Iron Supplementation Does Not Affect Cell Proliferation or Aberrant Crypt Foci Development in the Colon of Sprague-Dawley Rats 1,2,3

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ABSTRACT It has been suggested that high iron stores enhance colon carcinogenesis. The effect of high dietary iron (Fe) on indices of iron, copper (Cu) and manganese (Mn) status, lipid peroxidation using the thiobarbituric acid reactive substances assay, superoxide dismutase, glutathione peroxidase, glutathione transferase and ceruloplasmin activities, cell proliferation and development of preneoplastic lesions known as aberrant crypt foci (ACF) in rat colon was examined using a 3 × 2 factorial design. Male weanling Sprague-Dawley rats were fed adequate (AFe; 45 mg Fe/kg diet), moderately high (MHFe; 225 mg Fe/kg diet) and high (HFe; 450 mg Fe/kg diet) dietary Fe for 2.5 wk, then treated with azoxymethane (AOM; 2 injections, 1 wk apart; total dose 30 mg/kg body weight) or saline (n = 14–15 per group). Dietary treatment continued for another 6 wk after the second AOM dose. At the time of AOM injection, colon Fe concentrations were one- and threefold higher for MHFe and HFe rats, respectively, than for AFe rats. It was proposed that high dietary Fe would adversely affect Cu and Mn status, resulting in impaired antioxidant enzyme activity. However, neither indices of Cu and Mn status nor colonic mucosal antioxidant enzyme activities were affected by dietary Fe except for plasma ceruloplasmin activity, which was slightly lower in rats fed high iron diets than in rats fed adequate iron diets (P < 0.01). Dietary Fe had no significant effect on colonic mucosal lipid peroxidation, cell proliferation or ACF development. In conclusion, our findings suggest that dietary Fe concentrations that are ~5 and 10 times adequate do not enhance oxidative stress, cell proliferation and ACF development in the colon of rats. J. Nutr. 128: 764–770, 1998.

KEY WORDS: • iron • copper • antioxidants • colon cancer • rats

Although iron deficiency remains a common nutritional deficiency (Yip and Dallman 1996), it has been recognized that the incidence of iron overload is greater than previously thought and that iron intake may be excessive for some individuals (Lynch 1995). Some studies have led to conjecture that high body iron stores may enhance risk of cancer and heart disease (Lynch 1995, Yip and Dallman 1996); in a 1996 review regarding dietary iron recommendations, Lynch and Baynes (1996) suggested a need for studies examining the role of iron in chronic disease development. Our aim was to examine the proposed relationship between iron and colon carcinogenesis. Although hereditary hemochromatosis is associated with enhanced risk of hepatocellular carcinoma, there is little evidence of a relationship between genetic iron disorders and cancer at other sites (Lynch 1995). However, results of some epidemiologic and animal studies have suggested that high iron status or high iron intakes may enhance colon cancer risk (Nelson 1992). A 1988 analysis of the NHANES I Epidemiologic Follow-Up Study (NHEFS) 4 by Stevens et al. (1988) found a positive relationship between transferrin saturation and risk of cancer at all sites combined, as well as risk of colon cancer, for men only. However, a subsequent analysis of this NHEFS data with additional subject exclusions failed to confirm these results (Yip and Dallman 1996). A later NHEFS analysis (Wurzelmann et al. 1996) with additional follow-up years reported a positive relationship between serum iron and colon cancer risk in women, and another large prospective study by Knekt et al. (1994) revealed enhanced risk of colon cancer only in men and women with transferrin saturation >60%. Case-control studies (Bird et al. 1996, Nelson et al. 1994) have shown at least a weak positive relationship between serum ferritin levels and risk of adenoma of the colon. A positive association between dietary iron intake and risk of colon cancer (Wurzelmann et al. 1996) or adenoma (Bird et al. 1996) has also been reported, and epidemiologic and animal studies showing a possible relationship between high meat intake and risk of colon cancer have prompted speculation that this may be due, in part, to iron (Babbs 1990, Giovannucci et al. 1994). Interpretation of some of the epidemiologic studies is hampered by the following: 1) a limited number of disease cases; 2) quantitative selection bias due to case-control design; and 3) long duration of follow-up that could be associated with confounding factors. A number of prospective studies have been performed in which dietary iron intake was prospectively measured, and their results are inconclusive. Although iron deficiency remains a common nutritional deficiency (Yip and Dallman 1996), it has been recognized that the incidence of iron overload is greater than previously thought and that iron intake may be excessive for some individuals (Lynch 1995). Some studies have led to conjecture that high body iron stores may enhance risk of cancer and heart disease (Lynch 1995, Yip and Dallman 1996); in a 1996 review regarding dietary iron recommendations, Lynch and Baynes (1996) suggested a need for studies examining the role of iron in chronic disease development. Our aim was to examine the proposed relationship between iron and colon carcinogenesis. Although hereditary hemochromatosis is associated with enhanced risk of hepatocellular carcinoma, there is little evidence of a relationship between genetic iron disorders and cancer at other sites (Lynch 1995). 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2 Funded by University of Georgia Experiment Station, Hatch Project #H-757 and The University of Georgia Research Foundation.
3 4 Abbreviations used: AFe, adequate iron; AOM, azoxymethane; BrdU, 5-bromo-2'-deoxyuridine; Cu,ZnSOD, copper,zinc superoxide dismutase; HFe, high iron; MHFe, moderately high iron; MnSOD, manganese superoxide dismutase; NHEFS, NHANES I Epidemiologic Follow-Up Study.
IRON SUPPLEMENTATION AND ABERRANT CRYPT FOCI

