Research Communication

Effects of anthocyanin-rich tea “Sunrouge” on dextran sodium sulfate-induced colitis in mice

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Abstract.
Sunrouge, an anthocyanin-rich tea, has similar levels of catechins as “Yabukita,” the most popular green tea cultivar consumed in Japan. Green tea polyphenols (GTPs) have attracted interest due to their potent antioxidative activities combined with a lack of side effects in humans at normal consumption levels. However, we previously reported that high doses (0.5 and 1%) of dietary GTPs can result in deterioration of colitis and failed to prevent colon carcinogenesis in inflamed colons. In the present study, we determined the inhibitory effects of Sunrouge on colitis in dextran sodium sulfate (DSS)-treated and untreated control mice. Five-week-old female ICR mice were administered a single dose of Yabukita or Sunrouge (extracts in 1 mL distilled water) via a stomach tube for 3 weeks. After 1 week of treatment, the mice were divided into four groups (two Yabukita and two Sunrouge groups) and given drinking water with or without 3% DSS for 2 weeks, then they were euthanized. Those treated with DSS developed watery diarrhea and bloody stools, and showed body weight loss, spleen hypertrophy, and shortening of the colon, as well as deteriorations in survival rate, liver function, colon mucosal interleukin-1β level and expression of phase II detoxification enzyme mRNA. Sunrouge improved these DSS-induced symptoms, at least in part, whereas Yabukita showed either no effect or adverse effects in regard to some those parameters. It is suggested that the differences between Yabukita and Sunrouge on DSS-induced colitis might be due to the high levels of anthocyanins found in Sunrouge tea.

1. Introduction
Green tea has long been consumed as a beverage and there has been recent increased interest in green tea polyphenols (GTPs), which have potent antioxidative activities in combination with a lack of side effects in humans under normal consumption levels. In general, green tea has been found to be superior to black and oolong tea in terms of antioxidant and health promoting benefits owing to its higher (–)-epigallocatechin-3-gallate (EGCG) content. Several studies have demonstrated that GTPs exert their effects by scavenging reactive oxygen species (ROS) since excessive production of ROS has been implicated in the development of a variety of ailments, including cancer [1–4]. In numerous studies, such as human intervention and GTP bioavailability assessments, low or moderate doses of green tea preparations have been used [5–8]. We previously reported that high doses (0.5 and 1%) of dietary GTP can deteriorate colitis and fail to prevent carcinogenesis in inflamed colons [4].

Sunrouge (Camellia taliensis × C. sinensis) is a new green tea cultivar that has a few unique characteristics due to its red leaves [9]. It is rich in anthocyanins and includes similar levels of catechins as Yabukita (C. sinensis), the most popular green tea cultivar consumed in Japan. The red color is attributed to the anthocyanin pigments contained in the plant. Anthocyanins are glycosides of anthocyanidins and...
universally associated with attractive, colorful, and flavorful fruits. Recently, anthocyanins have attracted attention because of their potential biological and pharmaceutical benefits, such as antioxidant [10,11] and antiinflammatory [12–14] activities, and their potential for reducing the risk of cancer has been proposed [14,15]. Those reports suggested that anthocyanins increase the activities and mRNA expressions of antioxidant and detoxifying enzymes, such as glutathione (GSH)-reductase, glutathione peroxidase (GPx), and glutathione S-transferase (GST) [11], while they decrease those of cyclooxygenase 2 (COX2), a major mediator of inflammation. Its product, prostaglandin E2 (PGE2), also has been shown to have several critical roles in promoting carcinogenesis [16–18]. Thus, Sunrouge is speculated to have potential to ameliorate colitis and inflammation-associated colon carcinogenesis. In addition, it is also necessary to assess the possible adverse effects of Sunrouge, which have been reported for GTPs [4].

