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Dietary rutin, but not its aglycone quercetin, ameliorates dextran sulfate sodium-induced experimental colitis in mice: attenuation of pro-inflammatory gene expression

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Abstract

Oxidative stress has been shown to play a pivotal role in the onset of inflammatory bowel disease (IBD) and carcinogenesis. We evaluated the effects of two dietary anti-oxidants, rutin and its aglycone quercetin, on dextran sulfate sodium (DSS)-induced experimental colitis in mice. Female ICR mice were fed a diet containing 0.1% rutin or 0.1% quercetin for 2 weeks, and given 5% DSS in drinking water during the second week to induce colitis. We also examined the dose-dependency of rutin and quercetin (0.01% and 0.001% each) as well as their therapeutic efficacy, which was evaluated following DSS administration, on DSS-induced colitis. The protein level of interleukin (IL)-1β in both colonic mucosa and peritoneal macrophages was quantified by enzyme-linked immunosorbent assay. Further, mRNA expression levels of *IL-1β, tumor necrosis factor-α*, *IL-6*, *granulocyte macrophage-colony stimulating factor*, *inducible nitric oxide synthase*, and *cyclooxygenase (COX)-1* and *COX-2* in colonic mucosa were determined by reverse transcription-polymerase chain reaction. A diet containing 0.1% rutin, but not quercetin, attenuated DSS-induced body weight loss and shortening of the colorectum ($P < 0.01$ and <0.05, respectively), and dramatically improved colitis histological scores. Further, DSS-induced increases in colonic mucosal IL-1β levels were blunted significantly in rutin-, but not quercetin-, fed mice ($P < 0.01$), while dietary rutin attenuated the expressions of *IL-1β* and *IL-6* mRNA in colonic mucosa (each, $P < 0.01$). As for dose dependency, 0.01%, but not 0.001%, dietary rutin significantly reduced mucosal IL-1β levels ($P < 0.01$). Notably, a 0.1% rutin diet given 3 days after DSS treatment significantly suppressed both colorectal shortening and IL-1β production ($P < 0.05$ and <0.01, respectively). Dietary rutin ameliorates DSS-induced colitis, presumably by suppressing the induction of pro-inflammatory cytokines. Our results suggest that rutin may be useful for the prevention and treatment of IBD and colorectal carcinogenesis via attenuation of pro-inflammatory cytokine production.

Keywords: Inflammatory bowel disease (IBD); Dextran sulfate sodium (DSS); Oxidative stress; Rutin; Pro-inflammatory genes; Experimental colitis

1. Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic, relapsing, and remitting inflammatory condition of unknown origin that afflicts individuals of both sexes throughout life [1,2]. The disease is characterized by a pronounced infiltration of neutrophils into colonic lesions, accompanied by epithelial cell necrosis and ulceration. Although the exact pathogenesis of IBD is poorly understood, infection, environmental factors, heredity, and immunological abnormalities have been proposed as causes [3,4], and several models of experimental colitis have been developed to investigate the molecular and

Abbreviations: CD, Crohn's disease; COX, cyclooxygenase; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethylsulfoxide; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GM-CSF, granulocyte macrophage-colony stimulating factor; HPRT, hypoxanthine guanine phosphoribosyltransferase; IBD, inflammatory bowel disease; ICE, IL-1β-converting enzyme; IL, interleukin; iNOS, inducible nitric oxide synthase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; pMϕ, peritoneal macrophages; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; UC, ulcerative colitis

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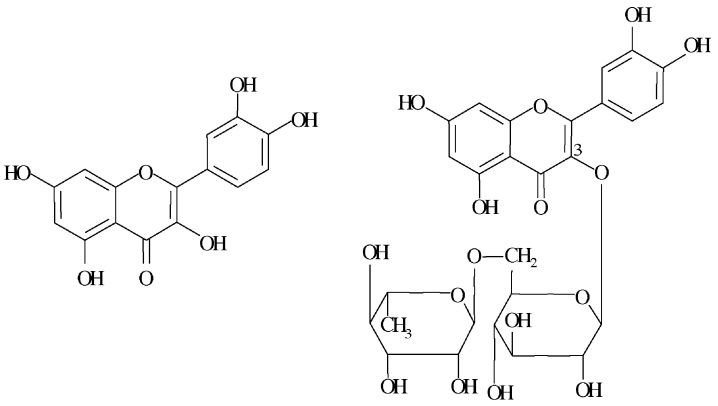


Fig. 1. Chemical structures of quercetin and rutin.

cellular mechanisms of inflammation and immunological disorder [5]. Currently, trinitrobenzene sulfonic acid (TNBS)- and dextran sulfate sodium (DSS)-induced colitis animal models are the most widely used for the study of IBD, with TNBS experimental model that exhibits many symptoms similar to those seen human CD, i.e., transmural edema and inflammtion. The DSS model exhibits many symptoms similar to those seen human UC, i.e., diarrhea, bloody feces, body weight loss, mucosal ulceration, and shortening of the colorectum [6]. In addition, DSS-induced colitis animal models have a number of advantages over others, such as simple experimental methods, reproducibility of the time course of development and severity of colitis among individual mice, and relative uniformity of the induced lesions [5–8]. Therefore, this model is thought to be reliable for studying the pathogenesis of UC and testing drugs for treatment [5–8].

Most of the current therapies for IBD involve treatment with glucocorticosteroids and 5-aminosalicylic acid, however, they display limited beneficial action [2,9]. Immunosuppressive drugs have also been used to control severe illness, regardless of the more serious complications and toxic side effects associated with them [4]. Although many types of treatment for IBD have been proposed and clinically conducted, additional therapeutic approaches are needed because many patients either do not respond to the currently available options or demonstrate significant side effects, thereby precluding their continued use. Remedy with food phytochemicals, on the other hand, is basically safe, sustainable and practical, and change of dietary habits has been implicated in the therapy of IBD [10].

