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ORIGINAL ARTICLE

Low-dose Oral Ferrous Fumarate Aggravated Intestinal Inflammation in Rats with DSS-Induced Colitis

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Background: Oral ferrous iron therapy may reinforce intestinal inflammation. One possible mechanism is by catalyzing the production of reactive oxygen species. We studied the effects of low-dose oral ferrous fumarate on intestinal inflammation and plasma redox status in dextran sulfate sodium (DSS)-induced colitis in rats.

Methods: Forty male Wistar rats were divided into 5 groups: no intervention, sham gavage (distilled water), ferrous fumarate, DSS, and ferrous fumarate + DSS. Ferrous fumarate was dissolved in distilled water (0.60 mg Fe^{2+}/kg per day) and administered by gavage on days 1 to 14. All rats were fed a standard diet. Colitis was induced by 5% DSS in drinking water on days 8 to 14. Rats were killed on day 16. Histologic colitis scores, fecal granulocyte marker protein, plasma malondialdehyde, plasma antioxidant vitamins, and plasma aminothiols were measured.

Results: DSS significantly increased histologic colitis scores ($P < 0.001$) and fecal granulocyte marker protein ($P < 0.01$). Ferrous fumarate further increased histologic colitis scores ($P < 0.01$) in DSS-induced colitis. DSS + ferrous fumarate decreased plasma vitamin A compared with controls ($P < 0.01$). Otherwise, no changes were seen in plasma malondialdehyde, plasma antioxidant vitamins, or plasma aminothiols.

Conclusion: Low-dose oral ferrous iron enhanced intestinal inflammation in DSS-induced colitis in rats.

Key Words: colitis, dextran sulfate, iron, oxidative stress, rats

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Iron deficiency anemia is a common problem of inflammatory bowel disease (IBD).[1] Oral iron supplements, generally ferrous (Fe^{2+}) salts, are poorly absorbed and lead to high fecal iron concentrations. In an inflamed bowel, iron may react with superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) from activated neutrophils in the mucosa, leading to production of the hydroxyl radical ($OH^{\bullet}$). Hydroxyl radicals can attack the mucosa, cause oxidative damage, and thereby enhance intestinal injury, as suggested by results obtained in animal models of IBD given iron-fortified diets.[2–4] In these studies, dietary iron content was 10 to 100 times the normal.

Dextran sulfate sodium (DSS) is a synthetic, sulfated polysaccharide that, given in drinking water to rodents, induces colitis, which is clinically and histologically reminiscent of human ulcerative colitis.[5] Granulocyte marker protein (GMP) extracted from feces is a noninvasive marker of gastrointestinal inflammation in rats,[6] and increased levels of GMP were previously found in DSS-induced colitis.[7] It was the object of this study to evaluate the effects of low-dose oral ferrous fumarate given by gavage on intestinal inflammation and plasma redox status in DSS-induced colitis in Wistar rats.

MATERIALS AND METHODS

Animals and Husbandry

Forty male Wistar rats (Mollegaard & Blomholtgaard, Ry, Denmark), which were 12 weeks old and had a mean weight of 375 g, were housed individually in Makrolon III cages in an open system. They were kept under standard laboratory conditions with a temperature of 22 ± 1°C, dark/light cycles of 12/12 hours, relative humidity of 55% ± 5%, and 20 air changes per hour. Access to food, Beekay rodent low protein diet (BK Universal AS, Nittedal, Norway), was ad libitum. Iron content in the diet was 130 mg/kg (ferrous

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carbonate, 40 mg/kg; the rest as organic iron). Tap water was given ad libitum if not otherwise stated. The Norwegian Animal Research Authority approved the protocol.

Induction of Colitis

Acute colitis was induced by 50 g/L of DSS (MW 44 000; TdB Consultancy AB, Uppsala, Sweden) given in distilled drinking water for 7 days.

Experimental Protocol

After 7 days of acclimatization, animals were divided at random into 5 groups of 8 rats: control (no intervention); sham gavage (1 mL distilled water once daily by gavage on days 1 to 14); iron; DSS; or iron + DSS.