influence the development of cancer (Nelson 1992, Toyokuni 1996) Animals, diet, and study design. In male NMRI mice fed 10±35 g iron fumarate/kg diet for 10 wk, the incidence of tumors was 4.6, 110, and 405 mg Fe/kg diet, respectively (Thompson and Zhang 1991). In contrast, it has been hypothesized that high amounts of unabsorbed fecal iron resulting from excessive dietary iron may catalyze production of oxygen radicals, thus increasing fecal mutagenicity, activating carcinogens or forming tumor promoters within the intestinal lumen. High dietary iron levels could also enhance oxidative stress by interfering with antioxidant system components. High dietary iron may interfere with absorption of other minerals, including copper (Johnson and Murphy 1988) and manganese (Davis et al. 1990). Iron-induced depletion of these minerals may result in decreased antioxidant enzyme activity, particularly that of copper-zinc (Cu,Zn) (Johnson and Murphy 1988) and manganese (Mn) (Davis et al. 1990, Kurakto 1997) superoxide dismutase (SOD, EC 1.15.1.1). In contrast, it has been suggested that oxidative stress may enhance activities of SOD (Davis et al. 1990), and glutathione S-transferase (EC 2.5.1.18, Toyokuni 1996). Although rodent studies conducted thus far provide supportive evidence that iron may enhance oxidative stress, cell proliferation and colon carcinogenesis, many investigators (Nelson et al. 1989, Siegers et al. 1992, Thompson and Zhang 1991, Younes et al. 1990) have used dietary iron concentrations ±15-fold higher than the recommendation of the American Society for Nutritional Sciences for rodents (35 mg/kg diet, Reeves et al. 1993); this amount may be too high to be representative of realistic human dietary intakes. Further, few studies (Nelson et al. 1989, Thompson and Zhang 1991) have examined the effect of high dietary iron intakes on early or intermediate stages of colon carcinogenesis. Current methods for studying colon carcinogenesis include the use of intermediate biological markers such as cell proliferation (Lee et al. 1993, Thompson and Zhang 1991) and the development of aberrant crypt foci (Bird 1987). Aberrant crypt foci develop only in carcinogen-treated animals (Bird 1987) and have been correlated with tumor development (Pretlow et al. 1992). Because it has been proposed that high iron status may alter tumorigenesis at many different stages, including initiation and promotion (Toyokuni 1996), we chose to examine the effect of iron on an early stage of colon carcinogenesis rather than on tumor development.

The purpose of this study was to establish whether high dietary iron concentrations affect intermediate biomarkers of colon carcinogenesis in a dose-response manner by using iron levels thought to be representative of possible human intake from both diet and supplementation. Ferrous sulfate was employed as the dietary iron source because it is a highly bioavailable form of iron and is often used in iron supplements and for food fortification (Yip and Dallman 1996). We examined the effect of dietary iron concentration on the following: 1) colon, liver and spleen iron levels; 2) indicators of copper and manganese status; 3) lipid peroxidation and antioxidative enzyme activities of colonic mucosa; 4) cell proliferation; and 5) development of aberrant crypt foci in the colon.