Flavonoids are plant secondary metabolites ubiquitously distributed throughout the plant kingdom, and numerous reports have shown their anti-oxidative and anti-inflammatory activities in cellular and rodent models. They are also known to be inhibitors of several enzymes that are activated in certain inflammatory conditions [11], while a variety of cell types associated with the immune system are down-regulated by certain flavonoids in vitro [12]. Further, most flavonoids show potent anti-oxidative/radical scavenging effects [13]. Rutin (3-*O*-rhamnosyl-glucosyl-quercetin, Fig. 1) widely occurs in various foods, including buckwheat, parsley, tomatoes, and apricots, and is one of the most common naturally occurring flavonoids with a wide range of biological activities [14]. It and its aglycone, quercetin (3,3′,4′,5,7-pentahydroxy-flavone, Fig. 1), have been reported to exert numerous biochemical and pharmacological activities such as free radical scavenging [15,16], suppression of cellular immune and inflammatory responses [17], and anti-carcinogenic activities in rodents [18,19]. In our previous study, we found that the suppressive effects of rutin and quercetin on DSS-induced interleukin (IL)-1β production from peritoneal macrophages (pMϕ) were substantially contrasting, i.e., while rutin attenuated production, quercetin increased it [20].

That background information led us to examine the effects of dietary supplementation with rutin and quercetin on DSS-induced experimental colitis in ICR mice.

2. Materials and methods

2.1. Mice

Female specific pathogen-free ICR mice were purchased from Japan SLC (Shizuoka, Japan). They were housed five per cage and given fresh tap water ad libitum and commercial rodent MF pellets (Oriental Yeast Co., Kyoto, Japan), which were freshly changed twice a week, and handled according to Guidelines for the Regulation of Animals, as provided by the Experimentation Committee of Kyoto University. The mice were maintained in a room controlled at 24 ± 2°C with a relative humidity of 60 ± 5% and a 12 h light/dark cycle (06:00–18:00). All mice at 7 weeks of age were quarantined for 1 week before starting the experiments.

2.2. Chemicals

Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). DSS with a molecular weight of 40,000 was from ICN Biomedicals (Aurora, OH). Mouse *inducible nitric oxide synthase* (*iNOS*) primer was from Maxim Biotech. Inc. (South San Francisco, CA), and other oligonucleotide primers were synthesized and purified by Proligo (Kyoto, Japan). A Qiashredder™ kit and RNeasy Mini Kit® were from Qiagen (Hilden, Germany), and an RNA PCR Kit (AMV) Version 2.1 was from TaKaRa Bio. (Shiga, Japan). A mouse enzyme-linked immunosorbent assay (ELISA) kit for IL-1β measurement was purchased from Endogen Inc. (Woburn, MA). Rutin and quercetin, as well as other chemicals, were from Wako Pure Chemicals (Osaka, Japan) unless specified otherwise.

2.3. Cell culture

pMϕ monolayers were prepared as described previously [21]. Peritoneal exudate cells were seeded on to a 96-well plate at a density of 4×10^5 cells/well in DMEM medium with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml), and then cultured at 37 °C for 24 h under a humidified atmosphere of 5% CO_2. After incubation for 24 h, pMϕ were treated with dimethylsulfoxide (DMSO) (0.5%, v/v), or various concentrations of rutin or quercetin (0.001–200 μM) dissolved in DMSO. Negative control cells were treated only with 0.5% DMSO. After incubation at 37 °C for 30 min, pMϕ were treated with DSS at a concentration of 1 μg/ml for 24 h for ELISA, as described below. Cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test [22]. The value for cell viability of the positive control cells, which were treated with 0.5% DMSO and DSS, was standardized as 100%.

2.4. Induction of colitis

Colitis was induced by a method previously reported with some modifications (Fig. 2) [23]. Following a 1-week quarantine, the mice were divided into 10 groups. In the control group (Group 1, $n = 20$), animals were given fresh tap water ad libitum and MF pellets, freshly changed twice a week, for 2 weeks. In the DSS group (Group 2, $n = 20$), the mice were fed with MF pellets for 2 weeks, with 5% DSS added to the tap water during the second week to induce colitis. The activities of quercetin and rutin were compared by administrating the identical milligram doses rather than molar ones. In the next six groups, 0.1% (17.7 μmol or 6 mg/day) quercetin (Group 3, $n = 15$), 0.1% (9.8 μmol or 6 mg/day) rutin (Group 4, $n = 15$), 0.01% (1.77 μmol or 0.6 mg/day) quercetin (Group 5, $n = 5$), 0.01% (0.98 μmol or 0.6 mg/day) rutin (Group 6, $n = 5$), 0.001% (177 nmol or 60 μg/day) quercetin (Group 7, $n = 5$), or 0.001% (98 nmol or 60 μg/day) rutin (Group 8, $n = 5$) was added to the MF pellets for 2 weeks, with the same 1-week DSS exposure as in Group 2. In Groups 9 and 10, the mice received the same dietary protocol as Group 2, then dietary feeding of 0.1% quercetin (Group 9, $n = 10$) or 0.1% rutin (Group 10, $n = 10$) was started 3 days after DSS was added until the end of the second week. The body weight of each mouse was recorded 0, 7, and 14 days after the start of the experiment, and the intake of food and drinking water was recorded every 2 days.

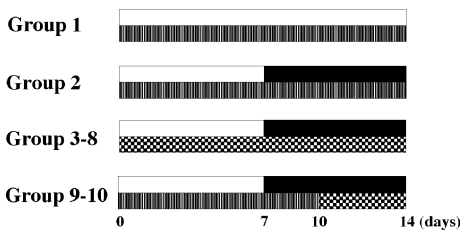


Fig. 2. Experimental protocol for DSS-induced colitis in mice. (□) Tap water, (■) 5% DSS in tap water, (▥) MF pellets, (▩) test chemical in MF pellets. Group 3, 0.1% quercetin (QER); Group 4, 0.1% rutin (RUT); Group 5, 0.01% QER; Group 6, 0.01% RUT; Group 7, 0.001% QER; Group 8, 0.001% RUT; Group 9, DSS → 0.1% QER; Group 10, DSS → 0.1% RUT.

2.5. Tissue harvest

At the end of each experiment, all mice were killed by cervical dislocation and the large intestines without the cecum were removed. After washing in ice-cold phosphate-buffered saline (PBS), they were placed on filter papers to measure their lengths. They were then opened with surgical scissors to remove their contents, and three specimens randomly selected from each of Groups 1–4 were subjected to histological analyses as described below. The colonic mucosa from all other mice were scraped off by razors in ice, then frozen in liquid nitrogen until use, according to a previous method reported by Perdue et al. with some modifications [24].

