

Dietary Red Raspberry Reduces Colorectal Inflammation and Carcinogenic Risk in Mice with Dextran Sulfate Sodium-Induced Colitis

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Abstract

Background: Ulcerative colitis causes recurring intestinal mucosal injury and sustained inflammation, increasing the likelihood of colorectal cancer (CRC) development. Dietary red raspberry (RB) is a rich source of phytonutrients known to have anti-inflammatory activity; however, the role of RB on CRC prevention in chronic colitis has not been examined.

Objective: This study examined the effects of dietary RB supplementation on inflammation, epithelium repair, and oncogenic signaling in dextran sulfate sodium (DSS)-induced chronic colitis in mice.

Methods: Six-week-old male C57BL/6J mice were fed a control or RB (5% of dry feed weight; $n = 12/\text{group}$) diet for 10 wk. Starting from the fourth week, mice were administered 2 repeated cycles of 1% DSS (7-d DSS treatment plus 14-d recovery) and were monitored daily for disease activity index (DAI) score. Colonic tissues were collected at the end of the study for histochemical, immunohistochemical, and biochemical analysis of inflammation, differentiation and proliferation markers.

Results: RB supplementation reduced the DAI score and histologic damage (by 38.9%; $P \leq 0.01$), expression of inflammatory mediators (by 20–70%; $P \leq 0.01$), infiltration of CD4 T cells (by 50%; $P \leq 0.05$), and $\alpha 4\beta 7$ integrin and related adhesion molecules (by 33.3%; $P \leq 0.01$). Furthermore, RB supplementation facilitated epithelium repair, as evidenced by enhanced goblet cell density, expression of transcription factors including Kruppel-like factor 4 (*Klf4*) and Hairy and enhancer of split 1 (*Hes1*), terminal differentiation markers, mucin 2 (*Muc2*), and intestinal alkaline phosphatase (by 20–200%; $P \leq 0.01$). Conversely, proliferating cell nuclear antigen (by 70%; $P \leq 0.01$), β -catenin, and signal transducer and activator of transcription 3 (STAT3) signaling (by 19–33%; $P \leq 0.05$) were reduced by RB supplementation. In addition, RB supplementation enhanced p53 stability (by 53%) and reduced oncogenic gene expression (by 50–60%).

Conclusion: RB supplementation reduced DAI score and the risk of CRC development during recurring colitis in mice, suggesting that RB is a possible dietary supplement for patients with ulcerative colitis and related gut inflammatory diseases. *J Nutr* 2018;148:667–674.

Keywords: colitis, colorectal cancer, inflammation, proliferation, intestine, red raspberry

Introduction

Ulcerative colitis, a common form of inflammatory bowel disease, is characterized by chronic recurring inflammation of the

colon, which is known to increase the risk of colorectal cancer (CRC) development (1). CRC is the third most common cancer in the United States and Europe and is becoming common around the world (2, 3). Repeated mucosal injury of the intestinal epithelium results in aberrant activation of inflammatory signaling and accumulation of immune cells, further amplifying the disease severity (1, 4). Epithelium repair or mucosal healing is a process of coordinated interplay between inflammation, cell proliferation, migration, and differentiation of epithelial cells in the intestinal epithelium (5, 6), which requires intricate regulatory mechanisms to prevent uncontrolled proliferation that eventually can lead to hyperplasia and CRC. Under chronic inflammation, Wntless and Int (*Wnt*)/ β -catenin and signal transducer and activator of transcript 3 (STAT3) signaling pathways are activated and promote epithelial cell proliferation

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Supplemental Tables 1–4, Supplemental Material, Supplemental References, and Supplemental Figure 1 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/jn/>.

Address correspondence to MJZ (e-mail: meijun.zhu@wsu.edu). Abbreviations used: *Bcl2*, B cell lymphoma 2; CON, control; COX-2, cyclooxygenase 2; CRC, colorectal cancer; DAI, disease activity index; DSS, dextran sulfate sodium; MDM2, mouse double minute 2; RB, red raspberry; STAT3, signal transducer and activator of transcription 3; *Wnt*, Wntless and Int.