Mice with dextran sulfate sodium (DSS)-induced colitis provide an experimental model that displays many symptoms similar to those seen in human ulcerative colitis (UC), such as diarrhea, bloody feces, body weight loss, mucosal ulceration, and shortening of the colorectum [19]. Some cytokines play key roles in the pathogenesis of inflammatory bowel disease (IBD), including UC and Crohn’s disease (CD), and increase the severity of DSS-induced colitis [20]. Interleukin (IL)-1β has also been reported to play a pivotal role in the early stage of the pathogenesis of IBD, resulting in upregulation of inflammatory responses [20,21]. Furthermore, IL-6 [21] and tumor necrosis factor (TNF)-α [22,23] have important roles in the initiation and amplification of inflammatory responses that lead to intestinal injury. In particular, TNF-α is involved in the expression of inducible nitric oxide synthase (iNOS), which has also been implicated in IBD [17,24]. Furthermore, phase II enzymes, such as NAD(P)H: quinone oxidoreductase 1 (NQO1) and heme oxygenase (HO-1), are regulated by the transcription factor, NF-E2-related factor 2 (Nrf2), which binds to antioxidant response element (ARE), a cis-acting enhancer sequence in the promoter of those enzymes, resulting in activation of gene transcription [25]. Interestingly, a depletion of phase II enzyme expression has been found in mice with DSS-induced colitis [26].

In the present study, we determined the effects of Sunrouge on proinflammatory cytokine levels in colonic mucosa, phase II detoxification enzyme mRNA expression in gastric and small intestine mucosa, and hepatotoxicity in DSS-induced colitis and untreated control mice.

2. Materials and methods

2.1. Animals and diets
Male-specific pathogen-free ICR mice (17–19 g, 4-weeks old) were purchased from Japan SLC (Shizuoka, Japan) and housed five per cage. All mice were fed an AIN-93G diet and given fresh tap water ad libitum, while being kept at 22–26°C with a relative humidity of 55–65% under a 12:12 (06:00–18:00) light/dark cycle for 6 days prior to the experiment. The mice were treated in accordance with the “Guidelines for the Treatment of Experimental Animals” of Kyoto University and the experimental protocol was approved by the Experimentation Committee of the same institution (approval number, #21-42).

Both Yabukita and Sunrouge leaves were collected by the National Institute of Vegetables and Tea Sciences and dried overnight in a warm wind. Yabukita and Sunrouge extracts were then prepared by incubating 1 g of dried leaves with 50 mL of distilled water at 100°C for 10 min with stirring. The extracts were filtrated through No. 2 filter paper and centrifuged, and the supernatant fractions collected. DSS with a molecular weight of 36,000–50,000 was purchased from MP Biochemicals, LLC (Aurora, OH). Mouse IL-1β, IL-6, and TNF-α ELISA kits were obtained from Endogen (Cambridge, MA).

2.2. Experimental design
After the 1-week of quarantine under the conditions described above, the mice were divided into six groups of 10 mice each, as shown in Fig. 1. Mice in two groups (C and D) received 1 mL day⁻¹ of distilled water (daily single dose) via a stomach tube for 3 weeks. Mice in the CY and DY groups were given Yabukita extract (0.13 mg mL⁻¹ day⁻¹ as total catechins), and those in the CS and DS groups received Sunrouge extract (0.16 mg mL⁻¹ day⁻¹ as total catechins). Those administrations were continued for 21 days. One week after beginning those administrations, mice in the D, DY, and DS groups were administered 3% DSS in their drinking water for 14 days.

![Fig. 1. Experimental protocol for determining the effects of Yabukita and Sunrouge on colitis induced by DSS.](image-url)
week thereafter, mice in the D, DY, and DS groups were administered 3% DSS in drinking water for 14 days. The mice had free access to food and drinking water. Body weight, food intake, and the amount of drinking water consumed were recorded every day during the experiment. After 3 weeks of treatment, all mice were euthanized, and then blood and tissue samples were collected for analysis.