2.6. Histopathological analysis

The excised large intestine specimens from Groups 1–4 were fixed in Mildform® (Wako Pure Chemicals) and embedded in paraffin. Sections (3 μm) were stained with hematoxylin and eosin, then photographed. Histological scoring was done in a blind fashion by a pathologist (T.T.) based on injury to the colonic mucosa, with particular attention paid to alterations of the colonic crypts, and the presence of edema and inflammation in the colon, as described previously [25]. In brief, edema scores were assessed as: Grade 0, absence of edema in the colon; Grade 1, mild edema in the mucosa; Grade 2, edema in the mucosa and submucosa; Grade 3, edema in the entire wall of the colon; and Grade 4, severe edema in the entire wall of the colon. Inflammation was scored as: Grade 0, a few

Table 1
List of primer sequences for RT-PCR

Gene	Primer	Sequence (5′–3′)	Product size (bp)
IL-1β	Forward	ATggCAACTgTTCCTgAACTCAACT	586
	Reverse	CAggACAggTATAgATTCTTTCCTTT	
TNF-α	Forward	TTgACCTCAgCgCTgAgTTg	402
	Reverse	CCTgTAgCCCACgTCgTAgC	
IL-6	Forward	TgCTggTgACAACAACggCC	298
	Reverse	gTACTCCAgAAgACCAgAgg	
GM-CSF	Forward	TgTggCTgCAgAATTTAC	374
	Reverse	gCTgTCTATgAAATCCgC	
COX-1	Forward	CTTTgCACAACACTTCACCCACC	402
	Reverse	AgCAACCCAAACACCTCCTgg	
COX-2	Forward	gCATTCTTTgCCCAgCACTT	304
	Reverse	AgACCAggCACCAgACCAAAg	
HPRT	Forward	gTAATgATCAgTCAACgggggAC	196
	Reverse	CCAgCAAgCTTgCAACCTTAACCA	

inflammatory cells; Grade 1, mild inflammation of the lamina propria and submucosa; Grade 2, severe inflammation of both the lamina propria and submucosa; Grade 3, severe inflammation in the entire wall of the colon; and Grade 4, more severe inflammation in the entire wall of the colon. Regenerative changes in the crypt cells surrounding erosion or an ulcer were scored as: Grade 0, no regenerative changes; Grade 1, slight regenerative changes; Grade 2, moderate regenerative changes; Grade 3, severe regenerative changes, and Grade 4, severe regenerative changes with fibrosis in the mucosa.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the frozen tissues using the Qiashredder™ and RNeasy Mini Kit®. Steady-state messenger RNA (mRNA) levels of *IL-1β*, *tumor necrosis factor* (*TNF*)-*α*, *IL-6*, *granulocyte macrophage-colony stimulating factor* (*GM-CSF*), *iNOS*, and *cyclooxygenase* (*COX-1*) and *COX-2* were detected by RT-PCR. A *hypoxanthine guanine phosphoribosyltransferase* (*HPRT*) transcript served as the internal control. The primer sequences used for PCR amplification and PCR product sizes are listed in Table 1. cDNA was synthesized using 1 μg of total RNA and an RNA PCR Kit (AMV). PCR amplification was then performed using a PTC-100™ thermal cycler (MJ Research Inc., Watertown, MA). The PCR conditions consisted of 35 cycles, with 30 s of denaturation at 94 °C, 30 s of annealing at 60 °C, and 30 s of primer extension at 72 °C. Amplified cDNA was separated by electrophoresis on 2% agarose gels and stained with SYBR Gold® (Molecular Probes, Eugene, OR). DNA band sizes were confirmed using a Gene Ruler 100-bp DNA ladder (Invitrogen, Grand Island, NY). Image analyses were performed using NIH image software. The ratios of expression levels of each gene were determined by dividing the band intensity of the product of interest by that of the corresponding *HPRT* band.

2.8. ELISA

The colonic mucosa was minced with surgical scissors and homogenized in ice-cold PBS using a homogenizer (Dr. Hielscher-UP 50H, GS, Germany). Tissue homogenates were then centrifuged at 1900 × *g* at 4 °C for 15 min to obtain the supernatant. Total protein concentrations in the tissue supernatant were determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) according to the protocol of the manufacturer (dilution factor = 30), with γ-globulin employed as the standard. IL-1β concentrations were determined using an ELISA kit, according to the protocol of the manufacturer (dilution factor = 20).

2.9. Statistical analysis

Data were analyzed by the difference between means and statistical significance was calculated using a non-parametric post hoc test (Kruskal–Wallis test with post hoc test).

3. Results

3.1. Symptomatic parameters and general observations

First, we observed the symptomatic parameters colorectum shortening and body weight loss caused by colitis 1 week after starting 5% DSS oral administration. Consistent with a previous report [26], the length of the colorectum in Group 2 was significantly shortened by 55% ($P < 0.01$) as compared with that of Group 1 (Table 2). Further, 0.1% rutin-fed mice (Group 4) showed a marked suppression of shortening by 73% ($P < 0.05$), whereas the 0.1% quercetin

Table 2
Symptomatic changes in each group

	Colorectum (mm)	Body weight (g)		
		0 days	7 days	14 days
Group 1 (control)	98.7 ± 10	30.2 ± 2.3	32.9 ± 2.1	34.3 ± 3.7
Group 2 (DSS)	44.2 ± 12[a]	30.3 ± 1.9	33.2 ± 2.6	24.7 ± 4.9[c]
Group3 (+0.1% QER)	52.3 ± ll[a]	30.1 ± 2.8	33.4 ± 2.5	26.8 ± 3.1[c,d]
Group 4 (+0.1% RUT)	84.1 ± 16[b]	30.0 ± 3.2	32.7 ± 3.1	29.9 ± 3.8[c,e]

In the control group (Group 1, $n = 20$), mice were given fresh tap water ad libitum and MF pellets for 2 weeks. In the DSS group (Group 2, $n = 20$), the mice were fed with MF pellets for 2 weeks, with 5% DSS added to the tap water during the second week to induce colitis. In 2 other groups, 0.1% quercetin (Group 3, $n = 15$) and 0.1% rutin (Group 4, $n = 15$) was added to the MF pellets for 2 weeks with the same 1-week DSS exposure as in Group 2. After the end of each experiment, all mice were killed by cervical dislocation and large intestines without the cecum were removed. After washing in ice-cold PBS, they were placed on filter papers to measure their lengths. The body weight of each mouse was recorded 0, 7, and 14 days after the start of the experiment. Statistical analysis was performed using a non-parametric post hoc test (Kruskal–Wallis test).

