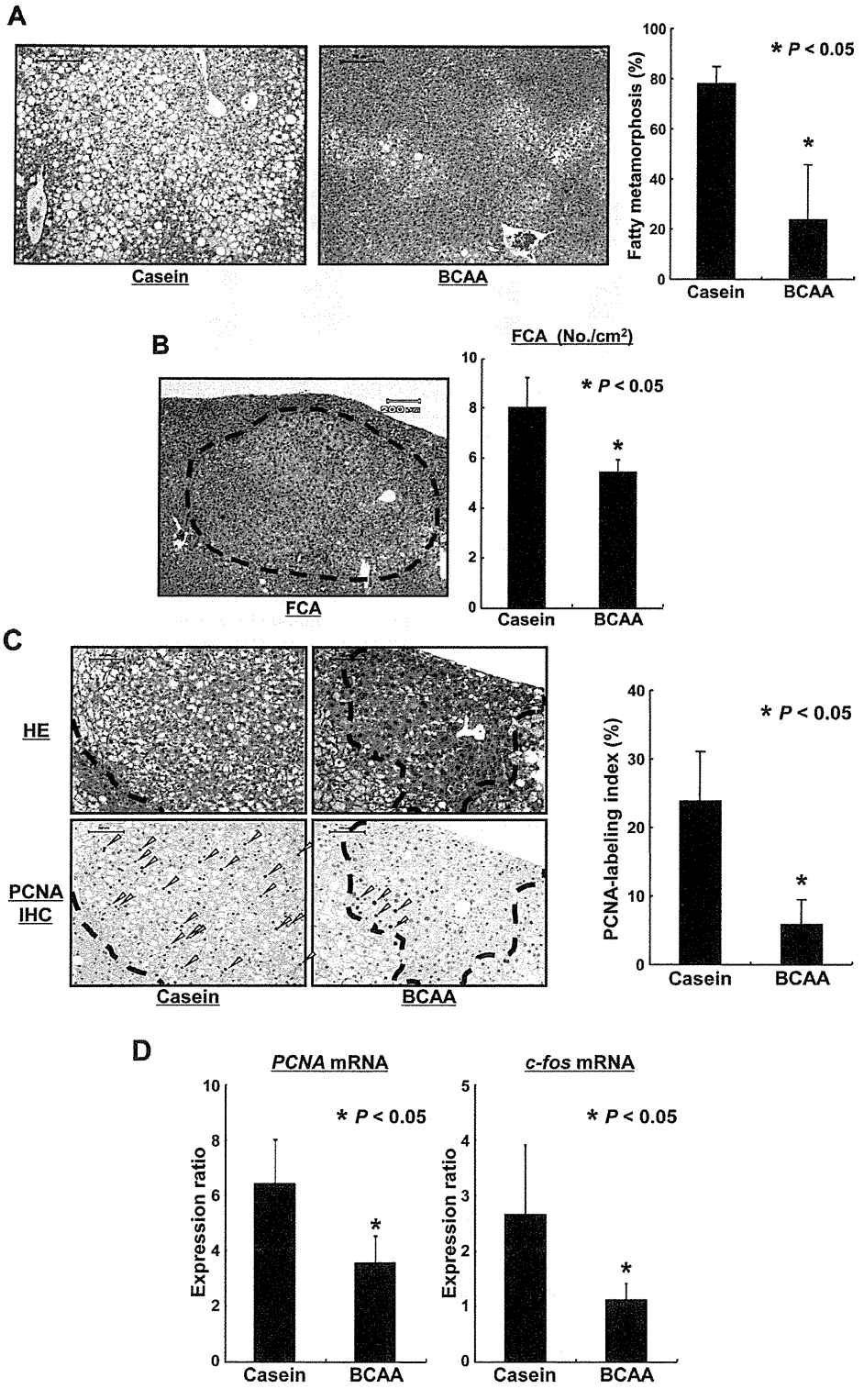

Fig. 1. Effects of BCAA supplementation on the hepatic steatosis, development of FCA, and on the expression of PCNA and c-*fos* mRNA in the livers of the *db/db* mice. (A) Histopatology (H&E staining) and a morphometric analysis of fatty metamorphosis in the liver of the casein-supplemented and the BCAA-supplemented *db/db* mice. (B) A representative photograph of FCA that spontaneously developed in the *db/db* mice (H&E staining) and the average number of FCA in the casein-supplemented and the BCAA-supplemented groups. (C) Representative photographs of H&E staining and the PCNA-immunohistochemical analysis of the FCA developed in the livers of the casein-supplemented and the BCAA-supplemented mice (left panels). The PCNA-labeling indices of the FCA developed in the livers of each group were determined by counting the PCNA-positive nuclei (arrowheads) in the FCA (right panel). * $P < 0.05$ versus the casein-supplemented group. (D) The expression levels of PCNA and c-*fos* mRNA in the liver of the casein-supplemented and the BCAA-supplemented mice were examined by quantitative real-time RT–PCR using specific primers. The values are expressed as the mean ± the SEM. *$P < 0.05$ versus the casein-supplemented group.

