoft, Tulsa, OK, USA) used $P<.05$ as the threshold of significance. Correlation between liver Fe and liver TBARS was examined post hoc using Statistica 6.1 software.

3. Results

The analyzed Cu and Fe concentrations of the test diets are shown in Table 2. Adding excess Fe to the diet resulted in a small but significant increase in diet TBARS in spite of the procedures used to minimize this. Most of the increase in diet TBARS may have occurred during diet mixing as the levels were already increased in diets stored immediately at −80°C (Table 2). Smaller additional increases noted after 1 week in the refrigerator or overnight at room temperature in the feed cup indicate that the storage precautions taken were at least partially successful in minimizing diet oxidation. Considerably larger increases in diet TBARS were found in samples stored at 4°C for the duration of the experiment (Table 2), which represents the usual storage conditions for nutritional experiments in many research centers [10]. Despite the observed increase in diet TBARS, palatability of the diets was apparently unaffected as feed intake was unchanged by the high dietary Fe treatments. Feed intake was slightly but significantly lower in Cu-deficient groups regardless of the level of dietary Fe, but body weight gain was not altered by dietary treatment over the 4-week experiment (Table 2).

Liver Cu and Fe concentrations responded as expected to changes in their respective dietary levels, Cu concentration being decreased by Cu deficiency and Fe concentration being increased by Fe overload (Table 3). Plasma Cu concentration decreased in Cu deficiency. Plasma Fe concentration was decreased in Cu deficiency but not affected by Fe overload (Table 4).

Cu,Zn-SOD activity in liver decreased by almost 50% with Cu deficiency and rose by 10–15% with Fe overload (Table 3). Liver Mn-SOD, Se-GSH-Px and catalase activities were not affected by dietary treatment in this study. Plasma reduced ascorbic acid concentration (ascorbate) was significantly lower in Fe-overloaded rats (Table 4) although the oxidized form DHAA was unchanged. Plasma Vitamin E was slightly but significantly elevated in Fe overload, while liver Vitamin E level was unaffected by dietary treatment (Table 3).

Plasma protein carbonyl level was decreased by approximately 20% in Cu deficiency and not altered by Fe overload (Table 4). Liver cytosolic protein carbonyl content was not affected by dietary treatment in this study (Table 5). Concentrations of several common oxysterol species in the liver were unchanged by either dietary Fe overload or Cu deficiency in this study (Table 5). Plasma oxysterol levels were similarly unaffected by the dietary treatments used (results not shown). Liver TBARS increased by almost 50% in rats fed the high Fe diets but were unaffected by dietary Cu deficiency (Table 5). There was a significant correlation between liver Fe and liver TBARS concentrations ($r=.54$; $P=.001$). Liver lipid

Table 4
Plasma concentrations of Cu, Fe, Vitamins C and E and protein carbonyl content in rats fed modified AIN-93G diets differing in Cu and Fe content for 4 weeks

	Control	−Cu	+Fe	−Cu/+Fe	Significance		
					−Cu	+Fe	Cu × Fe
Cu (mg/L)[a]	0.22±0.14	0.06±0.06	0.42±0.27	0.09±0.10	<.001	N.S.	N.S.
Fe (mg/L)	2.72±1.74	1.94±0.57	3.82±1.24	2.24±1.12	<.05	N.S.	N.S.
Ascorbate (mg/L)	9.5±4.5	7.4±2.2	4.6±2.0	3.8±1.2	N.S.	<.001	N.S.
DHAA (mg/L)	2.3±0.6	1.4±0.7	1.6±0.9	1.5±0.6	N.S.	N.S.	N.S.
Vitamin E (mg αT/L)	15.7±2.8	18.0±3.1	19.9±4.0	19.1±3.2	N.S.	<.05	N.S.
Protein carbonyl (nmol/mg protein)	1.27±0.34	1.01±0.17	1.35±0.33	1.05±0.21	<.01	N.S.	N.S.

[a] Mean±SD, $n=8$ for all values in this table.

