

Anti-inflammatory effects of freeze-dried black raspberry powder in ulcerative colitis

David C. Montrose, Nicole A. Horelik, James P. Madigan,
Gary D. Stoner¹, Li-Shu Wang¹, Richard S. Bruno²,
Hea Jin Park², Charles Giardina³ and Daniel W. Rosenberg*

Center for Molecular Medicine and Colon Cancer Prevention Program, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, USA, ¹Department of Internal Medicine and Comprehensive Cancer Center, Ohio State University, Columbus, OH 43240, USA, and ²Department of Nutritional Sciences and ³Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269, USA

*To whom correspondence should be addressed. Tel: +860 679 8704;
Fax: +860 679 7639;
Email: rosenberg@uchc.edu

Ulcerative colitis (UC) is a chronic inflammatory disease of the colonic mucosa that can dramatically increase the risk of colon cancers. In the present study, we evaluated the effects of a dietary intervention of freeze-dried black raspberries (BRB), a natural food product with antioxidant and anti-inflammatory bioactivities, on disease severity in an experimental mouse model of UC using 3% dextran sodium sulfate (DSS). C57BL/6J mice were fed either a control diet or a diet containing BRB (5 or 10%) for 7–14 days and then the extent of colonic injury was assessed. Dietary BRB markedly reduced DSS-induced acute injury to the colonic epithelium. This protection included better maintenance of body mass and reductions in colonic shortening and ulceration. BRB treatment, however, did not affect the levels of either plasma nitric oxide or colon malondialdehyde, biomarkers of oxidative stress that are otherwise increased by DSS-induced colonic injury. BRB treatment for up to 7 days suppressed tissue levels of several key pro-inflammatory cytokines, including tumor necrosis factor α and interleukin 1 β . Further examination of the inflammatory response by western blot analysis revealed that 7 day BRB treatment reduced the levels of phospho-I κ B α within the colonic tissue. Colonic cyclooxygenase 2 levels were also dramatically suppressed by BRB treatment, with a concomitant decrease in the plasma prostaglandin E₂ (276 versus 34 ng/ml). These findings demonstrate a potent anti-inflammatory effect of BRB during DSS-induced colonic injury, supporting its possible therapeutic or preventive role in the pathogenesis of UC and related neoplastic events.

Introduction

Ulcerative colitis (UC) is an inflammatory disease in which patients experience cyclical bouts of clinical symptoms including diarrhea, rectal bleeding and anemia resulting from intestinal inflammation, edema and ulceration (1). Persistent UC can also increase the risk of colorectal cancer development by ~10-fold (2). Individuals with active disease have been found to generate high levels of pro-inflammatory cytokines and reactive oxygen species (ROS) within their colonic mucosa (3,4). These inflammatory mediators cause tissue damage and are thought to be critical events in the pathogenesis of UC. Thus, limiting their production has become an important component of UC treatment. Additionally, limiting colonic inflammation with anti-inflammatory agents appears to reduce the risk of UC-associated cancer development (5,6). Identifying approaches to safely manage long-term inflammation in UC is therefore a key component of cancer prevention.

Abbreviations: BRB, black raspberries; COX, cyclooxygenase; DSS, dextran sodium sulfate; IL, interleukin; MDA, malondialdehyde; NF- κ B, nuclear factor-kappaB; P-I κ B α , phospho-I κ B α ; PGE₂, prostaglandin E₂; QRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; TNF α , tumor necrosis factor; UC, ulcerative colitis.

The incorporation of natural food products into therapeutic regimens is an attractive approach for improving disease treatment due to their generally low toxicity profiles and high patient compliance. The efficacy of these natural food products in the amelioration of symptoms of cardiovascular and inflammatory disease, as well as cancer, has been reviewed (7). Among the most promising agents, dark berries have been shown to contain high concentrations of antioxidant polyphenols, including ellagic acid and anthocyanins, which provide them their dark purple color (8,9). Black raspberries (BRB) in particular have been shown to contain the highest levels of these constituents (8,9). Components found in BRB have also been shown to modulate messenger RNA and protein expression levels of important inflammatory mediators in cell culture, including cyclooxygenase (COX) 2, interleukins (ILs) and nuclear factor-kappaB (NF- κ B) (10–12).

In addition to their ability to limit the inflammatory response, these agents have a strong antioxidant capacity due to the presence of polyphenols. For example, anthocyanins have been shown to directly scavenge free radicals, increase expression of detoxification enzymes and increase the radical-absorbing capacity of cells (13–17). The administration of freeze-dried BRB in the diet has been shown to limit the development of esophageal cancer in rats during and after *N*-nitrosomethylbenzylamine exposure (18–20). Analysis of gene expression patterns identified a number of genes that were altered by *N*-nitrosomethylbenzylamine exposure that could be restored to normal patterns of expression by treatment with BRB (19). Furthermore, BRB were found to inhibit carcinogenesis in several other animal tumor models. These include azoxymethane-induced colon cancer in rats, intestinal tumors in *Apc*^{Min/+} mice, UV-induced skin tumors in mice, and 7,12-dimethylbenz(a)anthracene-induced tumors in the hamster cheek pouch (21–24).

The effect of BRB on experimental UC induced by dextran sodium sulfate (DSS) has not previously been investigated. In this study, we found that BRB supplementation ameliorated DSS-induced injury, which was associated with reductions in pro-inflammatory cytokine production, I κ B α phosphorylation and suppressed induction and activity of COX-2.

Materials and methods

Preparation of BRB powder

BRB powder was prepared essentially as described before (18). Briefly, BRB of the Jewel variety were grown on an Ohio farm in 2006, picked mechanically when ripe and washed and frozen at –20°C within 1–2 h of the time of picking. They were freeze-dried and ground into a powder at Van Druen Farms (Mokenca, IL) and the powder was shipped frozen to the Ohio State University where it was kept frozen until mixed into American Institute of Nutrition-76A synthetic diet at concentrations of 5 and 10%. These concentrations were chosen based on previous studies (19,25,26). The starch in the diet was reduced by 5 and 10%, respectively, to maintain isocaloric diets. Control and berry diets were shipped to the laboratory of Dr Daniel Rosenberg at the University of Connecticut Health Center (Farmington, CT). The contents of 26 nutrients, including four anthocyanins and ellagic acid, in BRB from crop year 2006 were found to be within ~20% of their content from previous years (Supplementary Table 1 is available at *Carcinogenesis* Online) (27). No residual pesticides, herbicides or fungicides were detected in the berry powder.