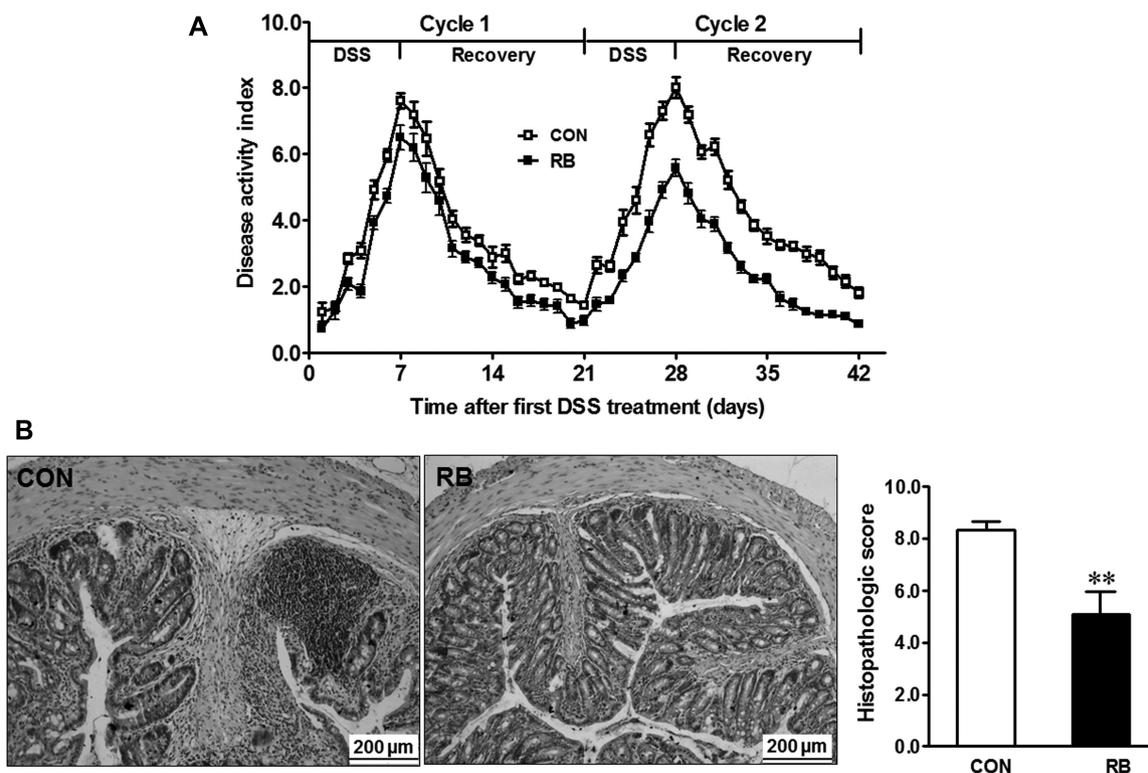


FIGURE 1 Disease activity index scores (A) and histopathologic scores (B) of male mice fed a CON diet or a 5% RB diet subjected to two 7-d cycles of 1% DSS treatment. Values are means \pm SEMs, $n = 12$. **Different from CON, $P \leq 0.01$. Panel B shows representative images of hematoxylin and eosin-stained colonic tissue. (Representative images are at 200 \times magnification.) CON, control; DSS, dextran sulfate sodium; RB, red raspberry.

(7). On the contrary, the key tumor suppressor protein p53 (8, 9) is reduced under chronic inflammation and is lost in $\sim 50\%$ of malignant tumors (10).

Among cancers, CRC etiology is unique and highly affected by diet. Western high-calorie diets with low dietary fiber and polyphenolic compounds are known to increase CRC incidence. Increasing fruit consumption reduces the risk of CRC and enhances the efficacy of anticancer drugs and therapies (11, 12), primarily mediated by fruit's bioactive components. Red raspberry (RB) contains ~ 50 different bioactive compounds (13), including anthocyanins, ellagitannins, sanguin H-6, and lambertianin C (14, 15). RB extract exerts antioxidative (16) and anti-inflammatory activities (17). Dietary RB reduces the severity of dextran sulfate sodium (DSS)-induced acute colitis in mice (18). In addition, *in vitro* studies showed that RB extract inhibits proliferation and induces apoptosis (19, 20), but the effects of dietary whole fruit RB on CRC prevention have not been tested. We hypothesized that dietary whole RB supplementation reduces the risk of CRC development in mice with chronic colitis, likely associated with its anti-inflammatory and antiproliferative properties on intestinal epithelium.

Methods

Animal care and experimental design. Six-week-old wild-type male C57BL/6J mice were randomly assigned into 2 groups ($n = 12/\text{group}$) and were fed a standard rodent diet [control (CON); Research Diets, Inc.] or a CON diet supplemented with RB (5% of dry feed weight; Research Diets, Inc.) for 10 wk. The pelleted diets with or without RB were stored at -20°C under vacuum package in the dark, and fresh diets were fed to mice weekly. The composition of RB powder has been published previously (18), which includes ~ 11 g gallic acid

equivalents/kg dry weight. The freeze-dried RB was powdered then shipped to Research Diets, Inc., overnight for customized rodent research diet preparation. Detailed information on the diet is provided in Supplemental Tables 1 and 2. The dietary RB dose is comparable to a 60-kg human dose of 29.0 g freeze-dried RB/d (21). The detailed calculation is shown in our recent publication (18), in which the same supplemental amount of RB was used.

For chronic colitis induction, after 4 wk of the dietary treatments, mice were treated with 1% DSS (Molecular weight = 36,000–50,000 Dalton; MP Biomedicals) in drinking water for 2 repeated cycles. Each cycle consisted of 7 d of 1% DSS treatment in water, followed by 14 d of recovery by providing normal drinking water. Repeated cycles of DSS exposure were used to mimic the recurring nature of colitis in human (22). We did not include mice that were not treated with DSS, because our previous study showed that RB supplementation has no effect on mice that do not receive DSS treatment (18).