2.3. Measurement of anthocyanin concentrations

Anthocyanin concentrations in tea, gastric mucosa, small intestine mucosa, colonic mucosa, brain, and serum samples were determined by high-performance liquid chromatography (HPLC). Tissue samples were homogenized in KCl solution (1.15% w/v, 1:1). The homogenates were diluted with 0.6 mL ice-cold acetone, vortexed (20 min), incubated at −20°C (20 min), and centrifuged (16,060g, 10 min). Supernatants from the acetone extractions were then allowed to evaporate until dry. The residues were reconstituted in water:trifluoroacetic acid (99:5:0.5, 20 µL) and centrifuged (16,060g, 10 min) prior to HPLC analysis. HPLC was performed with an L-7100 Intelligent pump system (Hitachi, Japan). The analytical HPLC was run on an ODS column (4.6 i.d. × 150 mm, YMC, Japan) with monitoring at 520 nm. The flow rate was 0.8 mL min⁻¹ with a linear gradient elution for 60 min with 0–100% solvent B (0.5% CF₃COOH in MeOH, v/v) in solvent A (1.0% CF₃COOH in H₂O, v/v). The anthocyanin concentration was determined as the combined value of cyanidin 3-galactoside (MW: 484.8) and delphinidin 3-galactoside (MW: 500.8), which were detected in the samples. Quantification was done by measuring the peak areas based on calibration plots of the peak areas of the two standards at various concentrations. Malvidin 3-glucoside was used as an internal standard for the recovery calculation.

2.4. Determination of biochemical biomarkers of hepatic injury

Blood samples were centrifuged and the supernatants used as serum samples, which were stored at −80°C until analysis. Serum aspartate transaminase (AST) and alanine transaminase (ALT) were measured using a commercial transaminase C II test kit (Wako Pure Chemicals, Osaka, Japan) according to the protocol described by the manufacturer.

2.5. Assay for cytokine production

Colonic mucosa samples were minced with scissors and homogenized in ice-cold PBS using a homogenizer (Hielscher-UP 50H, Hielscher, Stahndorf, Germany). Tissue homogenates were then centrifuged at 1,900g at 4°C for 15 min to obtain supernatants. Total protein concentrations in the tissue supernatants were determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA), as specified by the manufacturer’s protocol (dilution factor = 50), with γ-globulin as the standard. IL-1β, IL-6, and TNF-α concentrations were determined using ELISA kits in accordance with the manufacturer’s protocol (dilution factor = 5–20). The cytokine level was in the order of pg of IL-1β, IL-6, and TNF-α per mg of protein.

2.6. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from homogenized gastric mucosa, small intestine mucosa, and hepatic samples using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s specifications. The amount and purity of RNA were assessed by spectrophotometry. cDNA was synthesized using 1 µg of total RNA with an RNA PCR Kit (AMV). Thermal cycling was performed with a 7300 real time PCR system using SYBR green PCR mix (Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol. The primer sequences and PCR conditions were as follows: HO-1 (mouse), 240 bp (5'-GGATTTGGGCTGCTGGTTTC-3' and 5'-TCCCCAGACCCGTCTCCAG-3'), at 95°C for 5 Sec and 60°C for 31 Sec; NQO1 (mouse), 290 bp (5'-TCGGAGAACCCTTCTCCAG-3', at 95°C for 30 Sec, 55°C for 30 Sec, and 72°C for 60 Sec; HPRT (mouse), 196 bp (5'-GTAATGATCAGTCAACGGGGAC-3' and 5'-CCGCAAGCCTGCAACCTAACCA-3'), at 95°C for 30 Sec, 55°C for 25 Sec, and 72°C for 45 Sec. The expressions of HO-1 and NQO1 mRNA were normalized to that of HPRT mRNA.

2.7. Statistical analysis

The results are expressed as the mean ± SEM for each group of three to seven mice. After examining equality with Levene’s test, when a significant difference (P < 0.05) was found, each value was converted to a logarithmic value before performing ANOVA. Data were analyzed by two-way ANOVA to determine the effects of DSS treatment (D), Sunrouge administration (S), and their interaction (D × S). Tukey’s test was used to determine the significance of differences resulting from multiple comparisons among the groups, with differences considered to be significant at P < 0.05.

