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5-ASA and lycopene decrease the oxidative stress and inflammation induced by iron in rats with colitis

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Background. Supplementation of 5-aminosalicylic acid (5-ASA) and of iron are among the principal therapies in patients with inflammatory bowel disease. Therapeutic iron, as well as heme iron from chronic mucosal bleeding, can increase iron-mediated oxidative stress in colitis. This study was designed to examine the influence of iron supplementation on histological expression and oxidative status relative to 5-ASA treatment and antioxidant treatment. ***Methods.*** Colitis was induced using the iodoacetamide rat model, and rats were divided into different dietary groups of 6 rats each: 1, normal chow diet (control); 2, diet supplemented with iron; 3, iron supplementation and lycopene; 4, iron and β-carotene; 5, 5-ASA; 6, 5-ASA and lycopene; 7, 5-ASA and iron; 8, 5-ASA, iron, and lycopene. The animals were killed after 3 days and the weight of the ulcerated area recorded. Mucosal specimens were histologically evaluated. Myeloperoxidase (MPO) was measured to evaluate inflammatory status (U/g). Malondialdehyde (MDA) was measured in colonic tissue (μmol/g) and superoxide dismutase (SOD) in erythrocytes to assess the degree of tissue oxidative stress. ***Results.*** Significantly more severe colitis, including necrosis, ulceration, and hemorrhage, was seen in colonic biopsies of rats with colitis when iron was supplemented. This pathology was attenuated when iron was given in combination with 5-ASA and/or lycopene. There was no significant benefit from adding β-carotene. ***Conclusions.*** Iron supplementation can amplify the inflammatory response and subsequent mucosal damage in a rat model of colitis. We suggest that the resultant oxidative stress generated by iron supplementation leads to the extension and propagation of crypt abscesses, either through direct membrane disruption by lipid peroxidation or through the generation of secondary toxic oxidants. Simultaneous treatment with 5-ASA and/or lycopene minimizes the potential hazard of iron. Therefore, we suggest giving iron supplementation with 5-ASA or lycopene or both.

Key words: inflammatory bowel disease, lycopene, oxidative stress, 5-ASA, iron

Introduction

Inflammatory bowel disease (IBD) is a chronic, relapsing inflammatory disorder. Due to the idiopathic nature of the disease, treatment focuses on reducing symptom severity and the number of relapses through pharmacological intervention. Sulfasalazine was the mainstay treatment for IBD and has been used for the past 50 years. The drug is poorly absorbed in the upper gastrointestinal tract, allowing most of it to reach the colon. There, its diazo bond is reduced by colonic flora to liberate sulfapyridine and mesalamine, the latter being responsible for the therapeutic effects seen in IBD. The action of mesalamine may be mediated through the inhibition of cyclooxygenase and lipoxygenase,[1] thus attenuating the formation of potentially proinflammatory prostaglandins.[2] In addition to inhibiting arachidonate metabolism, high concentrations of sulfasalazine and 5-aminosalicylic acid (5-ASA) have been shown to inhibit certain functions of human neutrophils such as migration, degranulation, phagocytosis, and superoxide formation.[3] More recent work has demonstrated that 5-ASA has potent antioxidant or free radical scavenging properties in vitro, which has been suggested to account for some of its therapeutic efficacy in vivo.[4]

One of the hallmarks of active episodes of colitis is the infiltration of large numbers of phagocytic leukocytes into the mucosal interstitium. Concurrent with this enhanced inflammatory infiltrate is extensive mucosal injury, resulting in the production and release of large

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quantities of reactive oxygen metabolites (ROM) such as superoxide and hydrogen peroxide. Both interact in the presence of transition metals such as iron to generate the highly reactive and cytotoxic hydroxyl radical. The hydroxyl radical is one of the most potent oxidants produced in biological systems. It is capable of oxidizing and peroxidizing a wide variety of biomolecules, such as proteins, carbohydrates and lipids.[5] In addition to classic ROM, activated neutrophils and monocytes also secrete the hemoprotein myeloperoxidase into the extracellular medium, where it catalyzes the oxidation of chloride ions via hydrogen peroxide to yield the highly reactive oxidizing and chlorinating agent hypochlorous acid. The latter has been shown to degrade gastrointestinal mucin, enhance mucosal permeability, and injure intestinal epithelial cells.