MATERIALS AND METHODS

Animals, diet, and study design. Weanling male Sprague-Dawley rats (n = 105, initial weight 39±45 g, Harlan Sprague Dawley, Indianapolis, IN) were housed individually in stainless steel wire-bottomed cages in a room controlled for temperature (21 ± 1°C), humidity and light (12-h light:dark cycle, lights on 0700–1900 h). Rats were allowed free access to distilled water. All animal procedures were approved by the University of Georgia Institutional Animal Care and Use Committee. Upon arrival, rats were acclimated for 24 h before being randomly assigned to one of three groups (n = 35 per group), with adjustments made so that mean group weights were approximately equal (47.0±48.4 g). Rats were then given free access to adequate (AFe, 45 mg Fe/kg diet, n = 35), moderately high (MHFe, 225 mg Fe/kg diet, n = 35), or high (HFe, 450 mg Fe/kg diet, n = 35) iron diets. High fat diets are frequently used to enhance colon carcinogenesis in rodents (Lafave et al. 1994), and a modified AIN-93G diet (Reeves et al. 1993) with 200 g corn oil/kg diet was used (Table 1). Iron concentrations of AFe, MHFe and HFe diets, as determined by atomic absorption spectrophotometry (Johnson 1986), were 46, 210 and 405 mg Fe/kg diet, respectively. Diets were prepared weekly and stored at -20°C for no longer than 7 d to minimize lipid oxidation. Fresh diet was given to rats daily. Diet samples were stored at -20°C for 7 d, left at room temperature for 24 h and tested for lipid oxidation. Lipid oxidation of diets, determined as described by Tarladgis et al. (1960), was as follows: AFe, 0.51 mg malondialdehyde (MDA)/kg diet; MHFe, 0.65 mg MDA/kg diet; and HFe, 0.71 mg MDA/kg diet.

After 2.5 wk of dietary treatment, a base-line group (n = 5–6 rats per diet group) was killed to determine liver, colon and spleen iron concentrations for each dietary treatment group. Remaining rats within each dietary treatment group were assigned to carcinogen or saline treatments (n = 14–15 per group) so that mean treatment group weights were approximately equal. The carcinogen azoxymethane (AOM, Sigma Chemical, St. Louis, MO; 5 g/L sterile saline) was prepared immediately before injection. AOM (15 mg/kg body weight) or an equivalent volume of saline was injected subcutaneously once a week for 2 wk, resulting in a cumulative dose of 30 mg AOM/kg body weight. Dietary treatment was continued for an additional 6 wk after the second injection for a total study time of 10 weeks. Body weight was recorded daily for the first 3 wk of the study and once per week thereafter.

Tissue collection. Food was withheld from rats overnight before tissue collection. For cell proliferation studies, 5-bromo-2-deoxyuridine (BrdU, 25 mg/kg body weight) was injected intraperitoneally 2 h before rats were killed. After anesthesia with a 3:2:1 (v/v/v) ratio of ketamine/xylazine/xylazine (0.8 mg/kg body weight), blood was obtained via heart puncture with the use of heparinized syringes (Sarstedt, Princeton, NJ). Whole-body perfusion with ice-cold hepa-
TABLE 1
Composition of experimental diet1

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg diet</th>
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<tr>
<td>Casemine</td>
<td>240.0</td>
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<tr>
<td>Cornstarch</td>
<td>369.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>70.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>200.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>60.0</td>
</tr>
<tr>
<td>AIN-93 vitamin mix2</td>
<td>12.0</td>
</tr>
<tr>
<td>AIN-93G iron-deficient mineral mix3,4</td>
<td>42.0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3.6</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>3.0</td>
</tr>
<tr>
<td>TBHQ5</td>
<td>0.04</td>
</tr>
</tbody>
</table>

1 Modified AIN-93G diet (Reeves et al. 1993).
2 Harlan Teklad Diets (Madison, WI) Teklad #TD94047 AIN-93-VX.
3 Teklad #TD95028 iron-deficient mineral mix contained (g/kg): calcium carbonate, 357.0; potassium phosphate monobasic, 146.0; potassium citrate monohydrate, 70.78; sodium chloride, 74.0; potassium sulfate, 46.6; magnesium oxide, 24.3; zinc carbonate, 1.65; manganese carbonate, 0.63; cupric carbonate, 0.31; potassium iodate, 0.016; sodium meta-silicate, 1.45; sodium selenate, 0.01025; ammonium para-molybdate, 0.00795; chromium potassium sulfate, 0.275; lithium chloride, 0.0174; boric acid, 0.0815; sodium fluoride, 0.0635; nickel carbonate hydrate tetrahydrate, 0.0318; ammonium vanadate, 0.0066; sucrose, 226.8.
4 Diets were supplemented with ferrous sulfate (FeSO4·7H2O, Sigma Chemical, St. Louis, MO).
5 Abbreviation: TBHQ, tert-butylhydroquinone.

rinsed saline was performed before organ removal. Whole blood was collected for hematocrit determination.