[a] $P < 0.01$ vs. Group 1.
[b] $P < 0.05$ vs. Group 2.
[c] $P < 0.001$ vs. Group 1.
[d] $P < 0.05$.
[e] $P < 0.01$ vs. Group 2.

(Group 3) did not show a significant effect. As for body weight, that of mice in Group 2, following 5% DSS administration for 1 week, decreased by 9.6 g ($P < 0.001$) as compared with that in Group 1. In addition, dietary feeding of 0.1% quercetin (Group 3) and rutin (Group 4) attenuated body weight loss by 21% ($P < 0.05$) and 54% ($P < 0.01$), respectively, as compared with Group 2.

Food intake in Groups 2–4 began to decrease after day 9 (Fig. 3A), with a nearly identical reduction tendency in Groups 2 and 3 (79% and 69%, respectively, of Group 1 on day 13, $P < 0.01$ each). On the other hand, 0.1% rutin (Group 4) remarkably suppressed the reduction of food intake by 61% ($P < 0.05$). Further, as shown in Fig. 3B, the intake of drinking water among the three groups who received DSS declined 2 days after DSS administration (43–65% on day 13, $P < 0.01$ each), with no statistical differences observed among those groups.

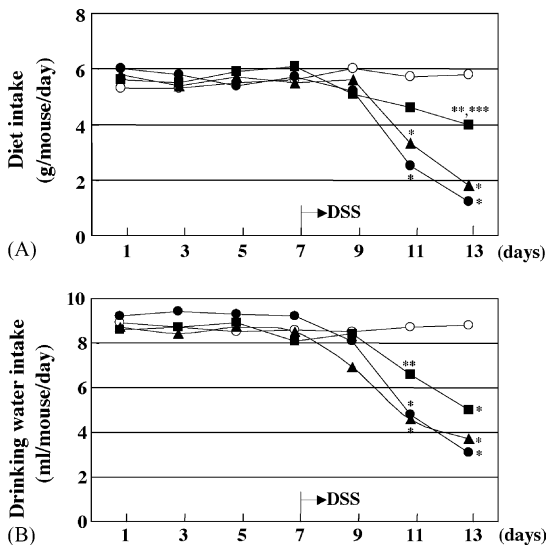


Fig. 3. Intake of diet (panel A) and drinking water (panel B) by each group. (○) Group 1; (●) Group 2; (▲) Group 3; (■) Group 4. Values are shown as mean ± S.D. of three replicate experiments. Statistical analysis was performed using a non-parametric post hoc test (Kruskal–Wallis test): $^{*}P < 0.01$, $^{**}P < 0.05$ vs. Group 1, $^{***}P < 0.05$ vs. Group 2.

3.2. Effects on IL-1β production in colonic mucosa and pMϕ

IL-1β has been well characterized as a pivotal pro-inflammatory cytokine, which is produced from both inflammatory cells and mucosal epithelial cells during colonic inflammation [27]. We quantified IL-1β protein levels in the colonic mucosa samples using ELISA. As shown in Fig. 4A, the IL-1β protein level in Group 2 was increased by 11-fold ($P < 0.01$) as compared to that in Group 1 after 1 week of DSS administration. This DSS-induced increase was attenuated by 74% in the 0.1% rutin-fed (Group 4, $P < 0.01$), however, not in Group 3 (0.1% quercetin) mice. In parallel, incubation of pMϕs from Group 2 samples for 24 h led to a marked increase in IL-1β production by 9.0-fold ($P < 0.01$), as compared to Group 1, whereas 0.1% rutin significantly suppressed it by 58% ($P < 0.01$), while quercetin was virtually inactive (Fig. 4B).

3.3. Contrasting effects of rutin and quercetin on IL-1β production in pMϕ

Next, the effects of rutin and quercetin on DSS-induced IL-1β production were evaluated in in vitro experiments using pMϕs prepared from non-treated mice by ELISA. As shown in Fig. 5, IL-1β protein was abundant in the media after 24 h of treatment with DSS (1 μg/ml) with no detectable cytotoxicity (13-fold greater than the control, $P < 0.01$). Rutin significantly suppressed DSS-induced IL-1β production at a range of 10–200 μM (65–74%, $P < 0.05$) without cytotoxicity. In contrast, quercetin

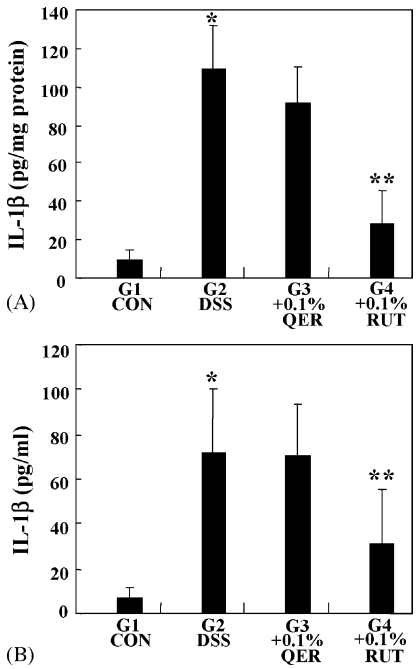


Fig. 4. Production of IL-1β protein in colonic mucosa (A) and pMϕ (B) from each group following DSS exposure. (A) IL-1β protein levels in colonic mucosa 7 days after 5% DSS administration were measured by ELISA, as described in Section 2. Values are shown as mean ± S.D. of five replicate experiments. Statistical analysis was performed using a non-parametric post hoc test (Kruskal–Wallis test): $^{*}P < 0.01$ vs. Group 1, $^{**}P < 0.01$ vs. Group 2. (B) pMϕ were obtained from female ICR mice in each group on day 14. Following incubation for 24 h, the resultant cell-free supernatants (50 μl) without dilution were subjected to ELISA for measurement of IL-1β concentrations, as described in Section 2. Values are shown as mean ± S.D. of five replicate experiments. Statistical analysis was performed using a non-parametric post hoc test (Kruskal–Wallis test): $^{*}P < 0.01$ vs. Group 1, $^{**}P < 0.01$ vs. Group 2.