Table 5
Protein, lipid and cholesterol oxidation parameters in livers of rats fed modified AIN-93G diets differing in Cu and Fe content for 4 weeks

	Control	−Cu	+Fe	−Cu/+Fe	Significance		
					−Cu	+Fe	Cu × Fe
Cytosolic protein carbonyl (nmol/mg protein)[a]	0.40±0.05	0.38±0.04	0.40±0.04	0.42±0.13	N.S.	N.S.	N.S.
LPO (nmol/mg protein)	0.45±0.09[b]	0.53±0.16[b]	0.66±0.23[b]	0.49±0.15[c]	N.S.	N.S.	N.S.
TBARS (nmol/mg protein)	0.54±0.07	0.55±0.09	0.78±0.19	0.81±0.08	N.S.	<.001	N.S.
Total cholesterol (mg/g wwt)	5.5±1.6	4.7±1.6	5.6±2.6	5.6±1.7	N.S.	N.S.	N.S.
Cholesterol β-oxide (μg/g wwt)	3.8±2.7	5.2±4.1[d]	4.1±2.3[d]	4.1±2.6[d]	N.S.	N.S.	N.S.
Cholesterol α-oxide +7β-hydroxycholesterol (μg/g wwt)	2.8±2.0	2.7±2.3[d]	2.7±2.3[d]	2.3±1.6[d]	N.S.	N.S.	N.S.
7-ketocholesterol (μg/g wwt)	1.1±0.6	1.0±0.8[d]	0.8±0.5[d]	0.9±0.6[d]	N.S.	N.S.	N.S.
25-hydroxycholesterol (μg/g wwt)	0.10±0.06	0.12±0.06[c]	0.12±0.08[b]	0.14±0.15[c]	N.S.	N.S.	N.S.
26-hydroxycholesterol (μg/g wwt)	0.21±0.11	0.24±0.14[d]	0.25±0.13[d]	0.24±0.15[d]	N.S.	N.S.	N.S.

LPO indicates lipid peroxides.
[a] Mean±SD, $n=8$ for all values except where noted.
[b] $n=5$.
[c] $n=6$.
[d] $n=7$.

peroxide levels were not affected by dietary treatment in this study.

4. Discussion

Liver Cu and Fe concentrations responded as expected to changes in their respective dietary levels. Plasma Fe was affected by Cu deficiency but not by Fe overload, suggesting that mobilization of Fe from tissue stores may have been compromised in Cu-deficient animals but that regulation of Fe distribution between tissues was not adversely affected by the level of Fe overload used here. Normal tissue Fe distribution depends on ceruloplasmin, a cuproenzyme [8]. Ceruloplasmin activity was not specifically measured in the present study due to the limited amount of serum sample available but has been shown previously to decline essentially to zero under similar experimental conditions of dietary Cu deficiency [23,24].

Cu,Zn-SOD activity in liver decreased with Cu deficiency, consistent with the critical catalytic role of Cu in this enzyme. A major adaptive response of rats to oxidative stress is the induction of antioxidant enzymes. Hence, the small but significant increase in SOD activity with dietary Fe overload may represent a partial compensatory response to induced oxidative stress, as an increase in oxidative stress has been shown to induce SOD biosynthesis in various tissues [25]. Liver catalase and Se-GSH-Px activities were not affected by dietary treatment in this study, suggesting that there was no generalized effect on the antioxidant enzymes.

Plasma reduced ascorbic acid concentration was significantly lower in Fe-overloaded rats, further suggestive of mild in vivo oxidative stress induced by this treatment. Experiments with guinea pigs have shown that ascorbic acid suppresses the effects of oxidative stress related to Fe overload [26]. Even the lower amount of ascorbate present in Fe-overloaded rats in the present study may have been sufficient to provide some protection from oxidative stress. As rats, unlike guinea pigs and humans, can synthesize Vitamin C [27], they may not be the most suitable experimental animal model for these investigations. Plasma Vitamin E was slightly but significantly elevated in Fe overload, which may represent another form of partial compensation in antioxidant defense.

Protein oxidation, measured as an increase in carbonyl groups, has been shown to be an early event in oxidative stress in vitro [28] and has also been used to show accumulation of oxidative damage to proteins over the longer term (e.g., in studies of aging) [29]. Free radical-mediated oxidation appears to play an important role in the generation of protein carbonyls in vitro [30] and in vivo [31]. Plasma protein carbonyls were decreased by Cu deficiency in the present study, consistent with our previous observations [3], and were not altered by dietary Fe overload. It is conceivable that tissue Fe concentrations need to be much higher than the concentrations observed in this work to cause protein damage in vivo. The lowered plasma protein carbonyls observed with the Cu-deficient diets could possibly have been due to a decrease in mixed function oxidase (MFO) isozyme activities, which have been linked to protein carbonyl production [32] and for the most part have been shown to decrease with Cu deficiency [33,34], although MFO activities were not assayed in the present work. Liver cytosolic protein carbonyl content, however, was not affected by the dietary treatments used in this study, although MFO activities would be expected to be much higher in the liver than in plasma. Further investigation will be necessary to elucidate the mechanism(s) behind the observed decreases in plasma protein carbonyls in Cu deficiency. In the meantime, these results suggest that protein oxidation was minimal in combined Cu deficiency and Fe overload under the conditions used.