3. Results

3.1. Body weight and survival rate

The effect of DSS treatment (D) was significant in regard to final body weight. The body weights of the D, DY, and DS groups were decreased at 6 days after initiation of 3% DSS oral administration, whereas they did not differ among the remaining three groups of untreated mice (Table 1). The survival rate was lower (50%) in the DY group and higher in the DS (70%) group as compared to the D group (60%). In the C, CY, and CS groups, none of the mice died and no significant effects on body weight were observed following administration of the Yabukita and Sunrouge extracts (Table 1).
3.2. Length of colorectum and organ weight

DSS significantly induced shortening of the colorectum length and spleen hypertrophy. Among the three groups administered DSS, the length of the colorectum was significantly longer (21%) in the DS group than in the DY group (Table 1). The two main effectors (D and S) and their interaction also had significant effects on spleen weight, as that was significantly lower in the DY and DS groups than in the D group (Table 1). There was no significant difference for liver weight among the six groups (Table 1).

3.3. Anthocyanin concentrations in tissues and serum

Anthocyanins were significantly increased in the gastric mucosa, small intestine mucosa, and serum following Sunrouge extract administration, while there was no significant difference between the CS and DS groups (Table 1). Anthocyanins were not detected in brain and colonic mucosal samples from any of the six groups (data not shown.).

3.4. Indicators of liver function and inflammatory cytokine levels in colonic mucosa

Although neither DSS nor Sunrouge had an effect on serum AST level, the two main effectors (D and S) and their interaction had significant effects on serum ALT, as that level was significantly higher in the D and DY groups than in the DS, C, CY, and CS groups. As compared with the D and DY groups, the DSS-induced increase of ALT was attenuated by 40 and 43%, respectively, in the Sunrouge treatment group (DS) (Table 2). Colonic mucosa IL-1β levels were significantly increased with DSS as the main effector (D). However, the IL-1β level in the DS group did not differ in comparison from that of the untreated groups (C, CY, and CS) (Table 2). There were no significant differences in colonic mucosa IL-6 and TNF-α levels among the six groups (Table 2).

3.5. Expressions of NQO1 and HO-1 mRNA

While the expression of NQO1 mRNA in the small intestine mucosa and liver did not differ among the six groups (Figs. 2B and 2C), it was significantly decreased in the gastric mucosa of the D group and tended to be decreased in that of the DY group (Table 1). As for the expression of HO-1 mRNA, two-way ANOVA showed that the main effectors (D and S) had significant effects on the gastric mucosa and liver (Figs. 3A–3C), while their interaction (D x S) was also significant in the liver (Fig. 3C). The expression of HO-1 mRNA in the gastric mucosa was significantly decreased (13%) in the D and DY groups compared with the C groups, but not in the DS group, while similar values were found in the gastric mucosa of the C, CY, and CS groups (Fig. 3A). Moreover, the expression of HO-1 mRNA in the small intestine mucosa was decreased in the DY group as compared with the D and DS groups (Fig. 3B). As for the liver, the expression of HO-1 mRNA was decreased by DSS-treatment,
consistent with previous reports [19–24], mice in the present study treated with DSS developed watery diarrhea, bloody stools, body weight loss, spleen hypertrophy, and shortening of the colorectum. Furthermore, after 2 weeks of treatment with 3% DSS, the survival rate decreased. Sunrouge, an anthocyanin-rich tea, showed inhibitory effects against DSS-induced colitis in mice, and abrogated the lowered survival rate and spleen hypertrophy, whereas Yabukita administration led to deterioration in survival rate of DSS-treated mice. Moreover, the length of the colorectum was shorter in Yabukita- than Sunrouge-treated mice (Table 1). We recently demonstrated that GTPs at dose levels of 0.5 and 1% profoundly enhanced DSS-induced acute colitis in mice, while those at a dose level of 0.01% alleviated colitis [4]. The total catechin dose administered via a stomach tube in the present study was similar to the higher doses in that previous study (0.5–1%), although the mice in our previous experiments would have received catechins from GTPs present in their food [4]. GTPs, especially EGCG, have antioxidative as well as prooxidative properties, and have been shown to activate the oxidative stress-signaling pathway in vitro [27].