(100 μM) enhanced the DSS-induced IL-1β production (94% at 100 μM, $P < 0.05$). Rutin or quercetin itself did not increase the spontaneous production of IL-1β from peritoneal macrophages at a concentration range of 0.001–200 μM (data not shown).

3.4. Effects on pro-inflammatory gene expression in colonic mucosa

To further provide insight into the molecular mechanism underlying the suppression of colitis by rutin, mRNA expression levels of pro-inflammatory mediators in colonic mucosa were measured by RT-PCR. As shown in Fig. 6, mRNA levels for both *IL-1β* and *IL-6* in samples from Group 2 were significantly increased 7 days after beginning DSS exposure ($P < 0.001$ and <0.01, respectively). Those increases were attenuated in the 0.1% rutin-fed mice, by 53% and 80%, respectively ($P < 0.01$ each), however, not in those given quercetin (Group 3). mRNA levels of three other genes (*iNOS*, *GM-CSF*, and *COX-2*) in Group 2 samples were also increased ($P < 0.01$ each), while mice in Group 4 showed significant suppression by 28% (*GM-CSF*) and 36% (*COX-2*) ($P < 0.05$ each), and suppression was also seen to a lesser extent in Group 3. On the other hand, *TNF-α*, *COX-1*, and *HPRT* were found to be expressed in a constitutive manner in non-treated colonic mucosa, and did not show level changes in any of the experimental groups.

3.5. Histopathological changes

One of the major hallmarks of DSS-induced colitis is profound colonic inflammation characterized by crypt destruction, mucosal ulceration, erosions, and infiltration of lymphocytes into the mucosal tissue. Representative histological images from each group are shown in Fig. 7. As shown in Table 3, the mean number of ulcers per colon was dramatically decreased by 81% in Group 4 as compared to Group 2 ($P < 0.01$), while edema formation was attenuated by 61% ($P < 0.01$) in Group 4. Tissue inflammation, based on histological observation, was increased in the DSS group (Group 2, $P < 0.001$) and reduced in Group 4 by 55% ($P < 0.01$). We also observed increased tissue regeneration in Group 3 (2.0-fold, $P < 0.05$)

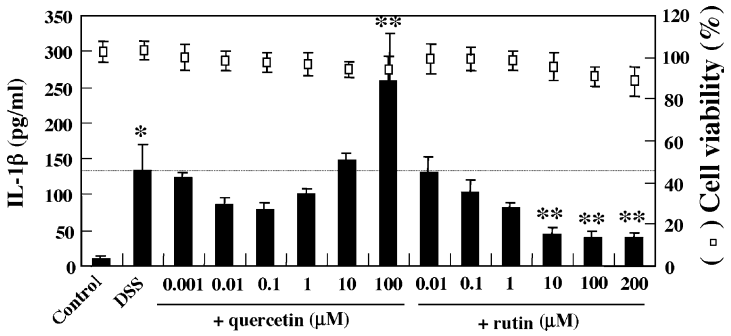


Fig. 5. Contrasting effects of rutin and quercetin on IL-1β production in pMϕ. Peritoneal exudates cells from non-treated female ICR mice were seeded onto a 96-well plate at a density of 4×10^5 cells/well, and then cultured at 37 °C for 24 h under a humidified atmosphere of 5% CO_2. After washing, the pMϕ were treated with the vehicle (0.5% DMSO, v/v) or various concentrations of rutin or quercetin (0.001–200 μM). Negative control cells were treated only with 0.5% DMSO. After incubating at 37 °C for 30 min, the pMϕ were then treated with DSS (1 μg/ml) for 24 h. The concentration of IL-1β in the supernatant of the medium (50 μl) without dilution was determined using a mouse IL-1β ELISA kit. Cell viability was determined with an MTT test. Each value is shown as mean ± S.D. of five replicated experiments. Statistical analysis was performed using a non-parametric post hoc test (Kruskal–Wallis test): $^{*}P < 0.01$ vs. control, $^{**}P < 0.05$ vs. DSS.

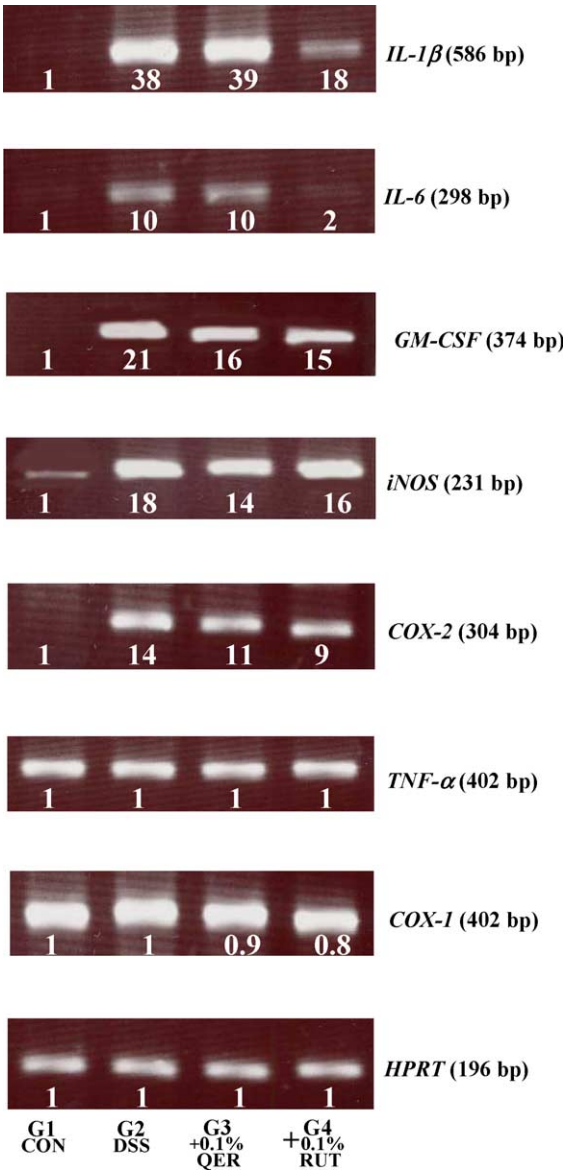


Fig. 6. Expression of pro-inflammatory genes in colonic mucosa from each group following DSS administration. Pro-inflammatory gene expression levels in colonic mucosa 7 days after 5% DSS administration were measured using RT-PCR, as described in Section 2. Representative photographs from three independent experiments with each gene are shown. *HPRT* served as the internal control. The expression seen in the Group 1 (control) was standardized as one-fold.

and Group 4 (2.5-fold, $P < 0.05$), as compared with Group 2.