For the base-line group of rats, liver, spleen and whole colon were removed after 2.5 wk, rinsed with saline, weighed and stored at −70°C before mineral analysis. At the conclusion of the study (10 wk), organs from AOM- and saline-treated rats were sectioned as needed for assessment of aberrant crypt foci development, cell proliferation, lipid peroxidation, enzyme activities and mineral analysis. Liver and spleen were removed, rinsed with cold saline, blotted dry and weighed. Pieces of liver (1 g) were homogenized in 4 mL of ice-cold buffer (pH 7.0, 0.05 mol/L potassium phosphate). Liver homogenates, spleen and remaining portions of the liver were frozen at −70°C before enzyme and mineral analysis. Colon was removed from the cecum to the anus, rinsed with ice-cold saline, and cut equally into proximal and distal sections. A 0.75-cm section was cut from the end of the distal colon directly adjacent to the proximal colon and placed in 10% buffered formalin for cell proliferation studies for rats in the AFe and HFe groups (n = 7 per group, randomly selected). The remaining distal colon sections from all rats treated with carcinogen (n = 15 per group) and randomly selected rats treated with saline (n = 10 per group) were placed briefly in cold PBS (pH 7.4) before final processing for aberrant crypt foci determination. Distal colon sections from remaining rats treated with saline (n = 5 per group) were rinsed, blotted dry, weighed and frozen at −70°C for mineral analysis. The proximal colon section (all groups, n = 7 per group, randomly selected) was cut open lengthwise and mucosa was scraped from the proximal colon tissue with a clean glass microscope slide, suspended in 2.15 mL buffer (pH 7.0, 0.05 mol/L potassium phosphate) and homogenized. Colon homogenate (1 mL) was used to assess lipid peroxidation. The remaining colon homogenate was frozen at −70°C until enzyme analysis could be performed.

Mineral, enzyme and lipid peroxidation analysis. Lipid peroxidation in fresh colon mucosa homogenate was measured by using the thiobarbituric acid reactive substances assay as described by Buege and Aust (1978). Total SOD, Cu,ZnSOD and MnSOD activities of colon mucosa (total SOD) and liver (Cu,Zn and MnSOD) homogenates were determined by the method of Marklund and Marklund (1974) with pyrogallol (4 mmol/L) as the substrate. To determine Cu,ZnSOD activity, a chloroform/ethanol mixture was added to undiluted liver homogenate before centrifugation for 30 min at 9400g to inactivate MnSOD as described by Johnson and Murphy (1988). To measure MnSOD activity, 1 mmol/L potassium cyanide was added to the final reaction mixture to inactivate Cu,ZnSOD. One unit (U) of SOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%. Glutathione peroxidase (EC 1.11.1.9) activity of colonic mucosa homogenate was determined at 25°C with tert-butyl hydroperoxide (0.3 mmol/L) as the substrate according to the method of Paglia and Valentine (1967). One unit of glutathione peroxidase activity is defined as one micromole of NADPH oxidized per minute. Glutathione S-transferase activity was determined according to the method of Habig et al. (1974) with 1-chloro-2,4-dinitrobenzene (10 mmol/L) as the substrate. One unit of glutathione S-transferase activity is defined as one nanomole of conjugate formed per minute. Plasma was frozen at −70°C until ceruloplasmin (EC 1.16.3.1) activity was determined as described by Schosinsky et al. (1974) with 7.88 mmol/L d-iodosulfinil dihydricloride as the substrate. Sample protein content was determined according to the method of Lowry et al. (1951), and all enzyme activities were expressed per milligram protein. All enzyme analyses were completed using a Beckman DU-650 spectrophotometer (Beckman Instruments, Fullerton, CA).

Atomic absorption spectrophotometry (model #5000, Perkin-Elmer, Norwalk, CT) was used to determine the iron concentrations of the diet, liver, colon and spleen, and liver copper concentration as described by Johnson (1979). Copper concentrations were determined by the method of the iron and copper content of a bovine liver standard obtained from the National Institute of Standards and Technology (Gaithersburg, MD) (mean recovery: Fe 104%, n = 33; Cu 100%, n = 12).

Aberrant crypt foci assay. Distal colon tissue was prepared and aberrant crypts were counted by using the method of Bird (1987) as modified by Gallagher et al. (1996). Briefly, distal colon sections were flushed with PBS (pH 7.4) from the cecal end, fixed in 10% formalin in PBS on a glass pipet for 6 min and slit open along the vascular line. Colon sections were then stored in filter paper in 10% formalin in PBS at 4°C until stained. To count aberrant crypt foci, distal colon sections were measured, stained with 0.2% methylene blue chloride in PBS for 10 min, rinsed with PBS and distilled water, and examined at 40× and 100× magnification. Aberrant crypts were identified by alterations in size, shape of luminal opening and stain intensity. The number of aberrant crypt foci and number of aberrant crypts per foci were counted and expressed per square centimeter of tissue. Foci having ≥4 crypts/foci were classified as large foci (Pretlow et al. 1992). To ensure count accuracy, one reader counted aberrant crypts in all tissue sections. A second independent reader counted aberrant crypts in 10% of tissue sections chosen randomly at the beginning, middle and end of the reading period to verify counts. Counts were not tabulated until all sections were read.