After receiving DSS treatment, the mice were monitored daily for colitis symptoms during both DSS cycles. At the end of the study, the mice were killed and colonic tissues were collected for histologic and biochemical analyses. Mice were housed in a temperature-controlled room with a 12-h light and 12-h dark cycle and had a free access to diet and drinking water. There was no difference in feed intake and water consumption. All animal procedures were approved by the Washington State University Animal Care and Use Committee (BAF 04316-001). Tissues were collected and processed according to the previously published process (18).

Colitis symptom assessment and disease activity index.

During DSS induction and the recovery stages, mice under treatment were monitored for body-weight loss, fecal consistency, and blood in the feces with the use of the previously published scoring criteria (18, 23). The disease activity index (DAI) score was calculated as the sum of the above 3 scores as shown in Supplemental Table 3.

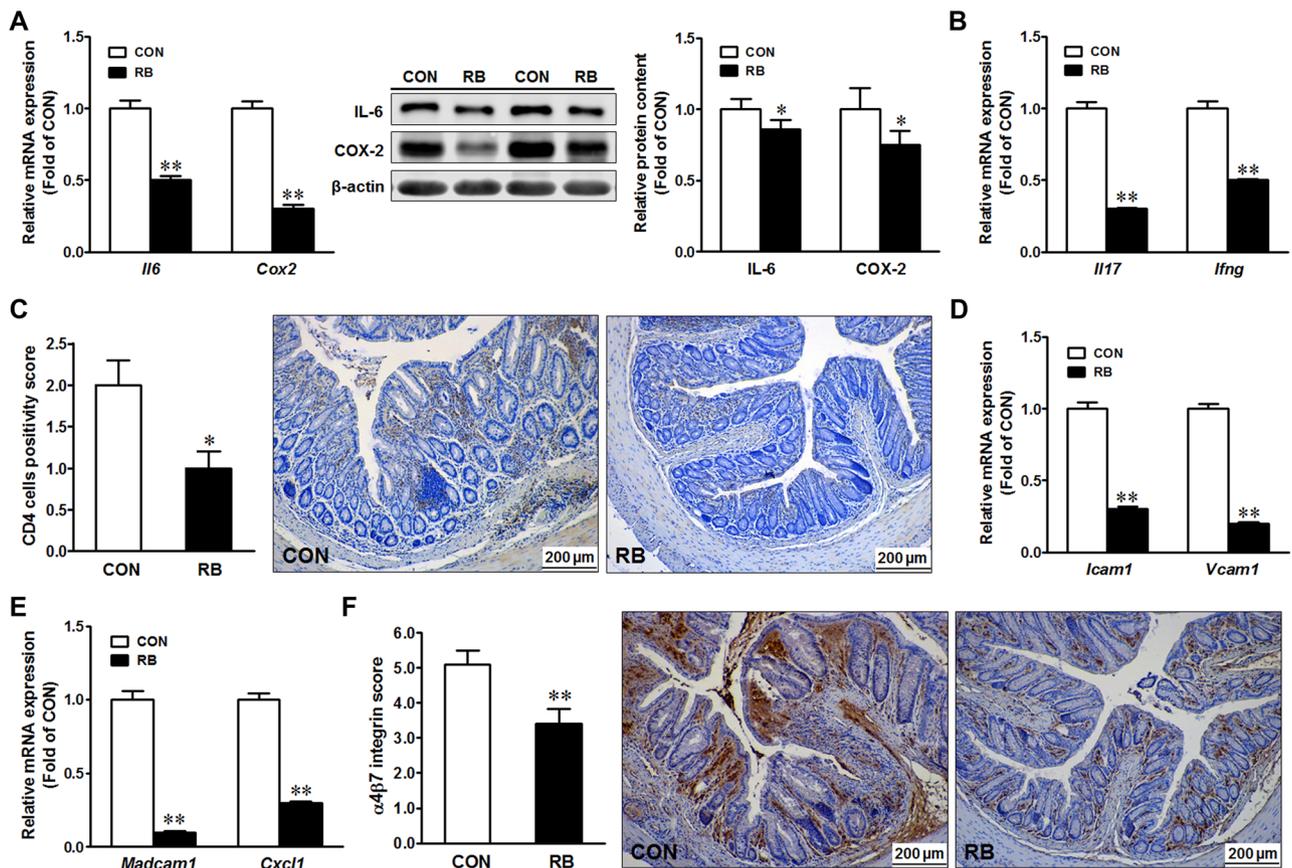


FIGURE 2 Relative protein content and mRNA expression of IL-6 and COX-2 (A), mRNA expression of *Il17* and *Ifng* (B) and CD4-positive cells (C), mRNA expression of adhesion molecules (D, E), and $\alpha 4\beta 7$ integrin filtration score (F) in male mice fed a CON diet or a 5% RB diet subjected to two 7-d cycles of 1% dextran sulfate sodium treatment. Values are means \pm SEMs, $n = 12$. ** $P \leq 0.01$. * $P \leq 0.05$. C and F are representative images of CD4 and $\alpha 4\beta 7$ integrin immunohistochemical stained colonic tissue, respectively. (Representative images are at 200 \times magnification.) CON, control; COX-2, cyclooxygenase 2; *Cxcl1*, chemokine (C-X-C motif) ligand 1; *Icam1*, intercellular adhesion molecule 1; *Ifng*, interferon γ ; *Madcam1*, mucosal vascular addressin cell adhesion molecule 1; RB, red raspberry; *Vcam1*, vascular cell adhesion molecule 1.