Clinical and histological analyses of experimental colitis

For the recovery study, 6- to 8-week-old C57BL/6J male mice were administered 3% DSS (MP Biomedical, Irvine, CA) in drinking water for 7 days and then switched to plain drinking water for 7 days. Mice were fed either control American Institute of Nutrition-76 powdered diet or diets containing freeze-dried BRB at 5 or 10% ($n = 10$ mice per group) for all 14 days of the experiment (Supplementary Figure 1A is available at *Carcinogenesis* Online). A control group of mice was placed on the same feeding protocol but given plain drinking water for the entire 14 days ($n = 10$ mice per group) (Supplementary Figure 1A is available at *Carcinogenesis* Online).

For the acute studies, 6- to 8-week-old C57BL/6J male mice were administered 3% DSS in drinking water or plain drinking water for 7 days and fed

either control American Institute of Nutrition-76 powdered diet or diets containing freeze-dried BRB at 5 or 10% ($n = 10$ mice per group) for the entire 7 day period and then euthanized (Supplementary Figure 1B is available at *Carcinogenesis* Online). The feeding protocol of concomitant BRB administration during active disease as well as during recovery from disease attempted to recapitulate a potential clinical scenario where patients could be administered a BRB formulation during an UC flare-up as well as during their transition to remission. Mice were weighed and assessed for evidence of gross rectal bleeding on a daily basis. At the end of each experimental period, mice were euthanized and colons were immediately excised and flushed with ice-cold phosphate-buffered saline. Colons were formalin fixed and Swiss rolled for histological analysis after hematoxylin and eosin staining. The percentage of ulcerated tissue was calculated as the amount of ulcerated tissue as a percentage of the whole length of the colon. Analysis was performed in a blinded manner.

Analysis of biomarkers of oxidative stress

Mice were administered 3% DSS-incorporated drinking water for 7 days along with either control diet or 10% BRB-incorporated diet ($n = 4-5$ per group) as shown in Supplementary Figure 1B (available at *Carcinogenesis* Online). A control group was given plain drinking water with either control diet or 10% BRB-incorporated diet ($n = 4-5$ per group). Immediately after being killed, blood was collected by cardiac puncture and transferred to blood collection tubes containing sodium citrate followed by centrifugation to isolate plasma. Colons were excised, flushed and snap frozen until use. Plasma nitrite levels were determined by a commercial nitrate/nitrite colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI) using the manufacturer's protocol.

Malondialdehyde (MDA) levels in the colon were determined by high-performance liquid chromatography-fluorescence as described earlier (28). Briefly, colons were homogenized in ice-cold phosphate-buffered saline containing 0.05% butylated hydroxytoluene and protease inhibitor cocktail. Following centrifugation for 5 min at 15 000g, supernatants were derivatized (30 min, 95°C) with 0.6% thiobarbituric acid and samples were extracted with butanol and injected onto a Beckman Gold 125 high-performance liquid chromatography system (Fullerton, CA) equipped with a Jasco FP-2020⁺ detector (Easton, MD; 520/553 nm, excitation/emission). Samples were separated isocratically at 0.8 ml/min on a Luna C18(2) column (250 × 4.6 mm, 5 μ ; Phenomenex, Torrance, CA) using 50:50 methanol and 25 mM potassium phosphate buffer (pH 6.5). MDA levels were quantified against standards prepared in parallel from 1,1,3,3-tetramethoxypropane. MDA was normalized to total colon protein determined by the Bradford assay (Bio-Rad, Hercules, CA) (29).

Quantitative real-time polymerase chain reaction

Mice were administered 3% DSS-incorporated drinking water for 7 days along with either control diet or 10% BRB-incorporated diet ($n = 4-5$ per group) as described in Supplementary Figure 1B (available at *Carcinogenesis* Online). A control group was given plain drinking water with either control diet or 10% BRB-incorporated diet ($n = 4-5$ per group). At sacrifice, colons were excised, flushed and snap frozen until use. Total RNA was extracted from colons using Trizol® Reagent (Invitrogen, Carlsbad, CA). Two micrograms of RNA was reverse transcribed into complementary DNA using Superscript III RT (Invitrogen). Messenger RNA expression was determined for tumor necrosis factor (TNF α) and IL-1 β by quantitative real-time polymerase chain reaction (QRT-PCR) using an ABI 7500 RT-PCR instrument (Applied Biosystems, Carlsbad, CA). QRT-PCR reactions were performed in duplicate in 20 μ l volumes using 1 μ l 20× TaqMan Assay-on-Demand for TNF α (ID = Mm00443258_m1) and IL-1 β (ID = Mm00434228_m1) (Applied Biosystems). The cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. QRT-PCR reactions were performed in duplicate for each sample with an endogenous control gene, *HPRT* (ID = Mm00446968_m1) used for normalization. All QRT-PCR reactions were performed with a 'no-template' control and a positively expressed sample was used for building the standard curve.