Cell proliferation. For cell proliferation studies, colon sections were fixed in 10% buffered formalin for 4–6 h, embedded in paraffin, sectioned at 4–5 μm and mounted on slides. Following standard dewaxing procedures, immunohistochemical methods were used to assess BrdU incorporation into DNA with the use of a cell proliferation kit from Amersham (Arlington Heights, IL). Anti-BrdU antibody was replaced with PBS on negative control slides. Slides were coded to prevent reader bias and one reader counted cells for all rats. Twenty full height, clearly oriented crypt columns were read for each rat. Crypt height (number of cells in crypt column from base to top of crypt), number of cells labeled per crypt column, labeling index (percentage of BrdU labeled cells in crypt column) and proliferative zone (location of highest labeled cell expressed relative to the height of the column) (Lee et al. 1993) were determined.

Statistics. Treatment means, SEM, medians, ANOVA, tests for normal distribution of data and nonparametric procedures were calculated using the statistical package SAS (SAS Version 6.10, SAS Institute, Cary, NC). The overall effects and interactions of iron and AOM on body weight, hematocrit, relative liver weight, lipid peroxidation, enzyme activity and cell proliferation were determined with two-way ANOVA. The effect of diet on liver and spleen iron concentrations after 2.5 wk of treatment was analyzed with one-way ANOVA, whereas the effect of diet and AOM on liver and spleen

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iron concentrations at the end of the study (10 wk) was determined with two-way ANOVA. A separate two-way ANOVA was also used to assess the effect of iron and length of study on liver and spleen iron concentrations using only saline-treated rats from the 10-wk time point in the analysis. Colon iron concentrations were measured in baseline rats at 2.5 wk, and in saline-treated rats only at 10 wk. Thus two-way ANOVA was used to examine the effect of iron and study time on colon iron. Fisher’s least significant difference test (Snedecor and Cochran 1980) was used to determine differences between treatment means. The effect of iron on aberrant crypt foci development was assessed using the nonparametric procedure, the Kruskal-Wallis test (Conover 1980), after it was determined that the data were not normally distributed. Differences were considered significant if \( P < 0.05 \).

**RESULTS**

**Body weight.** Neither diet nor carcinogen treatment significantly affected final body weight or total change in body weight during the study. Change in body weight (final body weight minus initial body weight) over the 10-wk study period for rats fed AFe, MHFe and HFe diets was 348 ± 36, 341 ± 33 and 337 ± 4 g, respectively. Body weight gain was attenuated in AOM-treated rats during the 24-h period after injection (\( P < 0.0001; \) data not shown), and weekly weight gain was slightly lower in AOM-treated rats than in saline-treated rats during the 2-wk injection period (\( P < 0.01; \) data not shown). Five days after the final AOM injection, body weight was lower in AOM-treated rats (217 ± 2 g) than in saline-treated rats (224 ± 2 g) (\( P < 0.05 \)); by the following week, however, an AOM effect on body weight was no longer apparent.

**Hematocrit, tissue iron concentrations and liver weight.** Hematocrit and relative liver weight were not significantly affected by dietary iron or AOM treatment (Table 2). Although liver iron concentrations were increased in a dose-dependent manner in response to dietary iron intakes at both the 2.5- and 10-wk time points, spleen iron was significantly affected by dietary iron level only after 10 wk of feeding (Table 2). Spleen iron was significantly higher at 10 wk than at 2.5 wk in all dietary groups (\( P < 0.001 \); base-line vs. saline-treated rats only). AOM administration did not alter liver or spleen iron concentrations. The effects of diet and time on colon iron concentrations are shown in Figure 1. Colon iron concentrations were higher in the MHFe and HFe groups compared with the AFe group after 2.5 wk of treatment, but after 10 wk of treatment were higher only in rats fed HFe diets compared with controls. At 10 wk, colon iron concentrations of rats in MHFe and HFe groups were lower than those of their respective groups after 2.5 wk.

**Copper and manganese status indicators.** Liver copper, and Cu,ZnSOD and ceruloplasmin activities were used to assess copper status (Johnson and Murphy 1988), whereas liver