Histologic assessment of colonic ulceration and goblet cells. The fixed distal colonic tissue sections were deparaffinized and subjected to hematoxylin and eosin staining. The pathological scores of the distal colon were evaluated and recorded blindly with the use of previously published scoring criteria (23, 24). For goblet cell staining, the colonic sections were subjected to Alcian blue (pH 2.5) staining, examined, and quantified with the use of the Image J 1.30v software (split color channels) (National Institute of Health, USA), as described previously (25).

Immunoblotting analysis. Immunoblotting analyses were conducted per our published method (26). Band density was quantified with the use of the Odyssey Infrared Imaging System and Image Studio Lite software (Li-Cor Biosciences) and normalized to the β -actin content. Antibodies used in the study are listed in the Supplemental Material.

Immunohistochemical analysis. Immunohistochemical analyses were carried out as previously described (26). CD4 and integrin $\alpha 4\beta 7$ antibodies are listed in the Supplemental Material. The CD4 T cell infiltration scores were assessed blindly according to the distribution and degree of CD4-positive cell staining per crypt, ranging from 0 to a maximum of 4 (0 = no cell staining, 1 = 0–25%, 2 = >25–50%, 3 = >50–75%, and 4 = >75–100% per crypt). The $\alpha 4\beta 7$ integrin filtration score was assessed blindly with the use of the distribution of $\alpha 4\beta 7$ integrin staining per crypt, ranging from 0 to 4, plus the intensity of the stain signal per whole section, ranging from 0 to 4 (0 = no staining signal and 1 = 0–25%, 2 = >25–50%,

3 = >50–75%, and 4 = >75–100% per whole section). This results in the total quantified score ranging from 0 to a maximum of 8/colonic section. Nine sections per mouse at a constant interval were used for microscopic examination and score assessment.

qRT-PCR analysis. Total RNA extraction, cDNA synthesis, and qRT-PCR were performed as previously described (18, 25). 18s Ribosomal RNA was used as the reference gene. Primer sequences are listed in Supplemental Table 4.

Statistical analysis. Data were analyzed with the use of a 2-tailed Student's t test. $P \leq 0.05$ was considered to be significant. Each mouse was considered as an experimental unit. Data are expressed as means \pm SEMs.

Results

Dietary RB prevents colitis symptoms and histologic ulceration. There was no difference in the weekly body weight between the treatment groups before DSS treatment (Supplemental Figure 1). RB supplementation decreased DAI scores during both DSS treatment cycles (Figure 1A). Morphologically, RB-supplemented mice showed improved colonic architecture with less inflammation and less crypt distortion than CON mice (Figure 1B), which was confirmed by histopathologic scores (Figure 1B).