Ferrous fumarate (Nycomed Pharma AS, Asker, Norway) was dissolved in distilled water to a concentration of 9.4 mM and saturated with nitrogen. To diminish oxidation, a new solution was made each day. A dose of 0.60 mg Fe^{2+}/kg rat (0.5 mL) once daily was given by gavage with a feeding tube (CH 06, 50 cm; Maersk, Lynge, Denmark) on days 1 to 14. DSS was given on days 8 to 14 and replaced the tap water.

On day 16, all rats were anesthetized by subcutaneous injection of a combination of fentanyl citrate + fluanisone (Hypnorm; Janssen Pharmaceutica, Beerse, Belgium) and midazolam (fentanyl citrate 0.079 mg/mL, fluanisone 2.5 mg/mL, midazolam 1.25 mg/mL; Dormicum; Roche, Oslo, Norway), at a dose of 0.2 mL/100 g. Thoracotomy, cardiac puncture, and exsanguination were performed.

Sample Collection and Analysis of Biochemical Parameters

Blood was drawn into a heparin-containing Vacutainer, placed on ice for 10 minutes, and centrifuged at 2000 rpm for 10 minutes at 4 °C. Plasma was stored at −80°C for future analysis. Plasma vitamins A, E (α-tocopherol), C, malondialdehyde,[8] and glutathione[9] were measured by high-performance liquid chromatography as previously described. Plasma iron was analyzed spectrophotometrically with ferrozine reagent on a Modular P800 analyzer (Roche Diagnostics, Mannheim, Germany).

Stools were collected on day 15 and kept below −20°C until analysis for GMP. Briefly, 1 g of fecal material was diluted in 4 mL of extraction buffer and thoroughly homogenized using an Ultra Turrax (20,000 rpm) before centrifugation at 45,000*g* for 20 minutes. The upper halves of the supernatants were carefully harvested and run on a standard 1-step enzyme-linked immunosorbent assay (ELISA) as described previously.[10]

Tissue Preparation and Histologic Examination

Colon from the colocecal junction to the anal verge was removed. The length of the colon was recorded. The colon was rinsed with phosphate-buffered saline buffer, opened longitudinally, and divided into one proximal and one distal segment, which were fixed in 10% formalin and embedded in paraffin. Eight pieces per segment were stained with hematoxylin and eosin. One piece per segment was stained with Prussian blue for qualitative determination of iron.

Crypt and inflammatory scores were determined according to a validated scoring system[2] and examined by a pathologist unaware of the experimental protocol. Crypt injury was scored as follows: grade 0, intact crypts; grade 1, loss of the bottom third of crypts; grade 2, loss of the bottom two thirds of crypts; grade 3, loss of the entire crypt with the surface epithelium remaining intact; grade 4, loss of the entire crypt and surface epithelium. The severity of inflammation was scored as follows: grade 0, normal; grade 1, focal inflammatory cell infiltration; grade 2, inflammatory cell infiltration, gland dropout, and crypt abscess. Both scores include a measure of involvement as follows: grade 1, 1% to 25%; grade 2, 26% to 50%; grade 3, 51% to 75%; grade 4, 76% to 100%. The score was the product of either the crypt or inflammation grade by the involvement grade. The final crypt and inflammation scores were the averages of all individual scores of 16 pieces per colon.

Statistical Analysis

Data were analyzed using the GraphPad Prism version 4 (GraphPad Software, San Diego, Calif.) statistical software package. Results are presented as mean ± SEM. Differences between means were evaluated with 1-way ANOVA and Bonferroni posttest for selected pairs of columns. Mean of differences and 95% confidence intervals (CIs) are given if not otherwise stated. P values less then 0.05 were considered statistically significant.

RESULTS

There was no difference in any parameter between the control (no intervention) group and the sham gavage group. These 2 groups were therefore merged into 1 group called controls. One rat in the iron group was excluded on day 2 because it bit off and swallowed the distal part of the feeding tube. Otherwise, no mortality was observed.