### Table 2

<table>
<thead>
<tr>
<th>Hematocrit (10 wk)</th>
<th>Relative liver weight (10 wk)</th>
<th>Spleen Fe (2.5 wk)</th>
<th>Spleen Fe (10 wk)</th>
<th>Liver Fe (2.5 wk)</th>
<th>Liver Fe (10 wk)</th>
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<tbody>
<tr>
<td>( g/100 \text{ g body wt} )</td>
<td>( \mu\text{mol/g wet organ} )</td>
<td>( \mu\text{mol/g wet organ} )</td>
<td>( \mu\text{mol/g wet organ} )</td>
<td>( \mu\text{mol/g wet organ} )</td>
<td>( \mu\text{mol/g wet organ} )</td>
</tr>
<tr>
<td>AFe</td>
<td>Saline</td>
<td>0.44 ± 0.01</td>
<td>3.0 ± 0.1</td>
<td>3.57 ± 0.22</td>
<td>9.64 ± 0.62c</td>
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<td>2.8 ± 0.1</td>
<td>3.39 ± 0.09</td>
<td>11.23 ± 0.54a,b,c</td>
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<tr>
<td>MHFe</td>
<td>Saline</td>
<td>0.44 ± 0.004</td>
<td>2.9 ± 0.1</td>
<td>10.39 ± 0.48b,c</td>
<td>3.61 ± 0.50b</td>
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<td>AOM</td>
<td>0.45 ± 0.01</td>
<td>3.0 ± 0.2</td>
<td>11.47 ± 0.55a,b</td>
<td>4.47 ± 0.31a</td>
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<td>HFe</td>
<td>Saline</td>
<td>0.45 ± 0.01</td>
<td>3.1 ± 0.1</td>
<td>4.08 ± 0.19</td>
<td>12.67 ± 0.70a</td>
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<tr>
<td></td>
<td>AOM</td>
<td>0.46 ± 0.01</td>
<td>2.9 ± 0.1</td>
<td>11.47 ± 0.55a,b</td>
<td>4.47 ± 0.31a</td>
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<td>ANOVA (P-value)</td>
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<tr>
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<td>NS</td>
<td>NS</td>
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<tr>
<td>AOM</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Fe × AOM</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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</table>

1 Mean ± SEM. At 2.5 wk time point, \( n = 5–6 \)/group. At 10 wk time point, \( n = 13–15 \)/group.

2 Abbreviations: AFe, adequate iron; MHFe, moderately high iron; HFe, high iron; AOM, azoxymethane.

3 Within a given column, means with different superscript letters differ significantly (\( P < 0.05 \)).

4 NS, nonsignificant, \( P = 0.05 \).
MnSOD activity was used as a functional indicator of manganese status (Davis et al. 1990). Liver copper concentrations were not significantly affected by iron intake or AOM treatment, although there was a trend toward an AOM × iron interaction (P < 0.16, Table 3). Although AOM treatment resulted in higher liver copper concentrations in rats fed control diets, liver copper was lowered by AOM in rats fed the MHFe and HFe diets. Iron intake significantly affected plasma ceruloplasmin activity (P < 0.01), and least significant difference tests showed that AOM-treated rats fed AFe diets had higher ceruloplasmin activity than AOM-treated rats fed MHFe and HFe diets and all rats fed HFe diets (Table 3). Liver Cu, ZnSOD and MnSOD activities were not significantly affected by dietary iron (Table 3), although there was a trend toward slightly lower liver MnSOD activity in HFe diets groups compared with controls (P < 0.13). Liver Cu, ZnSOD activity tended to be 13% lower (P < 0.10) in rats treated with AOM (3 saline vs. 3 AOM treatments, Table 3).

**DISCUSSION**

We tested the hypothesis that high dietary iron intakes (5 and 10 times normal) would increase colon iron concentrations, lipid peroxidation, cell proliferation and development of aberrant crypt foci in a dose-response manner. It has been proposed that iron enhances colon carcinogenesis either by elevating tissue iron (Toyokuni 1996) or by elevating fecal iron, which increases fecal mutagenicity (Babbs 1990). The percentage of iron absorbed decreases with oral iron overload in both humans and rats (Reddy and Cook 1991). Therefore, as dietary iron increases, both tissue iron and fecal iron increase. This experimental design provides the opportunity to study the effect of elevated tissue and fecal iron on colon carcinogenesis.

Tissue iron concentrations were assessed after 2.5 wk of treatment, which corresponded to the time of carcinogen administration, and after 10 wk, at the completion of the study. After 2.5 wk of treatment, liver iron concentrations were 100 and 200% higher, and colon iron was 100 and 300% higher in rats fed MHFe and HFe diets, respectively, than in controls. Previous studies of rats fed moderately high iron diets (242 mg Fe/kg diet) have shown comparable increases in liver iron (Kays et al. 1991). After 10 wk of dietary treatment, liver iron remained at high levels and accumulation of iron in the spleen became apparent. Interestingly, in this study, colon iron levels