Isolation of colonic lamina propria cells

Mice were administered either 3% DSS-incorporated drinking water or plain drinking water with either control diet or 10% BRB-incorporated diet for 7 days ($n = 2-3$ per group) as described in Supplementary Figure 1B (available at *Carcinogenesis* Online). At sacrifice, colons were removed and flushed with ice-cold phosphate-buffered saline, slit open longitudinally and cut into 1 cm pieces. Tissues were placed into a solution containing Ca/Mg-free balanced salt solution with 5 mM ethylenediaminetetraacetic acid and 0.15 mg/ml dithiothreitol and stirred at 37°C to remove the epithelium. The remaining tissue pieces were placed into a solution containing balanced salt solution, 1 mM CaCl₂, 1 mM MgCl₂, 0.5 mg/ml collagenase and 0.1 mg/ml DNase I and stirred at 37°C. Released cells were collected through a cell strainer and re-suspended in 44% Percoll, which was underlaid with 67% Percoll and centrifuged to collect relevant cells for fluorescence-activated cell sorting analysis. Isolated

cells were re-suspended in staining buffer consisting of balanced salt solution, 3% fetal bovine serum and 0.1% sodium azide. Non-specific binding was blocked by addition of anti-Fc monoclonal antibody followed by incubation with the following fluorescently conjugated monoclonal antibodies on ice for 30 min: F/480–allophycocyanin (1:100), CD11b–fluorescein isothiocyanate (1:200), GR 1–phycoerythrin (1:100), B220 peridinin chlorophyll protein complex (1:50), CD8b–phycoerythrin (1:300), CD4–fluorescein isothiocyanate (1:200) and CD11c allophycocyanin (1:150) (eBioscience, San Diego, CA). Flow cytometry was conducted on an LSRII flow cytometer (BD, Franklin Lakes, NJ) with data analyzed using FlowJo software (Tree Star, Ashland, OR). Comparisons of inflammatory cell infiltrate after DSS exposure in mice given control or BRB-incorporated diet were made by using the stains described above to determine the average percentage of inflammatory cells expressing these markers from colons of individual mice. For each respective diet, fold change was determined by dividing the average percentage of positively stained cells per surface marker in the colons from mice administered DSS for 7 days by that of the mice given plain drinking water for 7 days.

Western blot analysis

Mice were administered 3% DSS-incorporated drinking water for 7 days along with either control diet or 10% BRB-incorporated diet ($n = 5$ per group). At sacrifice, colons were excised, flushed and snap frozen until use. Frozen colonic tissue was homogenized in 1 ml Nonidet P-40 lysis buffer [50 mM Tris-HCl (pH 8.0) 150 mM NaCl, 1% Nonidet-40] supplemented with both Complete Protease and Complete Phosphatase Inhibitor Cocktail tablets (Roche, Indianapolis, IN), 0.5 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 12 000 r.p.m. and insoluble material was discarded. Protein concentrations were determined using a Bradford assay. Lysate proteins (50 μ g) in 2× Laemmli sample buffer were boiled for 5 min, resolved on sodium dodecyl sulfate–polyacrylamide gel (10% gel) and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked in 5% (w/v) non-fat dried skimmed milk powder in Tris-buffered saline–Tween 20 [20 mM Tris-HCl (pH 7.6) 137 mM NaCl, 0.2% Tween 20] and probed with appropriate primary antibodies for the following: phospho-I κ B α (P-I κ B α) (1:1000), total I κ B α (1:1000) (Cell Signaling, Beverly, MA); COX-2 (1:1000) (Cayman Chemicals) and β -tubulin (1:5000) (Sigma–Aldrich, St Louis, MO) followed by anti-mouse or rabbit immunoglobulin G–horseradish peroxidase-conjugated secondary antibody (Cell Signaling). Membranes were then incubated in Immobilon Chemiluminescent HRP Substrate (Millipore) and the signal developed on BioMax XAR film (Kodak, Rochester, NY). Band density was analyzed using ImageJ software and reported as signal intensity of protein normalized to signal intensity of β -tubulin for each sample.

Immunohistochemistry

Paraffin-embedded entire colons from five to six mice per group were reacted with primary antibodies for p65 and P-I κ B α . Slides with specimens were placed in a 60°C oven for 1 h, cooled, de-paraffinized and rehydrated through xylenes and graded ethanol solutions to water. All slides were treated for 5 min with a 3% H₂O₂ solution in water to block endogenous peroxidase. Antigens were retrieved by placing the slides in a vegetable steamer in Dako Target Retrieval Solution for 25 min, after which they were cooled for 15 min. The slides were then placed on a Dako Autostainer for automated staining. Primary antibodies, antibody dilutions, incubation times and temperatures used were as follows: rabbit anti-NF- κ B p65 (#4764, Cell Signaling, Danvers, MA), dilution 1:50, incubation for 1 h at room temperature and rabbit P-I κ B α (#NB100-92563, Novus Biologicals, Littleton, CO), dilution 1:400, incubation for 1 h at room temperature. Slides were then stained with their respective secondary antibodies, counterstained with hematoxylin, dehydrated through a graded series of ethanol and coverslipped.

Prostaglandin E₂ measurement

Mice were administered 3% DSS-incorporated drinking water for 7 days concomitantly with either control diet or 10% BRB-incorporated diet ($n = 5$ per group). At sacrifice, blood was collected by cardiac puncture and transferred to blood collection tubes containing sodium citrate and centrifuged to collect plasma. Prostaglandin E₂ (PGE₂) levels were determined from pooled plasma samples (diluted 1:5) from five mice per group using a commercially available enzyme-linked immunosorbent assay per manufacturer's protocol (Cayman Chemicals).

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 5). Data are expressed as the means \pm standard errors of the mean. Area under the curve analysis was performed to evaluate time-dependent changes in body mass resulting from DSS and BRB treatment. Area under the curve was calculated for each animal using the trapezoidal rule and then group mean differences

were evaluated by one-way analysis of variance with Bonferroni's post-test. All other analyses were performed using one-way or two-way analysis of variance with Bonferroni's post-test or two-tailed, unpaired *t*-tests as appropriate. Results for all analyses were considered to be statistically significant at an α -level of $P \leq 0.05$.

Results

BRB powder and its effect on acute intestinal injury

To determine the effects of BRB on acute intestinal injury, mice were given 3% DSS in their drinking water for 7 days and then switched to plain drinking water for 7 days with or without freeze-dried BRB (5 and 10%) added to the diet as described in Supplementary Figure 1A (available at *Carcinogenesis* Online). As shown in Figure 1A and B, BRB administration lessened DSS-induced weight loss. No differences in weight change were seen in mice given plain drinking water along with control, 5 or 10% BRB-incorporated diet for 14 days (data not shown).