The differences between the effects of the Yabukita and Sunrouge extracts may have been related to the concentrations, kinds, and combinations of anthocyanins found in the tea leaves, since the latter contains approximately an 8.4-fold greater quality of anthocyanins than the former [9]. This suggests that anthocyanins play major roles in the anti-inflammatory function of Sunrouge, though we cannot rule out the possibility that other component(s) are also responsible. In the present study, anthocyanins were detected in the gastric mucosa, small intestine mucosa, and serum samples of mice administered Sunrouge extract, while they did not appear in the tissues of mice administered the Yabukita extract (Table 1). Although anthocyanins were not detected in the colons of any mice in the present study, some metabolites of anthocyanins, such as anthocyanidins, phenolic acids, and aldehydes, have been detected in the distal gastro-intestinal tract, dependent on pH and microbial metabolism [28]. Anthocyanins are efficiently absorbed from the stomach and appear rapidly in blood, then show antioxidative and antiinflammatory effects [10–14]. However, Keppler and Humphf suggested that the major antioxidative properties would be preserved by the more stable phenolic degradation products [29]. In the present study, anthocyanins and their metabolites from Sunrouge might have played a key role in alleviation of colitis in DSS-treated mice. Nabae et al. reported a no-observed-adverse-effect-level (NOAEL) of 5.0% for purple corn color (PCC) from dietary sources in both male (3,542 mg kg$^{-1}$ of body weight per day) and female (3,849 mg kg$^{-1}$ of body weight per day) F344 rats after a 90-day feeding experiment [30]. Because PCC contains 26.4% anthocyanins, the NOAEL of the anthocyanins

while it was significantly lower in the DY group than in the D group (Fig. 3C).

4. Discussion

Consistent with previous reports [19–24], mice in the present study treated with DSS developed watery diarrhea, bloody stools, body weight loss, spleen hypertrophy, and shortening of the colorectum. Furthermore, after 2 weeks of treatment with 3% DSS, the survival rate decreased. Sunrouge, an anthocyanin-rich tea, showed inhibitory effects against DSS-induced colitis in mice, and abrogated the lowered survival rate and spleen hypertrophy, whereas Yabukita administration led to deterioration in survival rate of DSS-treated mice. Moreover, the length of the colorectum was shorter in Yabukita- than Sunrouge-treated mice (Table 1). We recently demonstrated that GTPs at dose levels of 0.5 and 1% profoundly enhanced DSS-induced acute colitis in mice, while those at a dose level of 0.01% alleviated colitis [4]. The total catechin dose administered via a stomach tube in the present study was similar to the higher doses in that previous study (0.5–1%), although the mice in our previous experiments would have received catechins from GTPs present in their food [4]. GTPs, especially EGCG, have antioxidative as well as prooxidative properties, and have been shown to activate the oxidative stress-signaling pathway in vitro [27].

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corresponds to 1,016 mg kg⁻¹ of body weight per day for females [30]. In the present study, even though the anthocyanin intake was 8.4-fold higher in the mice administered Sunrouge as compared to those that received Yabukita, the Sunrouge treated mice received much lower levels (i.e., <1,016 mg kg⁻¹ day⁻¹) of anthocyanins (200–300 mg kg⁻¹.

**Fig. 2.** Expressions of NAD(P)H quinone oxidoreductase (NQO1) mRNA in gastric mucosa, (B) small intestine and (C) liver. Values (rate of C) are shown as the mean ± SEM (n = 4–6). Bars without a common letter differ, P < 0.05.

**Fig. 3.** Expressions of heme oxygenase (HO-1) mRNA in (A) gastric mucosa, small intestine and (C) liver. Values (rate of C) are shown as mean ± SEM (n = 4–6). Bars without a common letter differ, P < 0.05.
In addition, our experimental period was shorter (3 weeks) than that of Naba et al. (90 days) [30]. Therefore, it is possible that the high level of anthocyanins in Sunrouge ameliorated the effects of DSS without any adverse effects, which GTPs at dose levels of 0.5 and 1% profoundly enhance DSS-induced acute colitis in mice.

We investigated two indicators of liver function in the present study. It is well known that leakage of cellular enzymes into plasma is a sign of hepatic injury in association with liver damage. AST and ALT are reliable markers of liver function, and increased levels of serum enzymes such as AST and ALT indicate increased permeability, damage, and/or necrosis of hepatocytes [31]. In the present study, serum AST levels did not differ between the DSS-treated and untreated mice. Although the serum ALT level was increased by DSS administration, Sunrouge had an inhibitory effect on that increase in the DSS-treated mice (Table 2).