Carotenoids are important plant pigments found in the photosynthetic pigment–protein complex of plants. Research on the antioxidant activity of carotenoids was sparked by a description of their singlet-oxygen-quenching properties and their ability to trap peroxyl radicals.[6] Singlet-oxygen quenching by carotenoids occurs via physical or chemical quenching, which has been discussed in detail in the literature. The efficacy of physical quenching greatly exceeds that of chemical quenching and involves the transfer of excitation energy from O^2 to the carotenoids, resulting in ground-state oxygen and excited triplet-state carotenoids. The quenching activity of the carotenoids depends mainly on the number of conjugated double bonds. Of the natural carotenoids, lycopene is among the most efficient singlet-oxygen quencher.[7] Pro-vitamin A activity is exhibited by β-carotene but not by lycopene. Lycopene is the major carotenoid in tomato and is responsible for the red color of the fruit. It is found in relatively high concentrations in all tomato products, the major source of lycopene for humans.

The aim of this study was to examine the effect of carotenoids with and without 5-ASA on the inflammatory and oxidative status of colitis in a rat model supplemented with iron.

Materials and methods

Male Wistar rats with an average weight of 190 g (range, 180–200 g) were used in the experiment. In the first experiment, we compared lycopene and β-carotene in terms of their effects on the inflammatory and oxidative status of rats with colitis treated with iron. Based on the results, we repeated the experiment with lycopene and used 5-ASA granules. Diets were prepared fresh for each group to avoid oxidation.

Each experiment lasted 10 days. Animals were fed specific diets. The iron-enriched diets included 300 mg Fe_2SO_4/kg chow diet, lycopene [powder containing 200 mg/kg diet of pure (98%) lycopene] and β-carotene powder (54 mg/kg diet) were obtained from Lycored Beer-Sheba. Mesalazine microgranules, an ethylcellulose-coated formulation from which mesalazine is released throughout the intestinal tract, were obtained from Falk Industries (Freiburg, Germany). These granules have been tested previously and were found to be effective for animal-induced (acetic acid) IBD.[8] Food and water were available ad libitum. The food intake of the various groups was weighed to ensure uniform intake among the groups, and there was no significant difference between the groups. Carotenoids were monitored in the diets and in the tissues by an HPLC GBC 1250 fluorodetector with a C-18 column. All animals were fed their specific diets 7 days before induction of colitis. Colitis was induced using the modified MacPherson method.[9] Seventy-two hours after induction, animals were killed using anesthesia with ether. The study was approved by the Animal Care Ethics Committee of the Faculty of Agriculture, The Hebrew University of Jerusalem.

The distal 10 cm of the colon was resected. The wet weight and width of the resected colon, as well as the area of colitis, were recorded. Tissues for histological evaluation were fixed in 10% formalin, and 3- to 5-μm slices were coated with paraffin and stained with hematoxylin and eosin. The inflammatory process was assessed by histological examination of the preparation and by measurement of myeloperoxidase (MPO) in the inflamed tissues. Two pathologists performed blind examinations of the preparations, and the degree of colitis was evaluated according to the scale described by Millar et al.[10]

MPO was assessed using the method described by Krawisz et al.[11]: 200 mg of colonic mucosal scraping was homogenized three times for 3 s at 4°C with a polytron in 1 ml ice-cold 0.5% hexadecyltrimethylammonium bromide (HTAB; Sigma) in 50 mmol/l phosphate buffer pH 60. The polutron probe was rinsed twice with 1.0 ml buffer, and the washings were added to the homogentate. The homogenate was sonicated for 10 s, frozen, and thawed three times, and centrifuged at 40000 *g* for 15 s at 4°C. The supernatant was assayed for MPO activity. We added 2.9 ml 50 mmol/l phosphate buffer pH 6.0 containing 0.167 mg/ml *O*-dianizidine hydrochloride and 0.005% hydrogen peroxide to 0.1 ml supernatant. Absorbance changes at 460 nm were measured with a Spectornic 601 spectrophotometer (Milton Ray, Rochester, NY, USA). One unit of MPO activity was defined as that degrading 1 μmol peroxide per unit at 25°C.