In order to determine the effects of BRB administration on acute DSS-induced pathological injury, mice were given 3% DSS in their drinking water for 7 days with or without freeze-dried BRB (5 and 10%) as described in Supplementary Figure 1B (available at *Carcinogenesis* Online). BRB afforded protection against colonic shortening, a well-established physiological consequence of DSS-induced injury (Figure 2A). No differences in colon length were seen in mice given plain drinking water along with control, 5 or 10% BRB-incorporated diet for 7 days (data not shown). Finally, the extent of

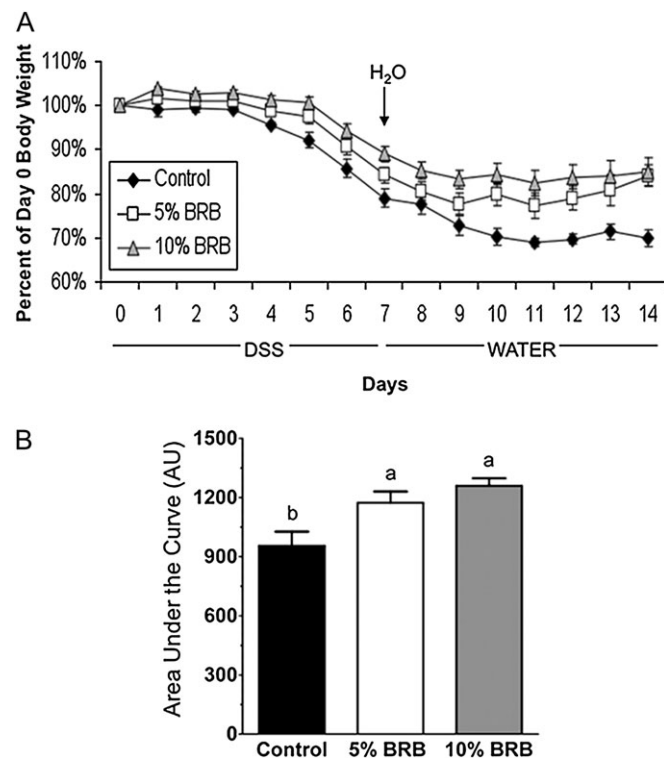


Fig. 1. The effects of BRB on DSS-induced body weight changes. Mice were administered 3% DSS in drinking water for 7 days and then switched to plain drinking water for an additional 7 days and fed either a control diet or a diet containing 5 or 10% BRB powder ($n = 10$ per group) for the entire 14 day period. (A) Body weights were measured daily and reported as a percentage of body weight at the start of the experiment (day 0). (B) Area under the curve (days 0–14) was determined for each animal using the trapezoidal rule (GraphPad Prism, version 5). The effects of DSS and BRB were evaluated using one-way analysis of variance with Bonferroni's post-test to evaluate group mean difference. An α -level of $P < 0.05$ was set for statistical significance. Means not sharing a common superscript are significantly different from each other.

colonic ulceration, the key index of DSS-induced inflammatory disease, was determined in hematoxylin and eosin sections prepared from each of the experimental groups. As shown in Figure 2B, BRB treatment resulted in a reduction in the percentage of colonic ulceration in each of the experimental groups that received BRB. Detailed histological examination of the colonic epithelium showed extensive areas of intense ulceration, with large amounts of edema present within the submucosa of the colons from DSS-treated mice (Figure 2C). Co-administration of dietary BRB elicited a profound reduction in the degree of mucosal ulceration. As shown in Figure 2D and E, only minimal areas of epithelial damage were evident within the colons of mice receiving 5 or 10% BRB. These areas contained many ulcerated regions that retained some intact crypts as well as a marked reduction in the edematous space within the muscularis mucosa (Figure 2D and E).

BRB does not affect reactive nitrogen species production or lipid peroxidation

The damaging effects of DSS have been shown to occur in part as a result of the increased production of free radicals within the colonic mucosa (30–32). BRB have been shown to function, in part, by scavenging free radicals, thereby increasing cellular antioxidant capacity (13–17). We therefore determined whether BRB protection occurs via its ability to attenuate nitric oxide production. Nitrite production was measured as a marker for nitric oxide in the plasma of mice exposed to 3% DSS, with or without lyophilized BRB (10%) added to the diet as described in Supplementary Figure 1B (available at *Carcinogenesis* Online). As shown in Figure 3A, 7 days of DSS exposure significantly increased nitrite production, but BRB failed to reduce this increase.

Many free-radical species elicit their damaging effects on cell membranes through increased lipid peroxidation (30–32). We therefore examined the extent to which BRB regulate the production of MDA, a biomarker of lipid peroxidation. As shown in Figure 3B, DSS treatment markedly increased MDA levels within the colonic mucosa after 7 days. Co-administration of BRB, however, did not protect against MDA production. Taken together, these data lead us to conclude that BRB protect against DSS-induced injury independently of oxidative/nitrative stress.

BRB administration does not affect colonic inflammatory cell infiltration

The observation that BRB failed to reduce oxidative stress responses prompted us to examine inflammatory cell infiltration within the colonic mucosa. Using fluorescence-activated cell sorting analysis, we evaluated the profile of inflammatory cells within the lamina propria of mice administered 3% DSS or plain drinking water for 7 days, with or without BRB treatment as described in Supplementary Figure 1B (available at *Carcinogenesis* Online). The fold induction of inflammatory cells was determined by dividing the number of surface marker-positive cells isolated from DSS-administered mice by the number of surface marker-positive cells isolated from mice administered plain water for each diet, as described in Materials and Methods. As shown in Figure 3C, DSS increased the infiltration of innate immune cells into the tissue (CD11b, GR1, F/480, CD11c and CD11b/GR1-positive cells), but inflammatory cell infiltration was unaffected by dietary supplementation of BRB.