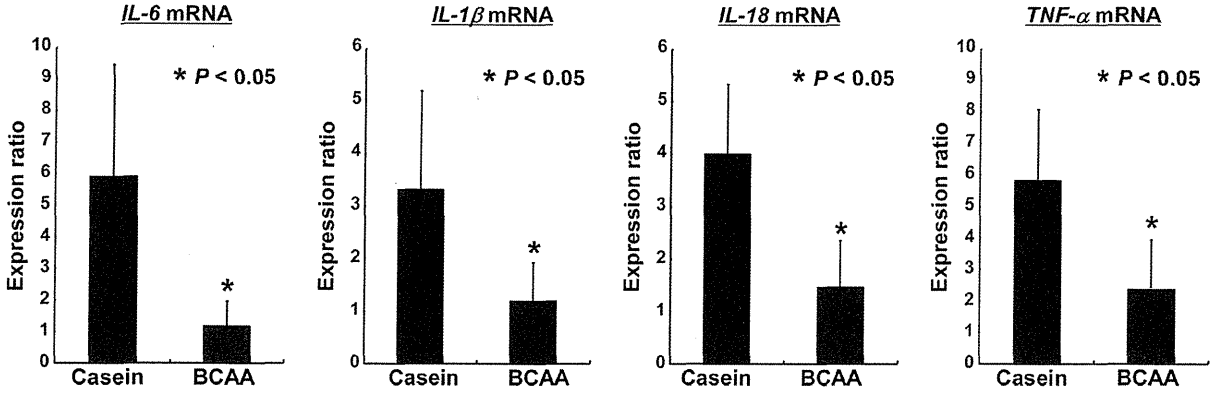

Fig. 2. Effect of BCAA supplementation on the expression of IL-6, IL-1β, IL-18 and TNF-α mRNA in the livers of the *db/db* mice. The expression levels of IL-6, IL-1β, IL-18 and TNF-α mRNA in the liver of the casein-supplemented and the BCAA-supplemented mice were examined by quantitative real-time RT–PCR using specific primers. The values are expressed as the mean ± the SEM. *$P < 0.05$ versus the casein-supplemented group.

findings suggesting any toxicity of BCAA to major organs, including the liver, kidney and spleen.

Effects of BCAA supplementation on the spontaneous development of FCA, the proliferation activity in FCA and the expression levels of PCNA and c-fos messenger RNA in the livers of the db/db mice

At sacrifice, FCA developed in the liver of all experimental mice regardless of the treatment. It was found that supplementation with BCAA significantly decreased the number of FCA when compared with casein supplementation ($P < 0.05$, Figure 1B). An immunohistochemical analysis to detect PCNA showed the mean PCNA-labeling index for FCA in the BCAA-supplemented mice to be significantly lower than that in the casein-supplemented mice ($P < 0.05$, Figure 1C). In the whole liver, BCAA supplementation also inhibited the expression levels of PCNA and c-*fos* messenger RNA (mRNA) in comparison with casein supplementation