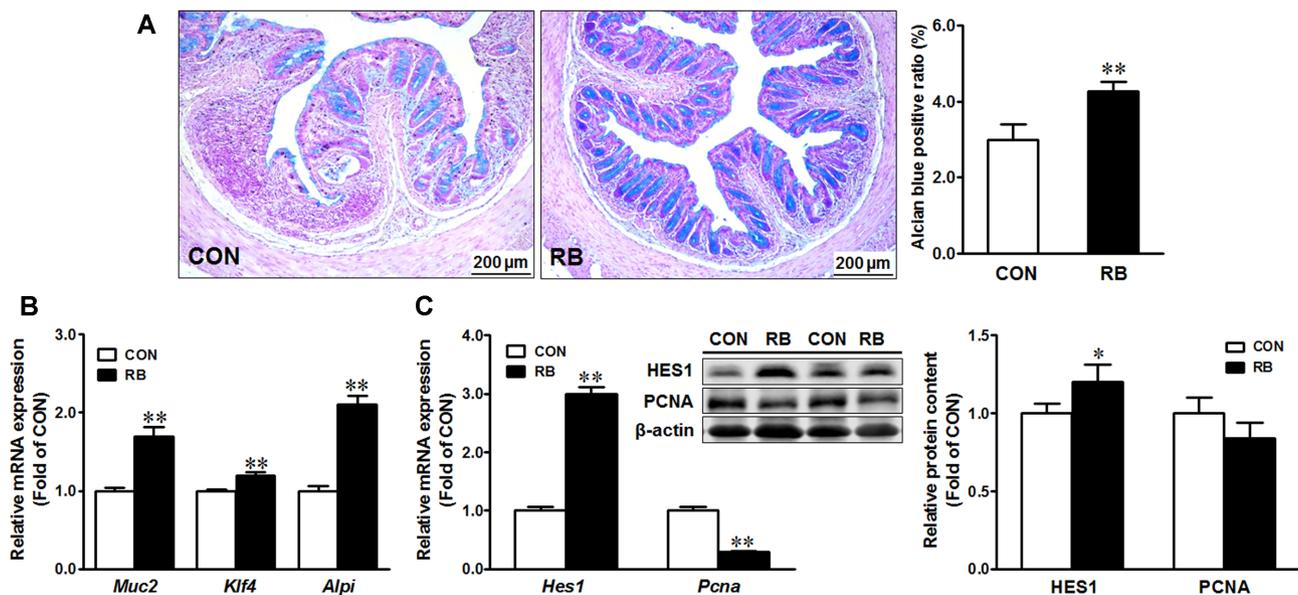


FIGURE 3 Goblet cell density (A); mRNA expression of *Muc2*, *Klf4*, and *Alpi* (B); and mRNA expression and protein content of HES1 and PCNA (C) in male mice fed a CON diet or a 5% RB diet subjected to two 7-d cycles of 1% dextran sulfate sodium treatment. Values are means \pm SEMs, $n = 12$. ***,**Different from CON: * $P \leq 0.05$, ** $P \leq 0.01$. Panel A shows representative images of Alcian blue–stained colonic tissue. (Representative images are at 200 \times magnification.) *Alpi*, intestinal alkaline phosphatase; CON, control; HES1, Hairy and enhancer of split 1; *Klf4*, Kruppel-like factor 4; *Muc2*, mucin 2; PCNA, proliferating cell nuclear antigen; RB, red raspberry.

Dietary RB reduces inflammation and associated signaling pathways. RB supplementation reduced both mRNA expression and protein levels of IL-6 and cyclooxygenase 2 (COX-2) in colonic tissues (Figure 2A). Consistently, the mRNA levels of *Il17* and interferon γ (*Ifng*) in colonic tissues were also reduced by RB supplementation (Figure 2B). The CD4 T cell infiltration and $\alpha 4\beta 7$ integrin immunohistochemical staining, mRNA levels of intercellular adhesion molecule 1 (*Icam1*), vascular cell adhesion molecule 1 (*Vcam1*), mucosal vascular addressin cell adhesion molecule 1 (*Madcam1*), and chemokine (C-X-C motif) ligand 1 (*Cxcl1*) were all reduced in the colonic tissues of DSS-treated mice supplemented with RB (Figure 2C–F). Collectively, these data show that RB supplementation reduced DSS-induced chronic inflammation and the resultant adaptive immune responses.

Dietary RB supplementation improves epithelium repair as indicated by enhanced differentiation of epithelial cells in chronic colitis. Dietary RB-enhanced goblet cell density in colonic tissues (Figure 3A), concomitant with elevated mRNA levels of mucin 2 (*Muc2*) and the goblet cell differentiation marker Kruppel-like factor 4 (*Klf4*), as well as intestinal alkaline phosphatase (*Alpi*), a differentiation marker of enterocytes in colonic tissues (Figure 3B). Furthermore, both mRNA and protein levels of Hairy and enhancer of split 1 (HES1) were enhanced, whereas those of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation, were reduced by RB supplementation (Figure 3C).

Dietary RB supplementation suppresses β -catenin and STAT3 signaling in chronic colitis. Cytosolic β -catenin has an important role in cell adhesion and intracellular signaling, including cell proliferation (27). RB supplementation reduced the protein content of phosphorylated β -catenin (Ser 552) and β -catenin and mucin 1, and enhanced E-cadherin in colonic tissues (Figure 4A–B). In addition, mRNA level of mucin 1 was reduced (Figure 4B, C), and phosphorylation of STAT3, involved

in proliferation and survival of tumor cells (7), were suppressed by RB supplementation (Figure 4D).

Dietary RB supplementation suppresses oncogenic signaling via enhancing tumor suppressor p53 expression. Dietary RB enhanced p53 and its phosphorylation at Ser 20 and Ser 15, as well as the content of p19^{ARF}, a small tumor suppressor protein that stabilizes p53, in colonic tissues (Figure 5A). The mRNA expression of *p21*, a cyclin-dependent kinase inhibitor that is a downstream target of p53, was higher in the RB-supplemented group (Figure 5B). Consistently, mRNA expression of the p53 downstream targets, antiapoptotic B cell lymphoma 2 (*Bcl2*), myeloid cell leukemia 1 (*Mcl1*), proliferative cyclin D1 (*Ccnd1*), and oncogenic myelocytomatosis (*Myc*) was reduced in the colonic tissues of RB-supplemented mice (Figure 5C, D).