BRB limits colonic pro-inflammatory cytokine expression

In cell culture systems, isolated components of BRB have been shown to suppress messenger RNA and protein levels of ILs, providing support for BRB as a potential anti-inflammatory dietary agent (10–12). To determine whether BRB afford protection to the colonic mucosa by limiting the production of pro-inflammatory cytokines, we measured TNF α and IL-1 β expression in the colons of 3% DSS-exposed mice concurrently given control diet or 10% BRB-incorporated diet. As shown in Figure 4A and B, 10% BRB attenuated the levels of TNF α and IL-1 β after 7 days that were otherwise increased by DSS treatment. These data suggest that BRB may lessen the damaging effects of DSS by suppressing the expression of pro-inflammatory cytokines.

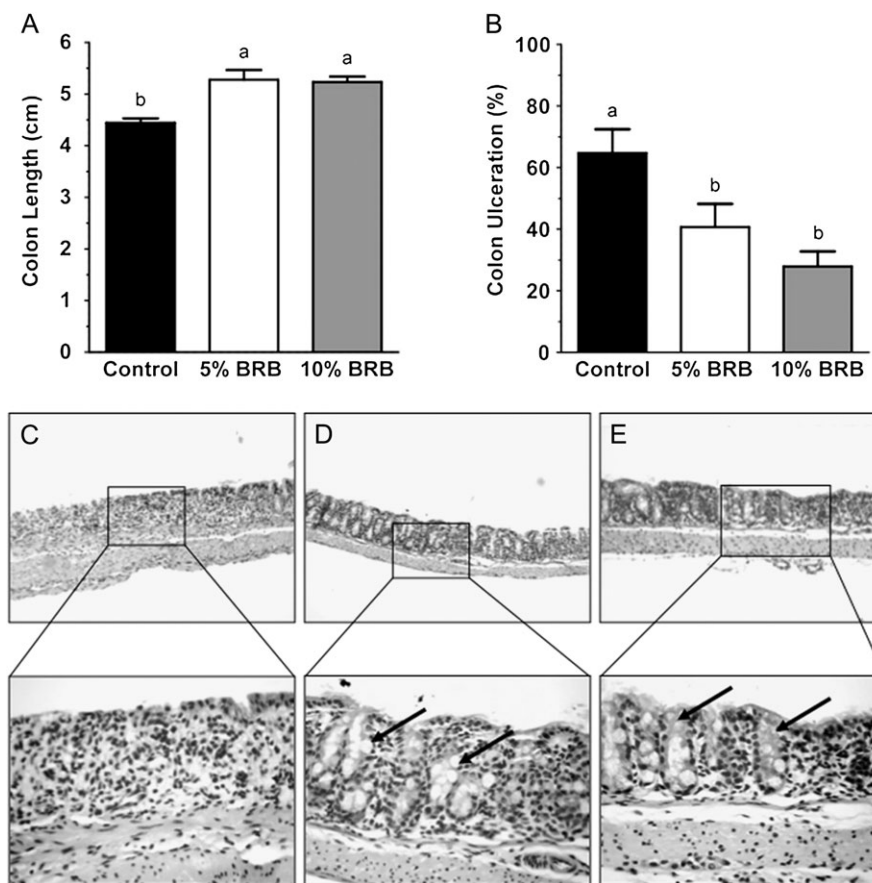


Fig. 2. The effects of BRB on DSS-induced colonic disease. Mice were administered 3% DSS in the drinking water and concomitantly fed either a control diet or a diet containing BRB powder (5 or 10%) until sacrifice at 7 days [$n = 10$ per group; means \pm standard errors of the mean (SEMs)]. (A) Colon lengths were determined immediately upon sacrifice and (B) percentage of colonic ulceration was quantified in hematoxylin- and eosin (H&E)-stained sections as described in Materials and Methods. Representative H&E staining of colonic ulcerations from mice given DSS plus control diet (C), 5% BRB diet (D) and 10% BRB diet (E) ($\times 100$); greater magnification of each panel is indicated by the boxes ($\times 400$); arrows indicate intact crypts retained within damaged mucosa. Data are means \pm SEMs, $n = 10$ mice per group. Significance was determined by one-way analysis of variance with Bonferroni's post-test. Groups not sharing a common superscript are significantly different ($P < 0.05$).

BRB inhibits NF- κ B and COX-2 activity in the colon

BRB has been shown *in vitro* to inhibit the activation of NF- κ B, a potent pro-inflammatory transcription factor (33). We tested the possibility that BRB may inhibit NF- κ B activity in the colons of DSS-treated mice. Levels of P-I κ B α (a marker of NF- κ B activity) were examined in tissue lysates prepared from DSS-exposed colons from mice concurrently given control diet or 10% BRB-incorporated diet. As shown in Figure 5A and B, after 7 days of DSS treatment, BRB markedly reduced ($\sim 50\%$) the levels of P-I κ B α , indicating reduced NF- κ B activation. Additionally, we measured the expression of total I κ B α as to further evaluate the amount of I κ B α turnover. As shown in Figure 5A and B, levels of total I κ B α were moderately but significantly increased in colons from those mice fed BRB-incorporated diet. Lower levels of P-I κ B α and increased levels of total I κ B α as found in the colons from those mice fed BRB-incorporated chow are indicative of reduced I κ B α turnover.

In order to identify which cell populations within the colonic tissue were expressing P-I κ B α , we performed immunohistochemistry for P-I κ B α in colonic sections from mice administered DSS or plain drinking water concurrently with either control diet or 10% BRB-incorporated diet for 7 days. As shown in Figure 5C–F, positively stained cells appeared in ulcerated areas and edematous space beneath the mucosa, likely to be infiltrating inflammatory cells in mice fed either diet.

In order to show that NF- κ B nuclear localization was occurring in the same cell population that stained positively for P-I κ B α , we per-

formed immunohistochemistry for NF- κ B p65 in colonic sections from the same groups of mice. As shown in Figure 5G–J, p65 nuclear positivity was also found in what appeared to be infiltrating inflammatory cells within both ulcerated areas of the mucosa as well as in the edematous space beneath the mucosa. Nuclear positivity was found neither in intact crypts nor in any epithelial cells (data not shown).