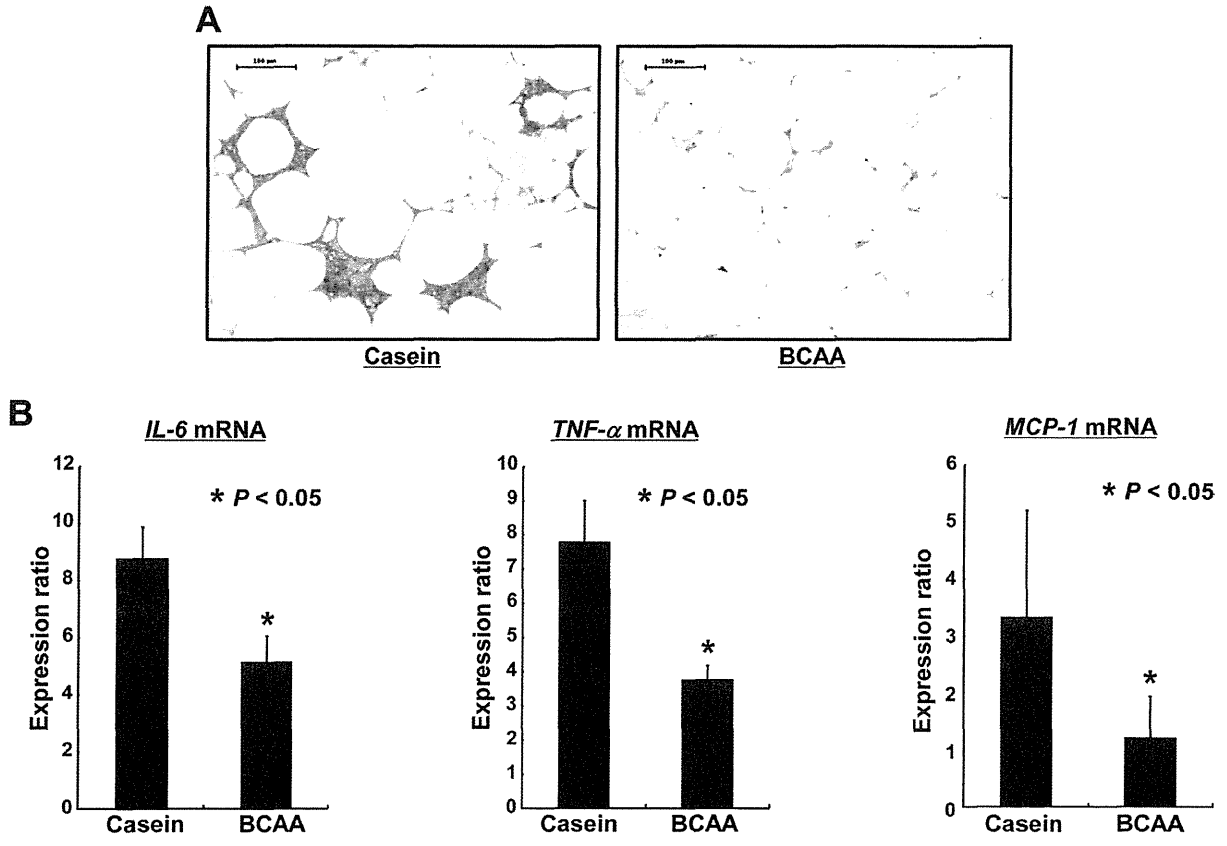

Fig. 3. Effect of BCAA supplementation on macrophage infiltration and the expression of IL-6, TNF-α and MCP-1 mRNA in the WAT of the *db/db* mice. (A) The F4/80 immunohistochemical analyses were performed in the periorchis WAT of the casein-supplemented and the BCAA-supplemented mice to show macrophage infiltration. (B) The expression levels of IL-6, TNF-α and MCP-1 mRNA in the periorchis WAT of the casein-supplemented and the BCAA-supplemented mice were examined by quantitative real-time RT–PCR using specific primers. The values are expressed as the mean ± the SEM. *$P < 0.05$ versus the casein-supplemented group.

($P < 0.05$, Figure 1D). These findings suggest that BCAA supplementation prevents the development of FCA, at least in part, by reducing cell proliferation.

Effects of BCAA supplementation on the expression levels of IL-6, IL-1β, IL-18 and TNF-α mRNA in the livers of the db/db mice

Chronic inflammation induced by the excessive production of storage lipids plays a role in obesity-related liver carcinogenesis (2,6–10). Therefore, the effects of BCAA supplementation on the expression levels of proinflammatory cytokines IL-6, IL-1β, IL-18 and TNF-α mRNA, which are central mediators of chronic inflammatory diseases (2,6–10), in the livers of the *db/db* mice were determined. Quantitative real-time RT–PCR revealed that in comparison with the casein-supplemented mice, the experimental mice showed significantly decreased expression levels of mRNA in the liver following BCAA supplementation ($P < 0.05$, Figure 2). These findings suggest that BCAA supplementation attenuates chronic inflammation in the livers of obese and diabetic *db/db* mice.

Effects of BCAA supplementation on macrophage infiltration and the expression level of IL-6, TNF-α and MCP-1 mRNA in the WAT of the db/db mice

Macrophages play important roles in inflammation in obese adipose tissue (21,22). Therefore, whether BCAA supplementation attenuates chronic inflammation or inhibits increased infiltration of macrophages in WAT was examined. Immunohistochemical analysis performed with an antibody to F4/80 revealed the presence of apparent macrophage infiltration in the periorchis WAT of the casein-supplemented *db/db* mice; however, the infiltration was markedly inhibited by BCAA supplementation (Figure 3A). The expression levels of IL-6 and TNF-α mRNA in the WAT were also reduced by BCAA supplementation. Additionally, supplementation with BCAA significantly inhibited the expression of MCP-1 mRNA ($P < 0.05$, Figure 3B), which plays a role in the recruitment of macrophages into obese adipose tissue (30,31). These findings suggest that inhibition of macrophage infiltration and subsequent attenuation of chronic inflammation in WAT by BCAA supplementation are, at least in part, associated with the suppression of MCP-1 expression.