### Table 3

<table>
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<tr>
<th></th>
<th>Liver Cu (μmol/g wet liver)</th>
<th>Ceruloplasmin (U/L)</th>
<th>Liver Cu, ZnSOD (U/mg protein)</th>
<th>Liver MnSOD (U/mg protein)</th>
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<tbody>
<tr>
<td>AFe</td>
<td></td>
<td></td>
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<tr>
<td>Saline</td>
<td>0.044 ± 0.002 (14)</td>
<td>105.5 ± 4.5 (9)</td>
<td>58.8 ± 6.8 (14)</td>
<td>3.9 ± 0.3 (14)</td>
</tr>
<tr>
<td>AOM</td>
<td>0.050 ± 0.004 (15)</td>
<td>118.8 ± 4.4 (13)</td>
<td>48.6 ± 2.3 (15)</td>
<td>3.9 ± 0.2 (15)</td>
</tr>
<tr>
<td>MHFe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>0.049 ± 0.003 (15)</td>
<td>105.2 ± 5.2 (11)</td>
<td>54.3 ± 7.1 (15)</td>
<td>3.4 ± 0.3 (15)</td>
</tr>
<tr>
<td>AOM</td>
<td>0.045 ± 0.003 (15)</td>
<td>104.9 ± 3.4 (14)</td>
<td>47.8 ± 3.0 (15)</td>
<td>3.4 ± 0.3 (15)</td>
</tr>
<tr>
<td>HFe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>0.046 ± 0.003 (15)</td>
<td>96.2 ± 6.6 (14)</td>
<td>56.7 ± 5.2 (15)</td>
<td>3.5 ± 0.3 (15)</td>
</tr>
<tr>
<td>AOM</td>
<td>0.041 ± 0.004 (15)</td>
<td>93.0 ± 5.9 (15)</td>
<td>51.3 ± 5.7 (15)</td>
<td>3.4 ± 0.3 (15)</td>
</tr>
</tbody>
</table>

1 Mean ± SEM (n).
2 Within a given column, means with different superscript letters differ significantly (P < 0.05).
3 Abbreviations: AFe, adequate iron; MHFe, moderately high iron; HFe, high iron.
4 One unit (U) of Cu, ZnSOD or MnSOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%.
5 NS, nonsignificant P ≥ 0.05.
of rats fed the MHe and HFe diets were lower after 10 wk of treatment than at the 2.5-wk time point and were only 30–80% higher than iron concentrations found in rats fed control diets. Similarly, Siegers et al. (1992) reported a normalization of colon iron in iron-supplemented rats after 20 wk, whereas liver iron remained significantly elevated. It is well established that as iron stores increase, iron absorption is reduced in genetically normal animals (Yip and Dallman 1996). Reddy and Cook (1991) reported that oral iron loading of rats fed 500 mg Fe/kg diet for 1 wk decreased basal iron absorption from >50 to 27%. This characteristic of iron metabolism may account for the reduction of colon iron levels seen at the 10-wk time point. Despite our observation that colon iron was high at the time of carcinogen administration (2.5 wk) and remained significantly elevated throughout the course of the study, iron did not alter biomarkers of tumor development.

Few studies have examined the effect of high dietary iron on early or intermediate biomarkers of colon carcinogenesis (Nelson et al. 1989, Thompson and Zhang 1991). In this study, neither cell proliferation nor aberrant crypt foci development were significantly enhanced by high iron intakes. Of the cell proliferation indices studied, only crypt height was significantly higher in AOM-treated rats than in saline-treated rats (P < 0.05), and it was not further elevated by high iron intakes. Morgan and Singh (1995) previously reported a lack of association between iron intake and colonic labeling index in a human population with an iron intake range of 3.5–127 mg/d. In contrast, Thompson and Zhang (1991) reported that iron supplementation of 535 mg/kg diet increased the colonic labeling index in female mice after only 2 wk of treatment. Thus, the lower iron concentration used in this study (450 mg Fe/kg diet) may not have been sufficient to produce a change in cell proliferation, or our results may have conflicted because of treatment time, species or gender differences. However, on the basis of recent research (Chang et al. 1997), this result may not be sufficient to rule out a modulatory role for iron in colon carcinogenesis. Cell proliferation is not consistently associated with tumor development, and the balance between cell proliferation and apoptosis may be more predictive of abnormal growth (Chang et al. 1997).

We found that total numbers of aberrant crypt foci were not enhanced by iron supplementation. Some have proposed that development of large foci (foci with >4 aberrant crypts/foci) is also a predictor of tumor incidence in the AOM model of colon carcinogenesis (Lafave et al. 1994, Pretlow et al. 1992). Our data were suggestive of a slight trend toward an elevation in large foci development as dietary iron increased. Although the differences were not significant (P = 0.29), the median number of large foci of both the MHe and HFe groups was 42% higher than that found in the AFe group. In comparison, rats fed high fat diets (50 g corn oil + 150 g beef tallow/kg diet), which are associated with colon tumor promotion in rodents, had ~72% more large aberrant crypt foci than controls in a study by Lafave et al. (1994). Previous rodent studies showing a significant relationship between high iron intake and colon tumorigenesis were of longer duration, the diets fed had higher iron concentrations and/or different rodent species were used than in the current investigation. In studies of at least 20 wk, Nelson et al. (1989) reported greater tumor development in rats fed 580 mg Fe/kg diet, and research by Siegers et al. (1992) showed elevated tumor rates in mice fed even higher iron levels of 10–35 g iron fumarate/kg diet. The trend toward enhanced large foci development may be consistent with these studies and suggests that the threshold at which dietary iron might affect colon carcinogenesis may be beyond levels typically consumed in the human diet. It is also possible that diets with 225–450 mg Fe/kg diet may influence colon tumorigenesis at a later stage of tumor development than was examined in this investigation. However, the overall results of our research do not support the hypothesis that feeding rats iron-supplemented diets in the range of 5–10 times adequate will result in a significant increase in development of aberrant crypt foci.