3.6. Effects of rutin and quercetin at low doses and under therapeutic protocol

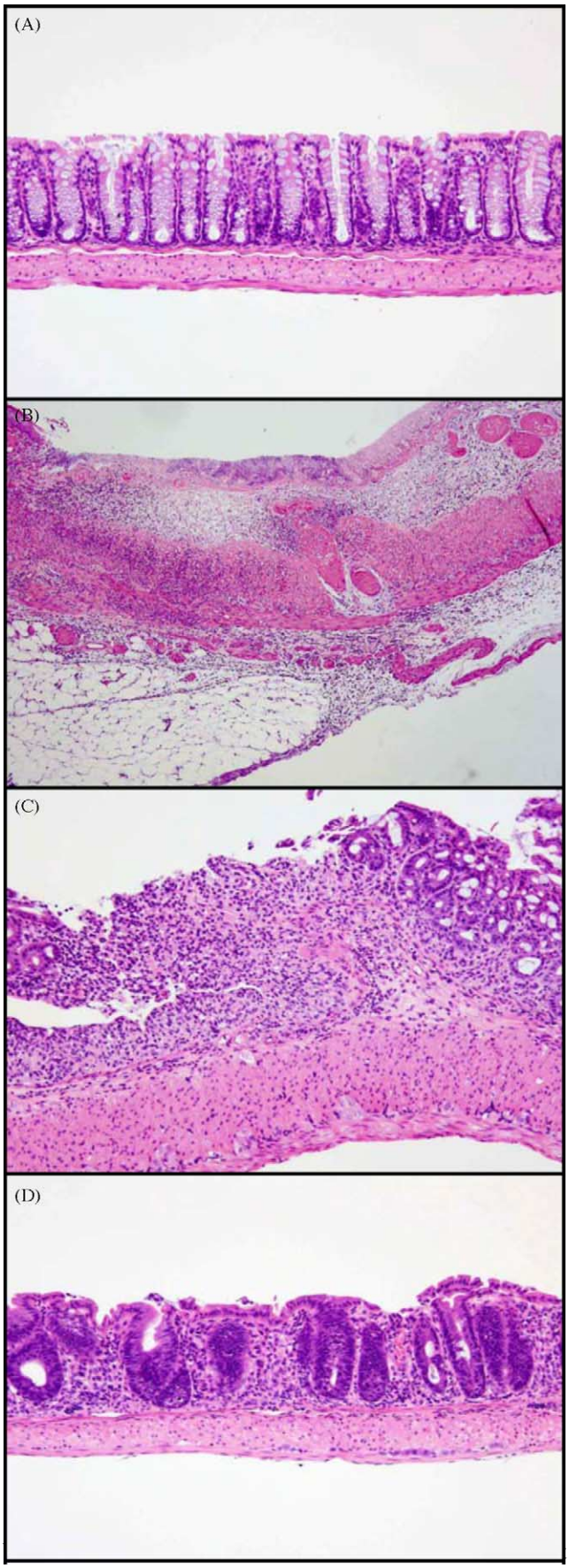
In another set of experiments, we examined the suppressive effects of quercetin and rutin at lower doses (0.01% and 0.001% in basal diet) on colorectum shortening and IL-1β production in colonic mucosa. As shown in Fig. 8A, colorectum shortening in 0.01% rutin-fed mice (Group 6) was significantly suppressed by 45% ($P < 0.05$), while the 0.001% rutin (Group 8) did not show such suppression. Further, the 0.01% (Group 5) and 0.001% quercetin-fed (Group 7) mice did not demonstrate attenuation of the shortening. In addition, dietary feeding of 0.01% rutin (Group 6) markedly decreased IL-1β production by 58% ($P < 0.01$), in contrast to the 0.001% rutin-fed mice (Group 8), while neither 0.01% nor 0.001% dietary quercetin groups (Groups 5 and 7) showed such suppression (Fig. 8B).

To determine whether rutin or quercetin could reverse DSS-induced colitis, we performed therapeutic experiments in which dietary feeding of 0.1% rutin or quercetin was started 3 days after exposure to 5% DSS. As shown in Fig. 8A and B, colorectum shortening was significantly ameliorated in 0.1% rutin-fed mice (Group 10), who also demonstrated suppression of IL-1β production by 43% ($P < 0.05$) and 52% ($P < 0.01$), respectively. In contrast, these parameters were not altered in Group 9 (0.1% quercetin).

4. Discussion

Rutin has previously been reported to attenuate TNBS-induced colitis in rats, presumably by mitigating intestinal oxidative stress [28]. Galvez et al. also found that oral administration of rutin suppressed colonic damage and inflammation associated with acetic acid-induced colitis in rats [29]. However, those studies presented scant experimental data regarding the molecular mechanisms underlying rutin-suppressed colitis. In the present study, we demonstrated for the first time that dietary rutin was profoundly effective for attenuating DSS-induced colitis in mice, which probably occurred through attenuation of pro-inflammatory gene expression, especially that of *IL-1β* and *IL-6*. Another important observation is that oral administration of rutin, 3 days after beginning DSS treatment, significantly reversed colitis, as shown by suppression of both colorectum shortening and IL-1β production. To our knowledge, there are only a few reports of natural compounds that can reverse experimental colitis when used in a therapeutic protocol [30]. In fact, most, if not all, of the suppressive efficacy by natural compounds reported thus far has been accomplished by simultaneous administration with the colitis-inducing agent [31]. In the present experiments, dietary rutin, even at a low dose (0.01%), was able to attenuate colorectum shortening and IL-1β production, which led to our hypothesis that rutin is effective not only as a prophylactic but also as a therapeutic agent for colitis. On the other hand, quercetin was virtually inactive to attenuate the DSS-induced colitis. This notion is supported by their dose settings (17.7 and 9.8 μmol/day for respectively quercetin and rutin, for instance).