Discussion

Chronic colitis is characterized by active inflammation during relapse separated by remissions, frequently with a progressive increase in wound region and duration of the disease, which might eventually lead to CRC occurrence (1). The consumption of fresh fruit, vegetables, and dietary fiber is associated with a reduced risk of CRC development (28); phytonutrients in plant foods potentially reduce the risk of cancer via multiple mechanisms (11, 12). RB is a rich source of polyphenolic compounds, vitamins B and C, folate, and dietary fibers (13–15, 29). Dietary RB reduced the severity of DSS-induced acute colitis (18). In the current study, we examined the protective effects of dietary RB against chronic colitis, which causes colonic mucosal inflammation and associated epithelial dysplasia, predisposing to CRC (22). We found that RB supplementation reduced the severity of chronic colitis and inflammation, modulated immune responses, enhanced epithelium repair, and reduced oncogenic signaling, suggesting that dietary RB can reduce the risk of CRC development in subjects with chronic colitis.

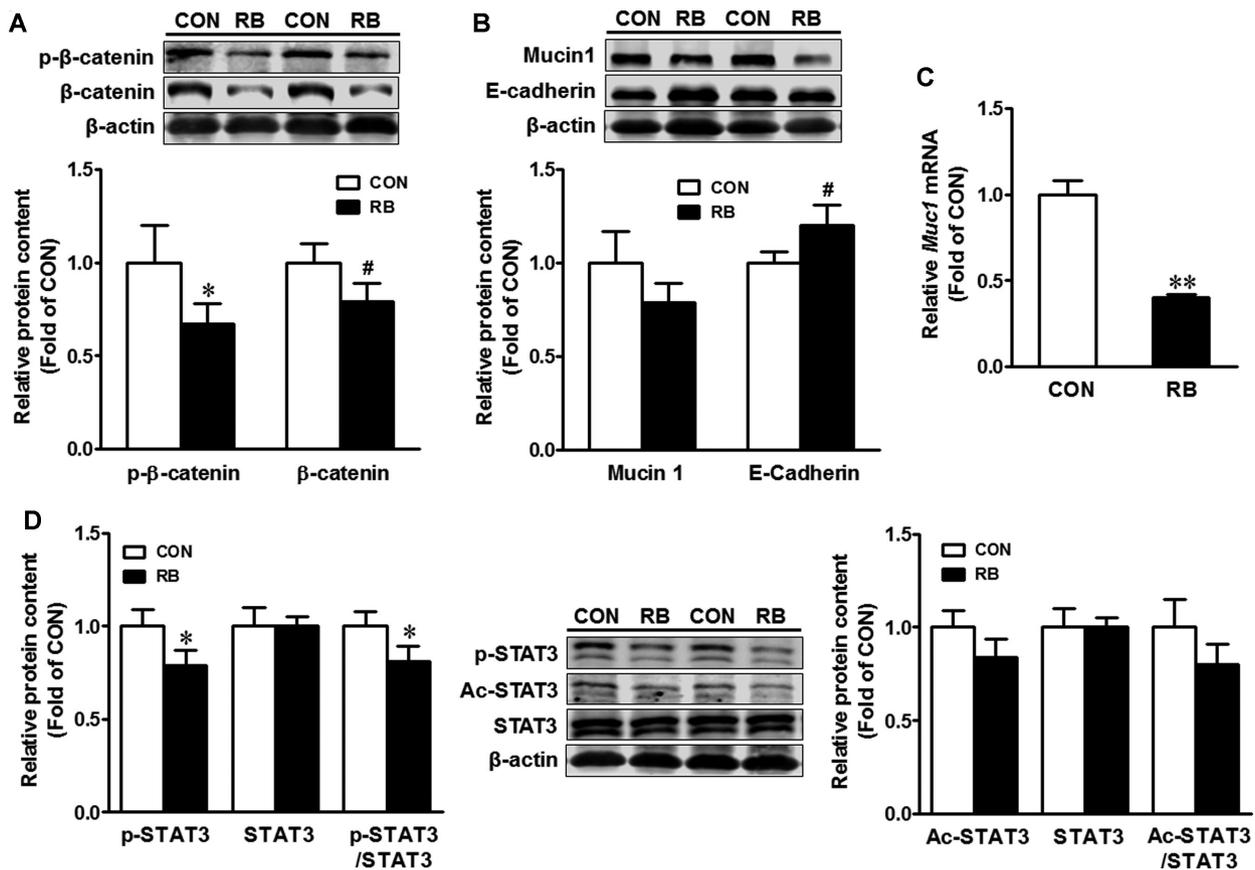


FIGURE 4 Protein contents of β -catenin and p- β -catenin (Ser 552) (A), mucin 1 and E-cadherin (B), mRNA expression of *Muc1* (C), and protein contents of total STAT3, p-STAT3 (Tyr 705), and Ac-STAT3 (Lys 685) (D) in male mice fed a CON diet or a 5% RB diet subjected to two 7-d cycles of 1% dextran sulfate sodium treatment. Values are means \pm SEMs, $n = 12$. #, **,*Different from CON: # $P \leq 0.10$, * $P \leq 0.05$, ** $P \leq 0.01$. Ac-STAT3, acetylated STAT3; CON, control; *Muc1*, mucin 1; p- β -catenin, phosphorylated- β -catenin; p-STAT3, phosphorylated STAT3; RB, red raspberry; STAT3, signal transducer and activator of transcription 3.