Oxysterols (COPs) have been described as a new, relatively stable index of oxidative stress that is recommended for further research [35]. In particular, 7-β-hydroxy- and 7-keto-cholesterol are produced nonenzymatically and are taken to indicate oxidative stress in vivo [36]. On the other hand, 25-hydroxy- and 26-hydroxy-cholesterol are

enzymatically formed in vivo [37]. Changes in skeletal muscle levels of 7-β-hydroxy- and 7-keto-cholesterol have been noted in alcoholic muscle disease, where muscle protein carbonyl levels were unchanged or even slightly diminished [38]. Serum ferritin level (an index of Fe status) has been shown to be positively correlated with levels of several oxysterols in humans [39]. Although tissue Fe levels (and hence Fe status of the animals) were affected by treatment in the present study, concentrations of several common oxysterol species in liver and plasma were unchanged by either dietary Fe overload or Cu deficiency. Levels of 7-β-hydroxy-, 7-keto- and 25-hydroxycholesterol in rat liver have been shown to increase in response to a similar level of dietary Fe overload when salmon oil was the dietary fat source, but not with lard [40], clearly indicating that other dietary factors can interact. Previous work has shown a lower sensitivity to Fe-induced oxidative stress in rats fed vegetable oil as opposed to fish oil-based diets [41], which could also have contributed to the unchanged hepatic oxysterol concentrations with Fe overload observed in the present study where soybean oil was used.

Liver TBARS increased with dietary Fe overload in the present study but were unaffected by dietary Cu deficiency. Although TBARS are a relatively nonspecific index of lipid peroxidation, the increase in hepatic TBARS values could be considered to indicate enhanced sensitivity of hepatic lipids to oxidation under the dietary condition of Fe overload. Dietary Fe (but not dietary Cu) has also been shown by others to be a weak but significant predictor of liver TBARS [24]. Given the observed increase in dietary TBARS in the present study, however, an exogenous origin for these substances in rat liver cannot be completely ruled out despite the specific measures employed to minimize this possibility. The much lower level of dietary TBARS measured in diets as fed to the rats in this study compared with diet samples held at 4°C for the duration of the experiment points out the necessity of appropriate diet storage conditions for experiments such as the one reported here. Lipid oxidation products have been shown to be absorbed from diet [42,43] and incorporated into tissues [44]. However, dietary TBARS concentrations in this study were increased 8- to 10-fold in the +Fe and −Cu/+Fe diets compared with controls after 1 and 7 days (at room temperature and 4°C, respectively), while concentrations in liver rose by only 50%, suggesting a poor correspondence between TBARS levels in diet and tissue. Previous work have shown that ingestion of lipid oxidation products from thermally stressed oils is normally associated with an increase in liver TBARS concentrations [45,46]. In the present study, however, activities of hepatic GSH-Px were unchanged with dietary Fe overload whereas hepatic GSH-Px activities were shown by others to rise following intake of lipid oxidation products [45]. Hence, the higher liver TBARS concentrations associated with Fe overload in the present study could be due to enhanced in vivo lipid peroxidation as opposed to dietary intake of secondary lipid oxidation products. In support of this contention, previous studies have shown enhanced lipid peroxidation in rats fed Fe overload diets [6,9]. Moreover, hepatic Fe concentrations in the present study were correlated positively with increases in TBARS, as has been reported previously with Fe overload [9]. Fe overload has been suggested to enhance tissue lipid peroxidation in vivo by accelerated production of peroxyl and alkoxyl radicals from lipid hydroperoxides [47].

As indicated previously, we have observed that Fe overload is associated with hepatic lipid peroxidation. The lack of significant changes in protein carbonyl or oxysterol concentrations, however, combined with the significant effect (either direct or indirect) of dietary Fe on liver TBARS suggests that there may be differences in the susceptibility of different biochemical substrates to the in vivo oxidative stress of dietary Fe overload. Likewise, Fischer et al. [9] showed that Fe overload diets did not significantly alter other oxidative stress indices such as DNA double-strand breaks or NF-κB activation despite observed increases in hepatic lipid peroxidation. Mild oxidative stress resulting from dietary Fe overload is further suggested by small compensatory changes in some antioxidant parameters (liver Cu,Zn-SOD activity, plasma Vitamin E). These differences were not affected by a combination with dietary Cu deficiency, thereby suggesting that dietary Fe overload and Cu deficiency do not act synergistically to increase hepatic oxidative stress.

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