Oxidative stress has been implicated in the pathogenesis of DSS-induced colitis [32]. Tardieu et al. reported that DSS increased the level of an oxidative DNA damage



colonic mucosa [32]. Sustained production of reactive oxygen species (ROS) during colonic inflammation may overwhelm the endogenous anti-oxidant defense system and, in accordance with that notion, there are several independent reports of decreased anti-oxidant levels in patients with IBD [33]. In parallel, decreased glutathione levels, which are indicative of oxidative stress, have been detected in humans [34] and colonic inflammation experiments [35]. Taken together, a strategy based on the use of anti-oxidants for compensating the disregulated anti-oxidative defense system in inflamed intestines is reasonable and may be effective for controlling IBD.

Most flavonoids, which occur widely throughout the plant kingdom [36], are well characterized as distinct anti-oxidative agents, with one such compound being rutin [37]. Flavonoid intake was recently estimated in some studies and reported to be in a range of 23–50 mg in a day, depending on the population investigated [38,39], while rutin intake by a Japanese population was calculated to be 1.5 mg/day (25 μg/kg/day) [39], which is approximately 20-fold less to the dose in the 0.01% rutin group. Our in vitro and in vivo results suggest that rutin markedly suppresses DSS-induced colitis by attenuation of *IL-1β* expression, which may be derived from its anti-oxidative property because IL-1β production is induced in response to oxidative insult, as described below. On the other hand, the poly-phenolic quercetin has been shown to act as a pro-oxidant in some models when used at high concentrations [40–42]. Laughton et al. reported that certain phenolic compounds, including quercetin, enhanced generation of the hydroxyl radical [40]. Smith et al. also reported that quercetin, as well as other related flavonoids, had the potential to serve as accelerative agents for DNA degradation via free-radical formation [41], while dietary quercetin-induced focal areas of dysplasia in the colon of 22% of normal mice [42]. In contrast to those observations, the pro-oxidative nature of rutin has yet to be shown, suggesting that the presence of glycosyl moiety is a notable determinant for exertion of its activity. To strengthen that hypothesis, it is notable that a free hydroxyl group at the 3-position, which is present in quercetin but masked in rutin, has been shown to be a prerequisite for pro-oxidative capability [43]. Our results showing a lack of ability by quercetin (0.001–0.1% in diet) to attenuate DSS-induced colitis may have been related to its potential pro-oxidative nature. Along a similar line, we recently found that quercetin enhanced DSS-induced IL-1β production in pMϕ, whereas rutin was highly suppressive [20]. Thus, additional studies of oxidative and anti-oxidative status of quercetin and rutin in the colonic mucosa of mice fed

Fig. 7. Histopathological changes in colonic mucosa from each group following DSS administration. Representative microphotographs of colonic mucosa stained with hematoxylin and eosin from three mice in each group are shown. Panel A, Group 1 (Control); panel B, Group 2 (5% DSS); panel C, Group 3 (5% DSS + 0.1% QER); panel D, Group 4 (5% DSS + 0.1% RUT). Original magnification: panels A, C, D, 100×; panel B, 40×.

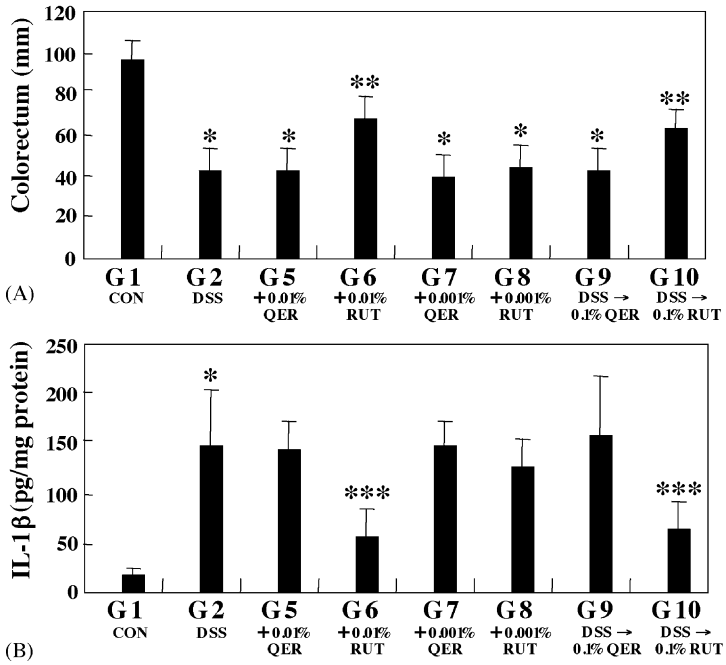


Fig. 8. Effects of dietary rutin and quercetin at low doses and under a therapeutic protocol. Suppressive effects on colorectum shortening (panel A) and IL-1β production in colonic mucosa (panel B) 7 days after 5% DSS oral administration in Group 5 (0.01% quercetin), Group 6 (0.01% rutin), Group 7 (0.001% quercetin), and Group 8 (0.001% rutin) were measured as described in Section 2. Dietary feeding of 0.1% quercetin (Group 9) and 0.1% rutin (Group 10) were started 3 days after beginning DSS exposure and continued for 4 days. Colorectum shortening (panel A) and IL-1β production in colonic mucosa (panel B) in Groups 9 and 10 were measured as described in Section 2. In panels A and B, the values are shown as mean ± S.D. of three replicate experiments. Statistical analysis was performed using a non-parametric post hoc test (Kruskal–Wallis test): $^{*}P < 0.01$ vs. Group 1, $^{**}P < 0.05$, $^{***}P < 0.01$ vs. Group 2.

diets containing each polyphenol are required. Meanwhile, rutin has been shown to be metabolized mainly to phenylacetic acids, such as 3-hydroxyphenylacetic acid in the human colon [44], though we could not determine whether rutin itself or its metabolized product(s) are responsible for attenuation of colitis. On the other hand, as it has been reported that rutin is absorbed from colon [45], it is possible that its ability to ameliorate DSS colitis results from an action within the colonic tissue itself or via rutin (or its metabolites) in the plasma. There are significant differences among the mouse, rat and human gut with respect to the mechanisms by which they utilize or exclude luminal flavonoids. We are now analyzing the bioavailability and metabolites of quercetin and rutin in the present mouse model. It is also important to compare their data with those in the rat model to validate the preventive and therapeutic efficacy for a potential patient population.