Weight Change

From the start of DSS treatment until death, weight loss was observed in rats on DSS (−11.3 ± 4.0 g) and iron + DSS (−15.4 ± 1.4 g). No significant weight change was observed in control rats (1.9 ± 1.4 g) and rats receiving only iron (1.7 ± 1.9 g). Weight change differed significantly between controls and rats on DSS (12.7 g; 95% CI, 4.5–20.9; $P < 0.01$), but not between rats on DSS and iron + DSS.

DSS Intake

Total intake of water with DSS was similar in the DSS group (157 ± 11.5 mL) and the iron + DSS group (146 ± 14.6 mL; $P = 0.57$).

Colon Length

Colon length did not differ significantly between groups, but there was a tendency for shorter colons in groups with colitis (DSS, 16.8 ± 0.4 cm; iron + DSS, 15.9 ± 0.8 cm) compared with those without (controls, 17.6 ± 0.4 cm; iron, 17.6 ± 0.6 cm).

Histology of the Colon

Microscopic evaluation showed indications of colitis in all rats receiving DSS. Changes were most prominent in the distal colon with areas of erosions, crypt distortion, and inflammatory infiltration as previously described[5] (Fig. 1). In rats with DSS-induced colitis, ferrous fumarate significantly increased mean crypt score by 3.0 (95% CI, 1.2–4.8; $P < 0.001$; Fig. 2) and mean inflammation score by 1.8 (95% CI, 0.6–3.1; $P < 0.01$; Fig. 2). Rats receiving only ferrous fumarate had normal colonic histology.

No intestinal iron deposition was found in any of the rats receiving ferrous fumarate.

Granulocyte Marker Protein

Compared with controls, DSS-induced colitis significantly increased fecal GMP by 55.8 mg/L (95% CI, 13.1–98.5; $P < 0.01$; Fig. 2). Ferrous fumarate did not further increase GMP in DSS colitis (difference between means, 24.2 mg/L; 95% CI, –25.1–73.4; $P > 0.05$; Fig. 2).

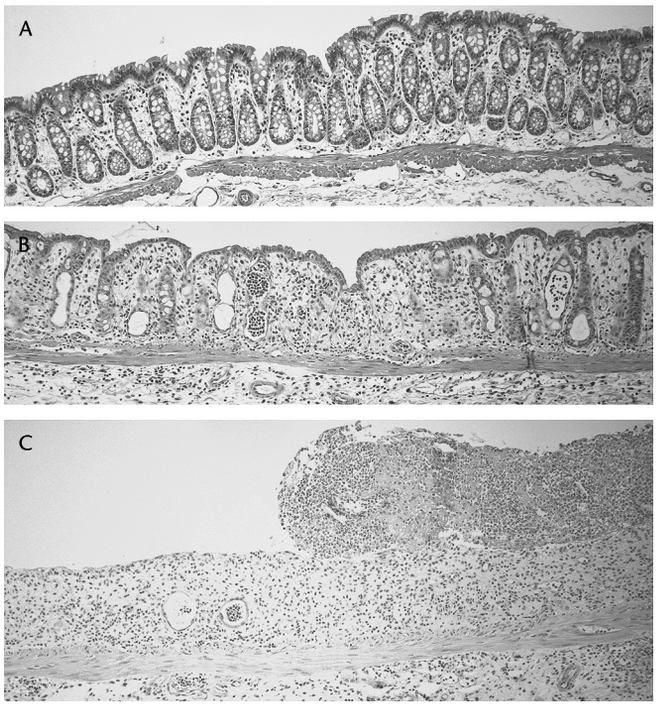

FIGURE 1. A, normal colonic mucosa of a healthy control rat. B, colonic mucosa of a rat with DSS colitis showing crypt loss, inflammatory infiltration, and a crypt abscess. C, colonic mucosa of a rat that received DSS and ferrous fumarate showing complete loss of mucosal architecture and 2 crypt abscesses. The inflammatory debris on top indicates ulceration in close vicinity (hematoxylin and eosin, ×10).

Laboratory Investigations

Laboratory investigations are shown in Table 1. Ferrous fumarate + DSS decreased plasma vitamin A compared with controls ($P < 0.01$). Otherwise, there were no differences in plasma vitamins, malondialdehyde, or aminothiols between groups. Plasma iron did not differ between rats receiving ferrous fumarate supplementation and those that did not.