The following parameters were used for assessment of oxidative stress: malondiadehyde (MDA) in the tissues using the method described by Buege and Aust[12]

and Esterbauer and Cheeseman[13] and superoxide dismutase (SOD) in the erythrocytes according to Fulbert et al.[14] MDA was measured by thiobarbituric acid (TBA): 2 ml fresh solution 15% w/v TEA, 0.375% TBA, and 0.25 mol/l HCl was added to 1 ml 10% colonic mucosol. The mixture was heated at 95°C for 15 min. The solution was cooled with tap water to room temperature and centrifuged at 3000 rpm for 10 min. Absorption of the pink supernatant was determined spectrophotometrically at 532 nm. CU–Zn superoxide dismutase (SOD) activity was measured by the reaction described by McCord and Fridovich (modified by Fulbert et al.[14]) in lysate of red cells from which hemoglobin was precipitated. The reaction was based on the capacity of SOD to inhibit the reduction of ferricytochrome C by the xanthine/xanthine oxidase system. A standard curve was prepared using commercially available SOD. Reaction development was monitored spectrophotometrically at 405 nm. One SOD unit is defined as the amount of enzyme inhibiting the rate of cytochrome C reduction by 50% under the respective conditions; activity is expressed per hemoglobin.

Statistical analysis: data are expressed as mean ± SD. Results were analyzed by two-tailed t test, $P < 0.05$.

Results

Macroscopic parameters

In terms of colon weight, there was no significant difference between the various study groups. The area of colitis in group 2 (iron supplementation) was significantly larger than in group 1 (normal diet) ($P < 0.05$). Group 8 exhibited a significantly smaller area of inflammation than group 7.

Microscopic scoring

Group 2 had the highest microscopic score, reflecting a higher inflammatory status than controls (group 1 and group 3) but a lower score than group 4 (Table 1). Groups 5 and 6 had the same microscopic score while group 8 scored lower than group 7. Although these results were not statistically significant, it should be noted that the lowest scoring was obtained in group 8.

MPO

This inflammation indicator was more prominent in group 2 than in groups 1 or 3 ($P < 0.05$) (Tables 2, 3). The addition of β-carotene to iron supplementation (group 4) increased the inflammatory status expressed by MPO relative to controls ($P < 0.05$). Lycopene was superior to β-carotene in attenuating the inflammatory status ($P < 0.001$). There was no advantage to adding lycopene in combination with 5-ASA. No difference was detected between groups 3 and 7. Group 8 had the lowest measured MPO, although this was not significantly different from group 7.

MDA

Lycopene, but not β-carotene (group 3 versus 4), attenuated the oxidative stress observed with iron supplementation in groups 3 and 4. There was no difference among the first four groups with respect to plasma. Addition of 5-ASA improved the MDA levels (groups 5–8 versus 1–4) (see Tables 2, 3), but there was no significant difference among groups 5–8.

SOD

Iron supplementation significantly reduced the SOD levels in erythrocytes (group 2 versus group 1) (see Tables 2, 3). Lycopene (group 3 versus 2) but not β-carotene annulled the changes induced by iron. When 5-ASA was added to the diet, SOD levels were lower in groups 5–8 versus groups 1–4 ($P < 0.05$). In group 6, higher levels were measured compared to group 5 ($P < 0.05$). SOD levels were also significantly higher in group 8 versus group 7.

Table 1. Micro- and macroscoring of colitis

Group	Colon weight (g)	Colitis area (cm^2)	Microscoring
[1] Colitis	1.62 ± 0.57[a]	1.53 ± 0.74[a]	2.9 ± 0.35[a]
[2] Ferrum	1.37 ± 0.39[a]	2.77 ± 1.74[a]	3.1 ± 0.99[a]
[3] Lycopene + ferrum	1.24 ± 0.21[a]	1.77 ± 0.79[a]	2.25 ± 0.71[a]
[4] β-carotene + ferrum	1.43 ± 0.35[a]	2.56 ± 1.46[a]	3.13 ± 0.83[b]
[5] 5-ASA	1.26 ± 0.65[a]	1.44 ± 1.78[a]	2.4 ± 0.74[a]
[6] 5-ASA + lycopene	1.4 ± 0.43[a]	1.41 ± 0.58[a]	2.4 ± 0.74[a]
[7] 5-ASA + ferrum	1.32 ± 0.35[a]	2.25 ± 1.25[a]	2.1 ± 0.99[a]
[8] 5-ASA + ferrum + lycopene	1.27 ± 0.66[a]	0.87 ± 1.03[b]	1.8 ± 1.16[b]