Chronic colitis is associated with increased production of inflammatory cytokine IL-6 (30) and other inflammatory mediators, including COX-2, IFN- γ , and CXCL1 (31, 32). IL-17-expressing CD4 T cells are present in the intestinal lamina propria and produce IL-17 (33). IL-17 stimulates IL-6, which activates STAT3, mediating CD4 T cell maturation, proliferation, and perpetuation into inflammatory intestinal tissues (33, 34). As a result, CD4 T cells are aberrantly activated in colitis (35, 36), and express $\alpha 4\beta 7$ integrin to further attract leucocytes (37). Vedolizumab, an integrin antagonist antibody, significantly induces mucosal healing and enhances remission in patients with ulcerative colitis (38). In agreement, RB supplementation reduced the expression of inflammatory mediators, CD4 T cell density, the content of $\alpha 4\beta 7$ integrin, and adhesion molecules in chronic colitis, clearly showing protective effects of RB on inflammation and pathological changes of DSS-induced colitis.

Intestinal epithelium wound healing is dependent on the homeostasis of 3 cellular processes, including restitution, proliferation, and differentiation of epithelial cells into functional cells in the injured epithelium (6). Goblet cells are one of the differentiated functional lineages of epithelial cells that secrete mucin 2, the major mucin of the mucosal layer shielding the underlying epithelium from pathogenic microbes and toxic luminal content (39). The depletion of goblet cells and mucin 2 is a characteristic of intestinal inflammation in inflammatory bowel disease (40–42). Goblet cell density and mRNA expression of *Muc2* and the lineage-specific differentiation factors

such as *Klf4* and *Hes1*, along with *Alpi*, were heightened in RB-supplemented mice, indicating enhanced epithelial cell differentiation. RB supplementation also reduced cell proliferation, thus decreasing the chance of CRC development. The total content of polyphenols could be the main contributor for these protective effects. Indeed, polyphenolic-rich grape seed extract protects epithelial integrity by reducing proliferation and enhancing differentiation in the intestine of IL-10-deficient mice (41, 42); berry extracts reduce the proliferation of LNCaP prostate cancer cells and HT-29 and HCT116 colon cancer cells (43).

Several major signaling pathways regulate cell proliferation and differentiation, including the Wnt/ β -catenin pathway. Intestinal inflammation is known to enhance Wnt/ β -catenin signaling, which further stimulates cell proliferation and CRC development (44). In the current study, we found that RB supplementation reduced the β -catenin content, which is consistent with a previous report in which dietary black raspberry reduced β -catenin concentration and colonic ulceration (45); grape seed extract supplementation downregulated the Wnt/ β -catenin pathway and proliferation in the colon of IL-10-deficient mice (42). STAT3 transcriptionally regulates β -catenin expression (7) and epithelial cell proliferation and survival (30); STAT3 is activated in 50–60% of CRC (46). RB supplementation reduced STAT3 activation, which could be partially explained by the reduced production of IL-6, an upstream activator of STAT3 signaling (30). As a result, the suppression of STAT3 and β -catenin signaling pathways reduces the risk of