NF- κ B has also been shown to regulate the expression of COX-2 (34). To determine the effects of BRB on COX-2 expression, western blot analysis was performed on the same tissue lysates used for experiments described in Figure 5. As shown in Figure 6A and B, BRB reduced COX-2 expression by $\sim 50\%$. In order to determine whether COX-2 activity may also be inhibited, we quantified PGE₂ levels in plasma from mice administered 3% DSS for 7 days and given either a control or 10% BRB diet. As shown in Figure 6C, PGE₂ levels were significantly reduced by $\sim 50\%$ in mice maintained on the BRB diet.

Discussion

The findings of this investigation demonstrate that BRB mitigates DSS-induced UC by suppressing colonic injury consistent with improvements in pro-inflammatory events. These protective effects occurred independent of changes in oxidative/nitritative stress, suggesting that BRB exerts anti-inflammatory, but not antioxidant, activity in this model. Indeed, BRB reduced inflammatory cytokine expression, as well as I κ B α phosphorylation and COX-2 expression. BRB and their

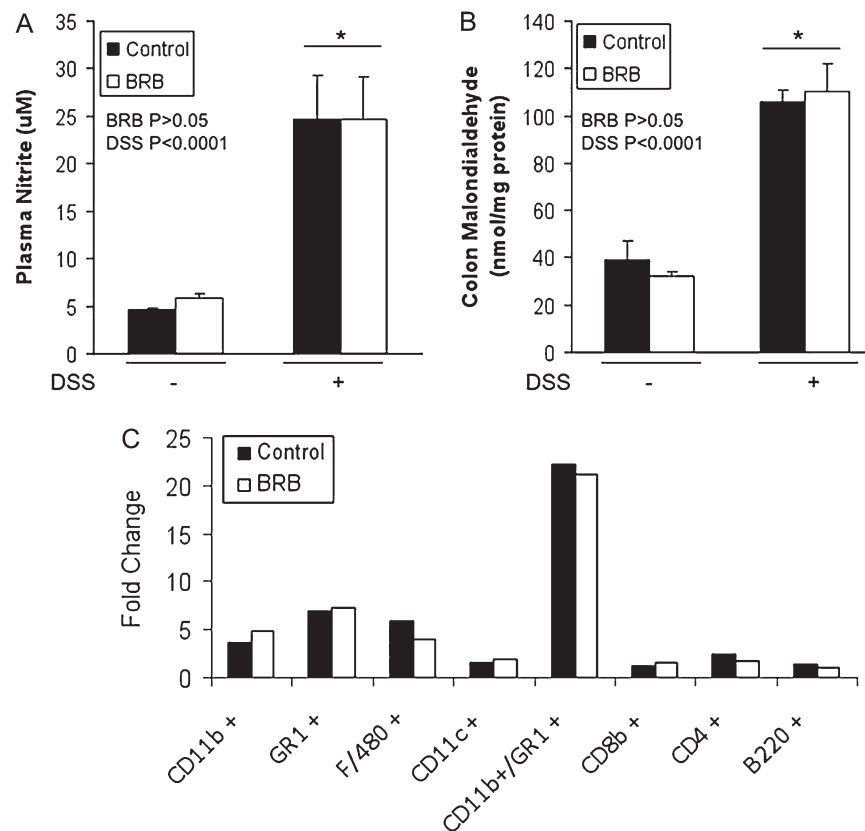


Fig. 3. The effects of BRB on DSS-induced activation of biomarkers of oxidative stress and inflammatory cell infiltrate in the colon. Mice were administered 3% DSS-incorporated drinking water and concomitantly fed either a control diet or a diet containing 10% BRB powder for 7 days. A control group of mice was administered plain drinking water along with either diet. Data are means \pm standard errors of the mean, $n = 4$ mice per group. (A) Plasma nitrite levels were determined by nitric oxide production using a colorimetric assay kit. (B) MDA concentrations were determined in colon tissue by high-performance liquid chromatography-fluorescence as described under Materials and Methods. Two-way analysis of variance with Bonferroni's post-test was used to evaluate the effects of DSS, BRB and their interaction on nitrite and MDA. Nitrite and MDA were affected, $P < 0.05$, by DSS only. (***) Indicates effects due to DSS. (C) fluorescence-activated cell sorting analysis was performed on cells isolated from the colonic lamina propria, as described in Materials and Methods. Cell populations are reported as fold changes after 7 days of DSS administration as compared with untreated mice for each diet.

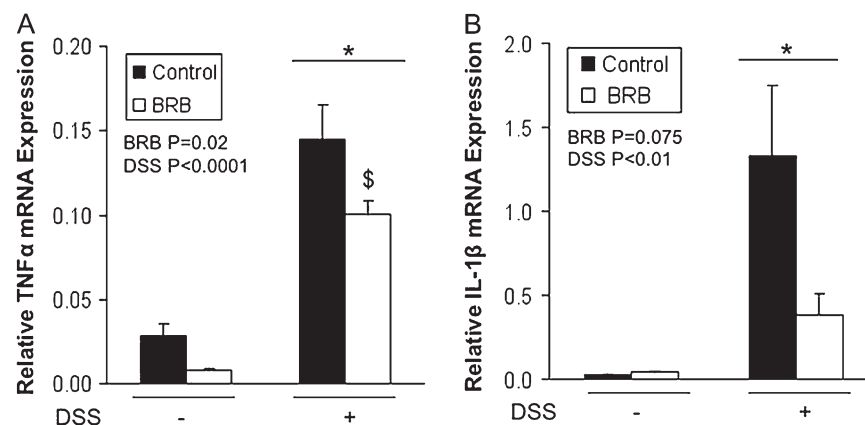


Fig. 4. The effects of BRB on DSS-induced pro-inflammatory cytokine production. Mice were administered 3% DSS-incorporated drinking water and concomitantly fed either a control diet or a diet containing 10% BRB powder for 7 days. A control group of mice was administered plain drinking water along with either diet. RNA was isolated from fresh-frozen colon samples and used for QRT-PCR analysis, as described under Materials and Methods. The relative messenger RNA expression levels of (A) TNF α and (B) IL-1 β are shown. Data are means \pm standard errors of the mean, $n = 5$ per group. Two-way analysis of variance with Bonferroni's post-test was used to evaluate the effects due to DSS, BRB and their interaction. TNF α was affected by DSS and BRB ($P < 0.05$). (***) Indicates differences due to DSS and (\$) indicates differences due to BRB. A significant increase in IL-1 β was observed in the control diet group after DSS administration. No significant increase in IL-1 β was observed in mice given BRB. A trend ($P = 0.075$) was found for BRB to reduce IL-1 β in comparison with control diet-fed mice after DSS administration.