DISCUSSION

The ferrous (Fe^{2+}) fumarate dose administered, 0.60 mg/kg daily, corresponds to low-dose supplementation in humans. Recommended dosage in iron replacement therapy for adults is 2 to 3 mg/kg daily. Administration by gavage allowed exact iron dosage. In previous studies in animal models of IBD, different iron formulations were administered by diet fortification.[2–4,11] Food ingestion should have been monitored to estimate iron intake accurately, but this was not done in any of the cited studies. The magnitude of diet fortification, 10 to 100 times the normal iron content, suggests that iron intake was considerably higher in the previous[2–4,11] compared with the present study. Furthermore, we found no change in plasma iron and no iron deposition in colonic mucosa of iron-supplemented rats. Others found significant increase in plasma iron[4] and iron deposition in inflamed mucosa[2,4,12] of animals receiving iron-fortified diets, indicating that higher doses were applied.

In this study, low-dose oral ferrous fumarate increased histologic colitis scores in DSS-induced colitis in Wistar rats. Control animals on iron supplementation had normal histology. Higher doses of iron lead to increase in intestinal inflammation, as assessed by histology, in several models of experimental colitis in rats, induced by DSS,[2,13] idioacetamide,[11,14] and trinitrobenzene sulfonic acid.[4] In interleukin-10 knock-out mice, developing an enterocolitis reminiscent of Crohn's disease, oral as well as rectal ferrous sulfate supplementation led to increased production of proinflammatory cytokines in the colonic mucosa.[3] No increase in cytokine levels was noted in control mice on a high-iron diet.[3]

Nonheme iron is transported into the enterocytes in the ferrous form, and most iron absorption takes place in the proximal duodenum, near the gastric outlet.[15] The gastric acid helps to keep iron in the more soluble ferrous form (Fe^{2+}). At the neutral pH of the duodenum, ferrous iron is rapidly oxidized to ferric iron (Fe^{3+}), which precipitates into poorly absorbed iron hydroxide and iron oxide polymers.[16] Oral iron supplementation therefore leads to high fecal iron concentrations. However, to participate in reactions leading to reactive oxygen species production, iron must be either freely water soluble or loosely bound to small organic compounds,

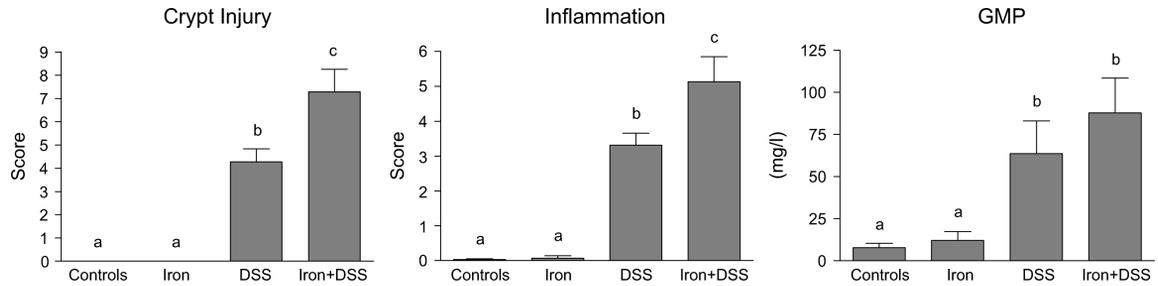

FIGURE 2. Effect of DSS and ferrous fumarate (iron) on histologic crypt and inflammation scores and fecal GMP. Results are expressed as mean ± SEM. Means with different letters differ ($P < 0.05$).

and only a fraction of the total iron concentration in feces is in such states.[17] On the other hand, only small amounts of free iron are needed to promote free radical generation. Lund et al[17] showed that low-dose ferrous sulfate supplementation markedly increased the concentration of weakly bound iron in feces of healthy volunteers.