Values were compared in the vertical columns: those values that are marked by superscript [a] do not differ significantly; those marked by [b] differ significantly from [a] at $P < 0.05$
5-ASA, 5-aminosalicylic acid

Table 2. Effect of β-carotene vs. lycopene on inflammatory and oxidative parameters

Group	MDA tissue (μmol/g)	MPO tissue (U/g)	SOD erythrocytes (U/g Hb)
[1] Colitis	35.9 ± 25.6[a]	2.9 ± 1.6[a]	0.63 ± 0.21[a]
[2] Ferrum	43.2 ± 33.6[b]	10.3 ± 7.2[b]	0.32 ± 0.09[a]
[3] Lycopene + ferrum	28.5 ± 8.5[a]	2.2 ± 1.6[a]	0.56 ± 0.13[b]
[4] β-carotene + ferrum	27.3 ± 7.9[b]	12.3 ± 5[b]	0.34 ± 0.13[b]

Values were compared in the vertical columns: those values with superscript [a] do not differ significantly; those with [b] differ significantly from [a] at $P < 0.05$
MDA, malondialdehyde; MPO, myeloperoxidase; SOD, superoxide dismutase

Table 3. Effect of 5-ASA and lycopene on inflammatory and oxidative parameters

Group	MDA tissue (μmol/g)	MPO tissue (U/g)	SOD erythrocytes (U/g Hb)
[1] Colitis	35.9 ± 25.6[a]	2.9 ± 1.6[a]	0.63 ± 0.21[a]
[2] Ferrum	43.2 ± 33.6[a]	10.3 ± 3.2[b]	0.32 ± 0.09[b]
[5] 5-ASA	16.4 ± 1.6[b]	1.7 ± 1.2[a]	0.46 ± 0.10[c]
[6] 5-ASA + lycopene	16.1 ± 2.01[b]	4.26 ± 4.2[c]	0.67 ± 0.15[a]
[7] 5-ASA + ferrum	15.6 ± 1.45[b]	2.3 ± 1.99[a]	0.32 ± 0.20[b]
[8] 5-ASA + ferrum + lycopene	17.04 ± 2.3[b]	1.6 ± 1.81[a]	0.53 ± 0.20[a]

Values were compared in the vertical columns: those with superscript [a] do differ significantly; those with [b] differ significantly from [a]; those with [c] differ significantly from [a] and [c] at $P < 0.05$

Discussion

This study was undertaken to determine the effects of interactions between 5-ASA, carotenoids, and iron on the generation of oxidation products, antioxidant status, and the inflammatory status in a rat model of colitis. We have been able to provide some evidence that susceptibility to lipid peroxidation can be overcome by use of either 5-ASA or lycopene.

Iron aggravated the inflammatory process, as reflected by the increase in MPO in the inflamed tissues and their histological appearance. Iron also significantly changed the oxidative status in the inflamed area, expressed by changes in both SOD and MDA. Lycopene and 5-ASA, but not β-carotene, attenuated these effects. 5-ASA neutralized the oxidative stress observed when only iron was supplemented to the diet in the colitic animals. Lycopene had similar effects. The effect of combining 5-ASA and lycopene was significant in several parameters but was not additive. The fact that attenuation was more impressive when iron was supplemented is not completely understood. It is possible that iron may have a potentiation effect on 5-ASA or lycopene.

Induction of colitis in the described study was done by an iodoacetamide enema. This substance is a sulphydryl alkylator that induces colonic injury due to the production of tissue inflammatory mediators and oxidative radicals.[9] We believe that the positive effect of 5-ASA and lycopene is not specific for the iodoacetamide model but is also applicable to other models of inflammation.