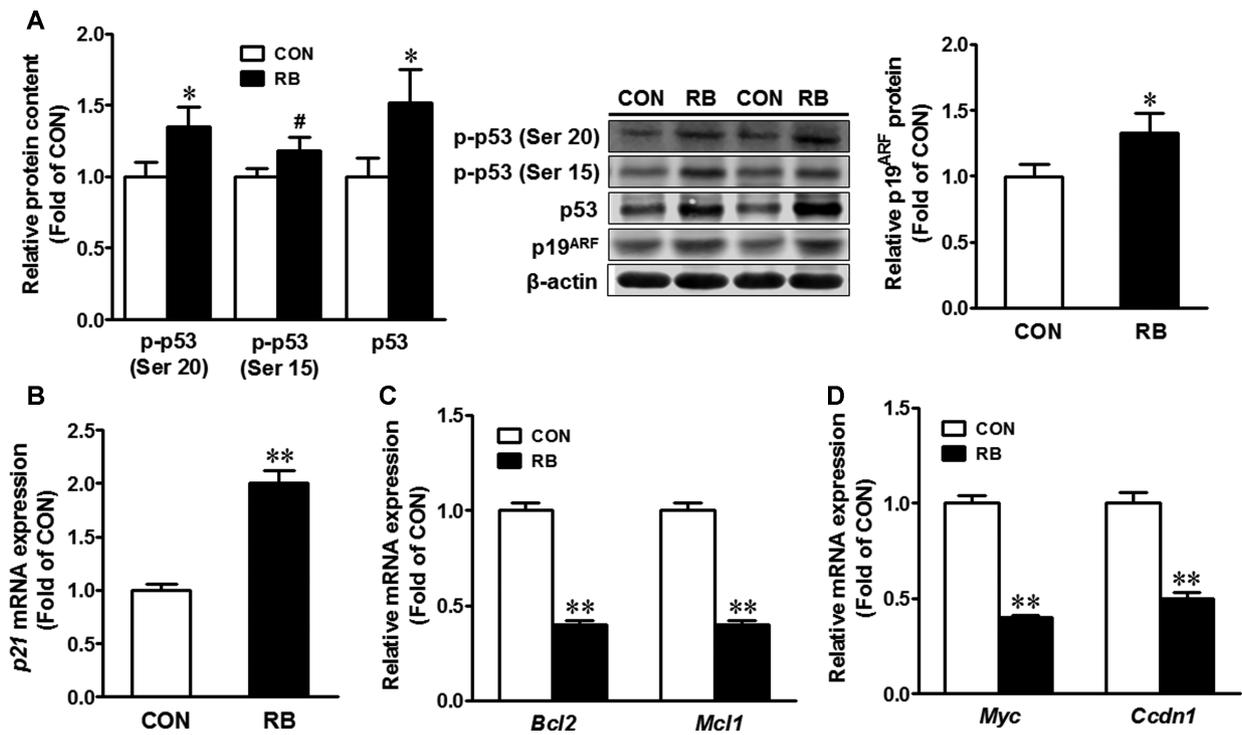


FIGURE 5 Protein contents of total p53 and p-p53 and p19^{ARF} (A), mRNA expression of *p21* (B), and antiapoptotic and oncogenic genes (C, D) in male mice fed a CON diet or a 5% RB diet subjected to two 7-d cycles of 1% dextran sulfate sodium treatment. Values are means \pm SEMs, $n = 12$. #, *, ** Different from CON: # $P \leq 0.10$, * $P \leq 0.05$, ** $P \leq 0.01$. *Bcl2*, B cell lymphoma 2; *Ccdn1*, proliferative cyclin D1; CON, control; *Mcl1*, myeloid cell leukemia 1; *Myc*, myelocytomatosis oncogene; p-p53, phosphorylated p53; RB, red raspberry.

CRC. In agreement with our results, the curcumin-derivative, small-compound FLLL32 inhibited STAT3 activity, which reduces CRC development (47). Triptolide (a diterpenoid triepoxide extracted from the traditional Chinese medicinal herb) inhibited CRC progression associated with STAT3 suppression (48).

Protein p53 is a tumor suppressor protein (8), which is reduced or lost in cancer cells (10). Phosphorylation of p53 at Ser 15 and Ser 20 stabilizes p53 by reducing its interaction with mouse double minute 2 (MDM2), which mediates p53 degradation (49). On the other hand, p14^{ARF} (p19^{ARF} in mice) stabilizes p53 by acting as an inhibitor of MDM2 (49). We found that RB supplementation stabilized the p53 protein correlated with increased phosphorylation at Ser 15 and Ser 20 and an upregulated level of p19^{ARF}. These changes could be due to the biological effects of polyphenols. Green tea polyphenols, epigallocatechin-3-gallate, stabilized p53 by inducing its phosphorylation at Ser 15 and Ser 20, and p14^{ARF} mediated downregulation of MDM2 in human prostate carcinoma LNCaP cells (49). p21 is a cyclin-dependent kinase inhibitor and one of the p53 downstream mediators, which initiates cell cycle arrest and interacts with PCNA to inhibit DNA replication (50). The p53 stabilization increases transcription of p21 and the expression of proapoptotic Bax, while reducing the antiapoptotic *BCL2* (9, 49). Consistently, dietary RB enhanced *p21* expression and reduced *Bcl2*, *Mcl1*, *Ccdn1*, and *Myc*, showing that RB suppressed epithelial cell proliferation and oncogenic signal activation in chronic colitis.

The bioactive components in whole fruit RB include vitamins, minerals, fibers, antioxidants, and polyphenols (51). RB freeze-dried powder contains anthocyanin, ellagitannins, sanguin H-6, lambertianin C, and other polyphenols (14, 15). RB anthocyanin supplementation reduced the production of

IL-6, IL-1 β , COX-2, and inducible NO synthase, and suppressed NF- κ B signaling in mice (17). RB ellagic acid showed immunoregulatory functions in various animal studies (52) and antiproliferative and apoptotic activities in Caco-2 cells (53, 54). In addition, RB contains 1.6% soluble fiber and 33.5% insoluble fiber (51), which provide substrates for gut fermentation to produce SCFAs and are expected to generate additional beneficial effects (55). By using the whole-fruit approach, beneficial effects of RB could be attributed to the combined effects of the high polyphenol and fiber contents in RB.

In summary, dietary RB reduced the severity of chronic colitis, colonic ulceration, inflammation, and associated signaling. RB facilitated epithelium repair by enhancing epithelial cell differentiation and mucin 2 production, while reducing cell proliferation, which were associated with reduced β -catenin and STAT3 signaling. Moreover, dietary RB supplementation stabilized the tumor suppressor p53 and reduced oncogenic signaling, suggesting that RB is a potential dietary strategy to reduce the risk of CRC development in subjects with chronic colitis.

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