It was theorized that by altering copper or manganese status, high iron levels would impair antioxidant enzyme activity, thus enhancing oxidative stress. Except for modest reductions of plasma ceruloplasmin activity in animals fed high dietary iron, there were no other indications of altered copper status. This is consistent with other studies, which showed that consumption of diets with moderately high iron (191 mg Fe/kg diet) resulted in lower ceruloplasmin activity without affecting liver copper status (Johnson and Murphy 1988). Whether slight decreases in plasma ceruloplasmin would influence colon carcinogenesis is unknown. DiSilvestro et al. (1992) reported that copper deficiency enhanced colon tumorogenesis in rats, but plasma ceruloplasmin was reduced to almost nondetectable levels in the animals, suggesting a severe impairment of copper status. Liver MnSOD activity, the manganese status indicator used in this study, was not significantly altered in rats fed high dietary iron.

We also examined the effect of high iron on key components of the antioxidant enzyme system in colon mucosa, including SOD, glutathione peroxidase and glutathione S-transferase. Iron intake had no effect on total SOD or glutathione peroxidase activities of colon mucosa, similar to results of Kuratko (1997). It has been suggested that activities of other antioxidant enzymes such as glutathione S-transferase might increase in response to iron-induced lipid peroxidation (Toyokuni 1996). Although colon glutathione S-transferase activity was not significantly altered (P = 0.35), mean activity was

### TABLE 4

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>Total ACF/cm² (≥1 crypt/foci)²</th>
<th>ACF/cm² (≥2 crypts/foci)²</th>
<th>Large ACF/cm² (≥4 crypts/foci)²</th>
<th>Aberrant crypts/foci³,⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFe</td>
<td>15</td>
<td>7.4 (2.8, 11.5)</td>
<td>5.5 (2.3, 8.8)</td>
<td>1.9 (0.9, 3.0)</td>
<td>2.93 ± 0.08</td>
</tr>
<tr>
<td>MHe</td>
<td>15</td>
<td>7.5 (6.3, 16.0)</td>
<td>6.3 (4.7, 13.2)</td>
<td>2.7 (1.5, 4.7)</td>
<td>2.88 ± 0.10</td>
</tr>
<tr>
<td>HFe</td>
<td>14</td>
<td>7.9 (4.3, 11.4)</td>
<td>6.8 (3.6, 10.6)</td>
<td>2.7 (1.5, 3.7)</td>
<td>3.02 ± 0.09</td>
</tr>
</tbody>
</table>

1 Abbreviations: AFe, adequate iron; MHe, moderately high iron; HFe, high iron.
2 Data were not normally distributed and medians (25%, 75% CI) are reported.
3 Mean ± SEM.
4 Total number of aberrant crypts/total ACF.
almost 30% higher in rats fed HFe diets compared with those fed AFe diets. Iron intakes >450 mg/kg diet may be necessary to induce significant changes in glutathione S-transferase activity, which would most likely serve to protect the colon. Thus, in this study, there was no indication of impaired colonic mucosal antioxidant enzyme status in rats fed high iron diets. However, total SOD may not accurately reflect changes in colonic antioxidant status, and examination of changes in Cu,ZnSOD and MnSOD activities may yield the best understanding of how iron administration affects the colonic mucosal SOD antioxidant system. Kuratko (1997) has reported decreases in colonic MnSOD activity in male Fisher rats fed high iron levels of 140 mg/kg diet.

In light of our finding that the activities of major antioxidant enzymes were not impaired, it was not surprising that dietary iron concentrations as high as 10 times control levels did not increase lipid peroxidation in colonic mucosa. Kuratko et al. (1994) have shown that lipid peroxidation is not easily induced in colonic mucosa. Previous reports of iron-enhanced lipid peroxidation in intestinal tissue (Younes et al. 1990) resulted from 4-wk feeding studies with mice and the use of much higher iron concentrations (5–35 g iron fumarate/kg diet) than were chosen for this study.

In conclusion, our data do not support the hypothesis that high amounts of dietary iron, ranging from 5 to 10 times adequate, will enhance oxidative stress or increase cell proliferation or aberrant crypt foci development, intermediate biomarkers of colonic neoplasia. Further research is necessary to determine if high dietary iron intake influences other intermediate biomarkers of colonic cancer or if studies of longer duration show a significant effect of iron intakes of this magnitude on colon carcinogenesis.

ACKNOWLEDGMENTS

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LITERATURE CITED