At the mucosal surface of inflamed bowel, reactive oxygen species from activated neutrophils may react with fecal iron to produce extremely reactive hydroxyl radicals. Hydroxyl radicals can attack any cell component and cause oxidative damage.[18] They can also lead to the formation of other reactive oxygen species and start chain reactions of lipid peroxidation.[19] The pro-oxidative imbalance created by overproduction of reactive oxygen species may directly enhance intestinal inflammation.

We did not find any changes in plasma redox status in rats with DSS colitis compared with control rats. Others report similar findings, with no increase in either colonic[2,4] or plasma lipid peroxide levels[2] or plasma antioxidants[2] in experimental colitis. On the other hand, iron supplementation to rats with colitis increased colonic[2,4,11] and plasma lipid peroxides and plasma 8-isoprostanes[13] and decreased plasma antioxidant vitamins.[2] These findings support the theory that oxidative stress is involved in the iron-induced aggravation of colitis. In this study, only one plasma parameter, vitamin A, decreased significantly after ferrous fumarate supplementation to rats with colitis. However, plasma parameters are not ideal for estimation of oxidative stress in the gastrointestinal tract because local changes have to be substantial to be recognized in plasma. The iron dose may have been too low to cause such changes. Fecal GMP is an unspecific marker of intestinal inflammation[20] and not a product of lipid peroxidation and does not necessarily relate to the production of free radicals in the mucosa.

Bacterial flora play an important role in the induction of DSS-induced colitis,[21] and treatment with antibiotics,[21] probiotics,[22] and prebiotics[23] ameliorate acute DSS colitis in rats. In contrast, oral iron supplementation could exacerbate intestinal inflammation by altering the microbial balance in an unfavorable manner. In line with this, ferrous sulfate supplementation in rats led to strong alterations in fecal flora such as to favor the growth of microorganisms with a pathogenic potential.[24] Iron is an essential element for bacterial growth and virulence.[25] Bacteria therefore produce ferric iron-chelating agents, called siderophores, which are capable of solubilizing and transporting iron into the microbe.[25] The affinity of siderophores to iron is remarkably strong, and they are capable of securing the metal from iron hydroxides.[25] As mentioned above, iron hydroxides are abundant in feces after ferrous iron intake and give bacteria plentiful access to iron.

The prevalence of anemia in patients with ulcerative colitis ranges from 9% to 67% depending on the patient subpopulation.[26] Iron deficiency and anemia of chronic diseases

TABLE 1. Plasma Levels of Vitamins A, E, and C, Malondialdehyde, Aminothiols, and Iron

Parameter	Controls	Ferrous Fumarate	DSS	Ferrous Fumarate + DSS
Vitamin A (μmol/L)	1.44 (0.05)	1.29 (0.08)	1.31 (0.07)	1.12 (0.03)*
Vitamin E (μmol/L)	15.7 (0.6)	14.6 (0.6)	14.9 (0.9)	15.3 (0.8)
Vitamin C (μmol/L)	35.0 (2.2)	31.8 (3.7)	26.8 (2.7)	34.7 (3.6)
Malondialdehyde (nmol/L)	1290 (70)	1167 (53)	1309 (94)	1189 (74)
Total cysteine (μmol/L)	138.2 (6.1)	150.7 (7.5)	140.6 (4.2)	153.3 (6.7)
Total glutathione (μmol/L)	37.5 (2.1)	41.5 (2.1)	38.6 (2.4)	38.1 (2.4)
Plasma iron (μmol/L)	43.6 (2.4)	43.1 (3.0)	41.1 (2.7)	41.3 (3.5)

Values are mean (SEM).
*$P < 0.01$ compared with controls.

are the most common causes of anemia in IBD, and iron therapy is routine. Tissue damage caused by oxidative stress[27] and the intestinal flora[28] are both thought to play pathogenic roles in human ulcerative colitis. Because iron may enforce these pathogenic mechanisms, caution should be exercised in the use of oral iron supplements in the treatment of iron deficiency anemia in patients with active colitis.

In conclusion, low-dose oral ferrous iron supplementation increased intestinal inflammation in experimental colitis. Iron-mediated oxidative damage and undesirable alterations in intestinal flora are both conceivable pathogenic mechanisms.

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