Iron is essential for maintaining proper cell function; it is normally tightly controlled by transport and storage proteins. Iron overload may result in deleterious reactions, such as protein and nucleic acid degradation and peroxidation of polyunsaturated fatty acids (PUFA). Although the exact mechanism by which iron is involved in initiating or promoting oxidative damage is not entirely clear, it is capable of catalyzing the transformation of hydrogen peroxide reductase via the Haber–Weiss reaction.[15] In addition, iron can catalyze the decomposition of lipid hydroperoxides to form alkoxyl peroxyl and other radicals.[16] Intraluminal iron may stimulate increased cell proliferation directly via participation in the Fenton reaction and hydrogen peroxide production[17] through an increase in oxidative stress in the dividing cells as a result of hydrogen peroxide exposure, or as a repair response to increased cell loss from the luminal surface.

The chronically inflamed intestine or colon may be subject to considerable oxidative stress. Furthermore, the colonic mucosa contains relatively small amounts of the antioxidant enzymes SOD and catalase. If reactive oxygen species play an important role in mediating mucosal injury in IBD, then it should be possible to attenuate the injury with antioxidants.

Although superoxide per se is not very reactive or cytotoxic, it does interact with iron to generate the toxic hydroxyl radical. Thus, any compound that decomposes

superoxide will attenuate superoxide-dependent formation of hydroxyl radicals. 5-ASA has potent SOD-like activity, as measured by its ability to inhibit the reduction of cytochrome *c* by xanthine oxidase-generated superoxide.[18] Sulfasalazine and 5-ASA are very effective in scavenging the hydroxyl radical,[19] which may explain the findings in our study whereby 5-ASA neutralized the prooxidant effect of iron. 5-ASA has also been shown to inhibit the iron-catalyzed hydroxyl radical-mediated degradation of deoxyribose by chelating iron and preventing its interaction with superoxide and hydrogen peroxide. 5-ASA, and to a lesser extent, sulfasalazine or sulfapyridine, selectively inhibit hemoglobin-catalyzed lipid peroxidation by acting as an alternative substrate for ferryl hemoglobin.[20] Carotenoids can accept energy from various electronically excited species, a property that serves an important protective function in plants exposed to excess light irradiation.[21] Part of this latter effect is attributed to the carotenoid ability to quench excited singlet oxygen, and this property served as the rationale for treating our animals with carotenoids.

Carotenoids are also capable of inducing intercellular communication via gap junctions associated with inhibiting proliferation of transformed cells.[7] Of the natural carotenoids, lycopene is among the most efficient singlet-oxygen quenchers.[22] The mechanism of lycopene's enhanced reactivity relative to other carotenoids remains to be elucidated. It has been speculated that the increased reactivity is related to the presence of the two nonconjugated double bonds.[24] We also observed the antiinflammatory effect of lycopene, which to date has received only little attention. We hypothesize that lycopene was superior to β-carotene in this instance because of its antiinflammatory effect, supplementing its antioxidative properties. Sustained production of ROM, seen in chronic inflammation, overwhelms the defense mechanism consisting of protective endogenous antioxidants, the prooxidative imbalance in this situation further aggravates the inflammatory process. Perhaps lycopene, as well as 5-ASA, act simultaneously as both antioxidants per se and direct downregulators of the inflammatory process.

We conclude that an increase in dietary iron causes significantly and disproportionately larger increases in both free and exchangeable iron in the large bowel of the rat. This pool of soluble, exchangeable iron may be available for reactions leading to adverse changes in free radical-mediated intraluminal chemistry. The colonic mucosa contains much smaller amounts of SOD, catalase, and glutathione peroxidase than other tissues. Most of the mucosal enzyme activity is associated with colonic epithelial cells, so the lamina propria is devoid of significant enzymatic defenses against ROM. The imbalance created by the overproduction of ROM within the inflamed interstitium suggests that antioxidant supplementation may prove useful in the treatment of IBD. More studies, and further delineation of the carotenoid mechanism of action, are needed before this can be translated into human practice.

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