antioxidant components, in particular the anthocyanins and simple phenols such as ellagic acid and quercetin, may have applications in the treatment of a host of human diseases (7). As reviewed by Stoner *et al.* (35), freeze-dried berries have been used in prevention trials for

a variety of cancers of the gastrointestinal tract and their efficacy is markedly improved by increasing their concentration through freeze-drying (35). In this study, we present evidence that freeze-dried BRB can attenuate colonic damage associated with DSS-induced UC. The

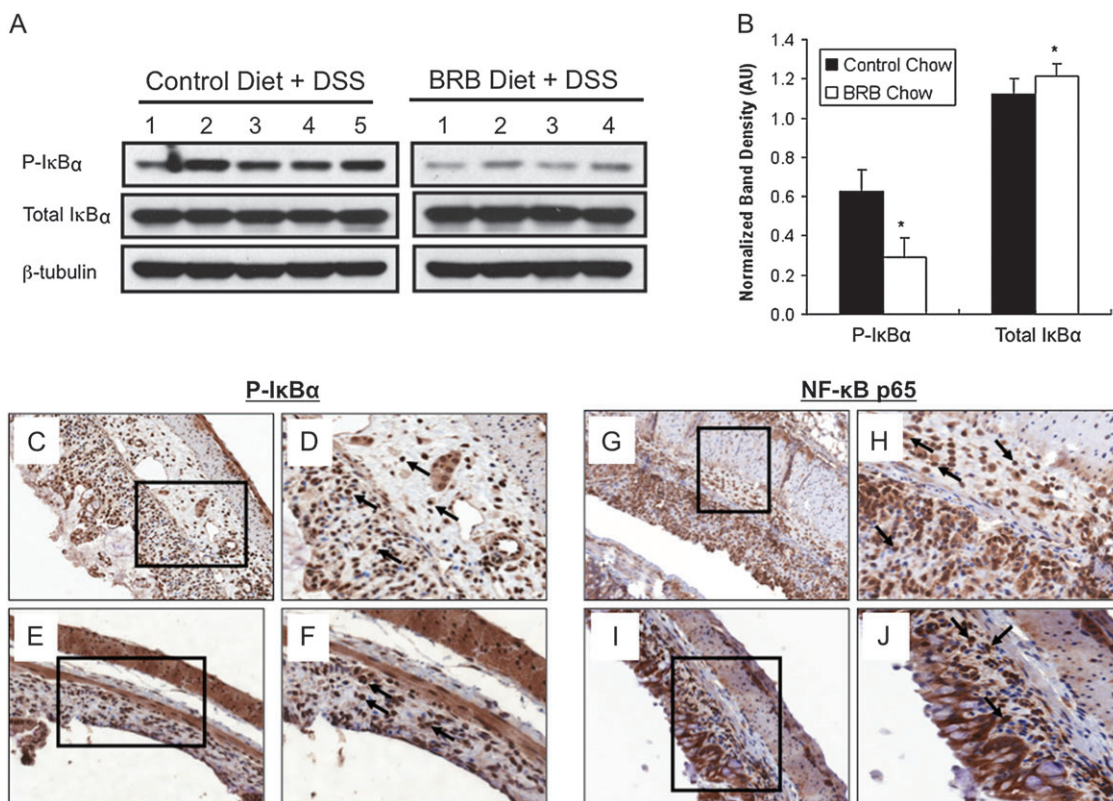


Fig. 5. The effects of BRB on DSS-induced changes in IκBα phosphorylation. Mice were administered 3% DSS in the drinking water concomitantly with either control or 10% BRB-incorporated powder diet. After 7 days, colons were harvested and protein lysates prepared for (A) immunoblotting analysis of P-IκBα and total IκBα protein expression. Numbers over lanes represent individual mice within treatment groups. (B) Band intensities were quantified as described in Materials and Methods. (C) Representative immunohistochemical staining for P-IκBα in a colonic section from a DSS-exposed mouse given control diet (×10). (D) Magnification of panel (C) (box), arrows indicate positively stained cells (×20). (E) Representative immunohistochemical staining for P-IκBα in a colonic section from a DSS-exposed mouse given BRB-incorporated diet (×10). (F) Magnification of panel (E) (box), arrows indicate positively stained cells (×20). (G) Representative immunohistochemical staining for NF-κB p65 in a colonic section from a DSS-exposed mouse given control diet (×10). (H) Magnification of panel (G) (box), arrows indicate positively stained cells (×20). (I) Representative immunohistochemical staining for NF-κB p65 in a colonic section from a DSS-exposed mouse given BRB-incorporated diet (×10). (J) Magnification of panel (I) (box), arrows indicate positively stained cells (×20). Images of entire slides containing immunohistochemically stained colon tissue sections we captured using ImageScope software (Aperio, Vista, CA). The magnifications listed for all representative images of stained sections are those designated by the software. Data are means ± standard errors of the mean, $n = 5$ mice per group. * $P < 0.05$, comparing control diet group and BRB diet group (unpaired t -test).

protection afforded to the colon by BRB is associated with a reduction in key clinical features of the disease, including reduced weight loss, colonic shortening and colonic ulceration. Unexpectedly, the protection occurred without a direct effect on inflammatory cell infiltration into the colonic mucosa nor via suppression of reactive nitrogen species production or ROS-induced lipid peroxidation.