A previous study reported that a decrease in plasma ALT level was observed in mice fed an anthocyanin-rich diet, and noted that the protective effect against oxidative damage associated with liver injury might be linked to the antioxidative and antiinflammatory efficacy of anthocyanins [32]. IL-1β, IL-6, and TNF-α contribute to increased severity of DSS-induced colitis [20–23]. In the present study, the level of IL-1β in colonic mucosa was increased by DSS administration (Table 2), confirming previous reports [4,20,21]. IL-1β has been shown to play a pivotal role in the early stage of the pathogenesis of IBD, resulting in upregulation of inflammatory responses [33]. We recently showed that GTPs at dose levels of 0.5 and 1% profoundly enhanced DSS-induced acute colitis in mice, presumably through increased IL-1β expression [4]. In that study, IL-1β was significantly enhanced by administration of 0.5 and 1% GTP, whereas 0.1% GTP suppressed IL-1β expression. In the present study, sunrouge did not show any effects on IL-1β expression, while Sunrouge tended to decrease it in the colonic mucosa with colitis (Table 2).

The expressions of phase II enzymes are regulated by the transcription factor Nrf2, which binds to ARE, resulting in activation of gene transcription [25]. Numerous phase II proteins are regulated as a group in a coordinated manner through the Nrf2 ARE pathway. Those include enzymes such as γ-glutamylcysteine ligase and glutathione synthetase, which regulate the key steps in glutathione biosynthesis, as well as HO-1, which catalyzes the breakdown of heme proteins into iron, carbon monoxide (a vasodilator), biliverdin (further reduced to the antioxidant bilirubin), NAD(P)H:quinone oxidoreductase 1 [NQO1] (reducing agent that plays role in antioxidant defences with cofactor NADH or NADPH), and thioredoxin (key cellular antioxidant), among others [34–36]. Depletion of phase II xenobiotic metabolizing enzyme activity is found in DSS-induced colitis [26]. Yabukita extract in the present study did not affect the tendency of decreased NQO1 expression due to DSS treatment, whereas Sunrouge extract showed a much higher tendency to ameliorate this effect (Figs. 2A–2C). DSS treatment reduced the expression of HO-1 mRNA in the gastric mucosa (Figs. 3A). In the gastric mucosa, Sunrouge extract showed an inhibitory effect on decreased expression of HO-1 mRNA induced by DSS treatment (Fig. 3A). In the small intestine mucosa and liver, the expression was relatively worse in DSS-treated mice administered Yabukita extract as compared with mice that did not (Figs. 3B and 3C). Furthermore, ingestion of Yabukita, which has GTPs, resulted in decreased expression of HO-1 mRNA, while that of Sunrouge, which has both GTPs and anthocyanins, had inhibitory effects on phase II enzymes (Figs. 3A–3C). From a biological perspective, there are key advantages for a cell to induce phase II proteins to fight oxidative stress. Phase II proteins are induced by a variety of compounds for their detoxification and degradation, including prooxidant xenobiotics, chemically reactive carcinogens, chemopreventive agents such as sulforaphane and oltipraz, antioxidants, and specific bioflavonoids [37–40]. Several investigators have suggested that both EGCG and anthocyanins induce phase II enzymes, HO-1, and NQO1 [38–40]. In the present study, anthocyanins in Sunrouge extract might have ameliorated the effects of the expression of phase II enzyme mRNA in DSS-treated mice while the GTPs may have had no or an adverse effect on this expression. However, it should be noted that the adverse and beneficial effects of Yabukita and Sunrouge, respectively, were only revealed in DSS-treated mice in the present study.

In conclusion, we showed that Yabukita and Sunrouge extracts had different effects on the development of DSS-induced colitis in mice. This is the first study to report that Sunrouge, which is rich in anthocyanins, may have inhibitory effects on typical DSS-induced symptoms of hepatotoxicity and increased colonic IL-1β, while it also might induce phase II enzyme expression in DSS-treated mice.

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References