Physiologically active IL-1β is produced from its precursor pro-IL-1β by activation of IL-1β-converting enzyme (ICE, also known as caspase-1) [46], while ICE

Table 3
Histological scores in each group

	No. of ulcers/mouse	Scores of		
		Edema	Inflammation	Regenerative changes
Group 1 (control)	0	0	0	0
Group 2 (DSS)	16 ± 4.0^{b}	3.3 ± 0.5^{c}	3.6 ± 0.5^{c}	1.3 ± 0.5^{b}
Group 3 (+0.1%QER)	10 ± 3.5^{b}	2.6 ± 1.1^{b}	2.6 ± 0.5^{c}	$2.6 \pm 0.5^{c,d}$
Group 4 (+0.1% RUT)	$3.0 \pm 2.0^{a,e}$	$1.3 \pm 0.5^{b,e}$	$1.6 \pm 0.5^{b,e}$	$3.3 \pm 1.1^{b,d}$

In the control group (Group 1, $n = 3$), mice were given fresh tap water ad libitum and MF pellets for 2 weeks. In the DSS group (Group 2, $n = 3$), the mice were fed with MF pellets for 2 weeks, with 5% DSS added to the tap water during the second week to induce colitis. In two other groups, 0.1% quercetin (Group 3, $n = 3$) and 0.1% rutin (Group 4, $n = 3$) was added to the MF pellets for 2 weeks with the same 1-week DSS exposure as in Group 2. After the end of each experiment, large intestine sections were excised from three mice in each group, then fixed in Mildform® and embedded in paraffin. Sections (3 μm) were stained with hematoxylin and eosin, and photographed. Histological scoring was done in a blind fashion by a pathologist (T.T.) based on injury to the colonic mucosa, with particular attention paid to alterations of the colonic crypts and the presence of edema and inflammation in the colon, as described previously [25]. Statistical analysis was performed using a non-parametric post hoc test (Kruskal–Wallis test).

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$ vs. Group 1.
[d] $P < 0.05$.
[e] $P < 0.01$ vs. Group 2.

activation and the resultant IL-1β production are thought to be mediated by oxidative stress [47]. Bhanoori et al. reported that thiol alkylation induced the expression of ICE and p21 (waf1/cip1) [48], and silica-induced ROS and reactive nitrogen species generation resulted in the activation of cell signaling pathways, including extracellular signal-regulated kinase phosphorylation, and increased the expression of IL-1β [49]. Those findings support our hypothesis that rutin attenuates IL-1β production by mitigating DSS-induced oxidative damage [33].

As noted above, IL-1β is activated by ICE at the early stage of the cascade that leads to intestinal inflammation [50]. Accordingly, Siegmund et al. reported essential evidence that ICE-deficient mice with DSS-induced colitis showed significantly attenuated body weight loss, diarrhea, rectal bleeding, and shortening of colon length [51]. In addition, Youngman et al. demonstrated increased IL-1 expression in surgically resected intestinal specimens, as well as isolated lamina propria mononuclear cells from patients with both UC and CD [52]. Therefore, IL-1β is implicated as a primary target for therapeutic intervention as treatment for several types of inflammatory disease, including IBD, which is supported by the finding that a neutralizing antibody for IL-1β attenuated DSS-induced colitis in mice [26]. In addition, the reduction of IL-1β production by inhibition of ICE has been promoted as a promising strategy because of its key roles in many inflammatory diseases [53].

Elevation of several other cytokines with important immunoregulatory and pro-inflammatory activities has been demonstrated during active IBD [54]. These cytokines, including IL-6, TNF-α, and GM-CSF, may have important roles in the initiation and amplification of inflammatory responses that lead to intestinal injury [55]. Lymphocytes, typical activated T cells, are known to secrete growth factors such as IL-6 and GM-CSF [56], and these growth factors have been shown to stimulate the proliferation of hematopoietic cells, followed by differentiation to granulocytes or macrophages [57]. Noguchi et al. have reported that pro-inflammatory cytokines such as IL-1 released from fibroblasts, endothelial cells, and mesenchymal cells stimulate intestinal epithelial cells to secrete GM-CSF production [58]. Moreover, IL-6 is strongly induced by IL-1 via activation of transcription factors, including nuclear factor-κB and activator protein-1 [59]. Therefore, IL-1β may induce up-regulation of IL-6 and GM-CSF in DSS-induced colitis models, and rutin may regulate these cytokines by an IL-1β blockade. These issues need to be addressed in the near future.

Recently, iNOS-generated nitric oxide was implicated in the pathogenesis of IBD, including UC and CD [60]. Although we did not determine whether IL-1β up-regulation is associated with colonic iNOS induction in the present study, it should be noted that IL-1β may be one of the cytokines responsible for the induction of iNOS in enterocytes [61], while superoxide-mediated oxidative stress increased IL-1β-induced iNOS protein synthesis in another model [62]. In addition, COX expression may also be one of the key steps in various inflammatory pathogeneses in the digestive tract, such as UC [63]. However, the relative contributions of COX-1 and COX-2 isoforms in large intestinal inflammation remain controversial [64,65].

In conclusion, dietary rutin, even at a low dose, was found to attenuate the production of the critical pro-inflammatory mediator genes *IL-1β*, *IL-6*, *GM-CSF*, and *iNOS*, thereby ameliorating DSS-induced colitis in mice. This flavonoid also notably ameliorated colitis under a therapeutic protocol. Importantly, because rutin is a common phytochemical found in a variety of fruits and vegetables, the feasibility of a therapeutic approach for IBD and colon carcinogenesis using a rutin-supplemented diet may be reasonable and promising. Further mechanistic and toxicity studies to explore the effectiveness of this phytochemical for preventing and treating IBD and colorectal carcinogenesis are expected.

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