Anthocyanins and ellagic acid, found in high concentrations in dark berries, have been shown to have strong antioxidant properties. These effects include direct scavenging of free radicals, increasing the oxygen radical-absorbing capacity of cells and stimulating the expression of detoxification enzymes (13–17). These effects may be associated with the suppression of inflammatory ILs and NF-κB activation (30–32). Since DSS treatment is associated with increased ROS generation, we tested the effects of BRB on plasma nitrite and tissue MDA levels at 7 days after the start of treatment (36). As expected, DSS caused an increase in the concentration of nitrite in the plasma, consistent with elevated nitric oxide production resulting from a severe inflammatory response. MDA, a marker of lipid peroxidation, was also increased in colon following DSS treatment. Treatment with BRB, however, failed to suppress either of these markers of oxidative stress. Given that this is the first study examining the effects of BRB on DSS-induced UC, it is possible that the levels of DSS used in this study may simply be too high, and the effects of BRB on these disease index markers may be

masked by the severe disease phenotype in the sensitive C57BL/6 mice. It is possible that the antioxidant properties of BRB may only afford protection when modest levels of ROS damage are being incurred.

The mechanisms by which components of BRB affect signaling pathways have been studied in cell culture systems and were reviewed by Stoner *et al.* and Wang *et al.* (35,37). For example, a fraction of BRB was demonstrated to inhibit transactivation of the activator protein 1 transcription factor and NF-κB in cells exposed to the carcinogenic metabolite, benzo(a)pyrene diol-epoxide. This effect appeared to be mediated through inhibition of mitogen-activated protein kinase activation and IκBα phosphorylation (33,38). In another study, the use of OptiBerry, a product made of extracts from six different berries, inhibited IL-8 production from gastric cancer cells exposed to *Helicobacter pylori* (7). Consistent with these earlier studies, we found that freeze-dried BRB inhibit the expression of several key pro-inflammatory cytokines, including TNFα and IL-1β, 7 days after DSS exposure, supporting the ability of dietary BRB to suppress the expression of inflammatory mediators.

To determine how BRB may affect NF-κB regulation, the expression levels of phosphorylated IκBα were examined by immunoblotting. A critical step in NF-κB activation is the phosphorylation of IκBα, which leads to its ubiquitinylation and proteasomal degradation (39–41). Degradation of IκBα allows NF-κB to translocate

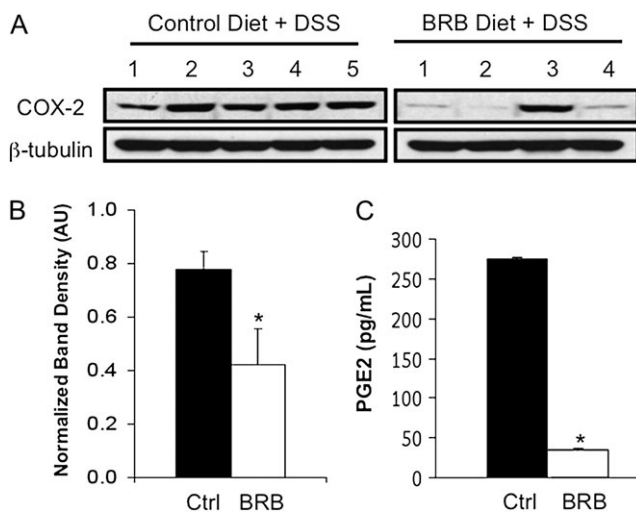


Fig. 6. The effects of BRB on DSS-induced changes in COX-2 and PGE₂ levels. Mice were administered 3% DSS in the drinking water along with either control or 10% BRB-incorporated powder diet. After 7 days, colons were harvested and protein lysates were prepared for (A) immunoblotting analysis of protein expression. Numbers over lanes represent individual mice within treatment groups and (B) band intensities were quantified as described in Materials and Methods. (C) Plasma was also prepared from mice and PGE₂ levels were determined by enzyme-linked immunosorbent assay. Data are means \pm standard errors of the mean, $n = 5$ mice per group. * $P < 0.05$, comparing control diet group and BRB diet group (unpaired t -test).

from the cytoplasm to the nucleus where it can activate target genes, including a number of pro-inflammatory genes and I κ B α (as part of a negative feedback loop) (42,43). We found that the level of P-I κ B α was significantly reduced in the BRB-fed animals, with a modest but significant increase in total I κ B α . In summary, our findings are consistent with the ability of BRB to suppress I κ B α phosphorylation and potentially I κ B α turnover resulting in inhibition of NF- κ B activity. This may lead to a reduction in the expression of NF- κ B target genes, including COX-2, TNF and IL-1 β (44–46). BRB may also provide protection from cancer development by suppressing the expression of other anti-apoptotic and growth regulatory NF- κ B target genes (47).

The effects of BRB on COX-2 expression and activity have also been studied. Two separate studies demonstrated that BRB was able to suppress COX-2 expression in rat esophageal tissue after *N*-nitrosomethylbenzylamine exposure (25,26). This suppression was also shown to correlate with a 50% reduction in PGE₂ levels (48). COX-2 expression and PGE₂ production are markedly increased during inflammation, including DSS-induced colonic injury, although the role of these mediators in the initiation and progression of the disease is not fully understood (3,4,49,50). Although we report a reduction in COX-2 and PGE₂ levels by BRB treatment, it is not known whether these effects are simply related to suppression of ulceration or a central feature of the protective response afforded by this approach.

In summary, the present study demonstrates the efficacy of freeze-dried BRB to protect the colonic mucosa from the acute injury induced by DSS exposure. Further studies to assess the role of COX-2 suppression should provide insight into the mechanisms by which this effect occurs. Given the non-toxic nature of these natural food substances and their accessibility, these results provide support for the incorporation of freeze-dried BRB into therapeutic regimens for UC, which could reduce disease severity and associated colon cancer risk.

Supplementary material

Supplementary Table 1 and Figure 1 can be found at <http://carcin.oxfordjournals.org/>

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