Effects of BCAA supplementation on adipocyte size and expression levels of PPAR-α, PPAR-γ, and adiponectin mRNA in the WAT of the db/db mice

The induction of inflammation in obese adipose tissue is associated with increased adipocyte size (21,22). Therefore, whether BCAA supplementation alters the histology of WAT was next examined. Histological analysis showed that in addition to the inhibition of macrophage infiltration, BCAA supplementation reduced the size of adipocyte (Figure 4A). The average adipocyte size observed in the BCAA-supplemented mice was significantly smaller than that observed in the casein-supplemented mice ($P < 0.05$, Figure 4B).

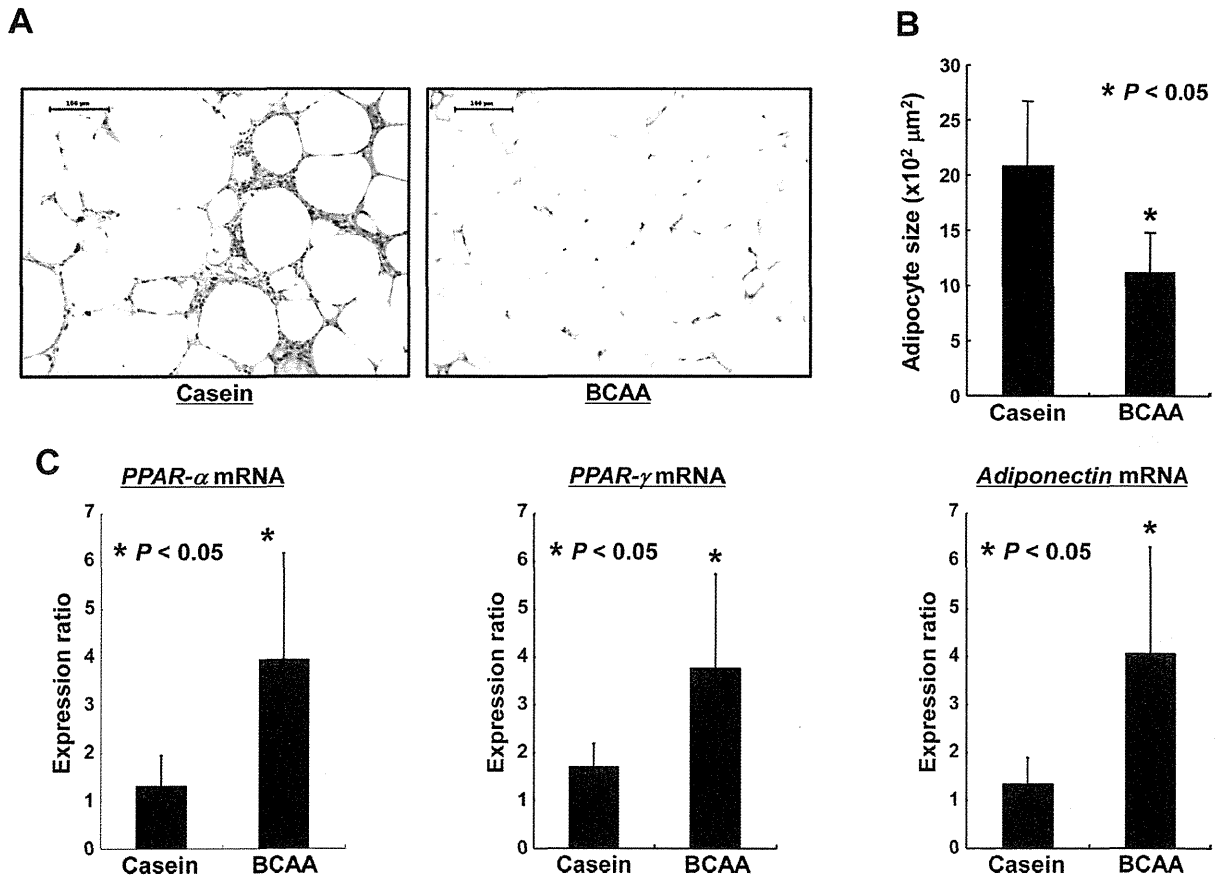

Fig. 4. Effect of BCAA supplementation on adipocyte size and the expression of PPAR-α, PPAR-γ and adiponectin mRNA in the WAT of the *db/db* mice. (A) The histopathology of the periorchis WAT of the casein-supplemented and the BCAA-supplemented mice (H&E staining). (B) The H&E staining images of the adipose tissues were analyzed using a fluorescence microscope BZ-9000, and adipocyte size was measured using a BZ-Analyzer-II. (C) The expression levels of PPAR-α, PPAR-γ and adiponectin mRNA in the periorchis WAT of the casein-supplemented and the BCAA-supplemented mice were examined by quantitative real-time RT–PCR using specific primers. The values are expressed as the mean ± the SEM. *$P < 0.05$ versus the casein-supplemented group.

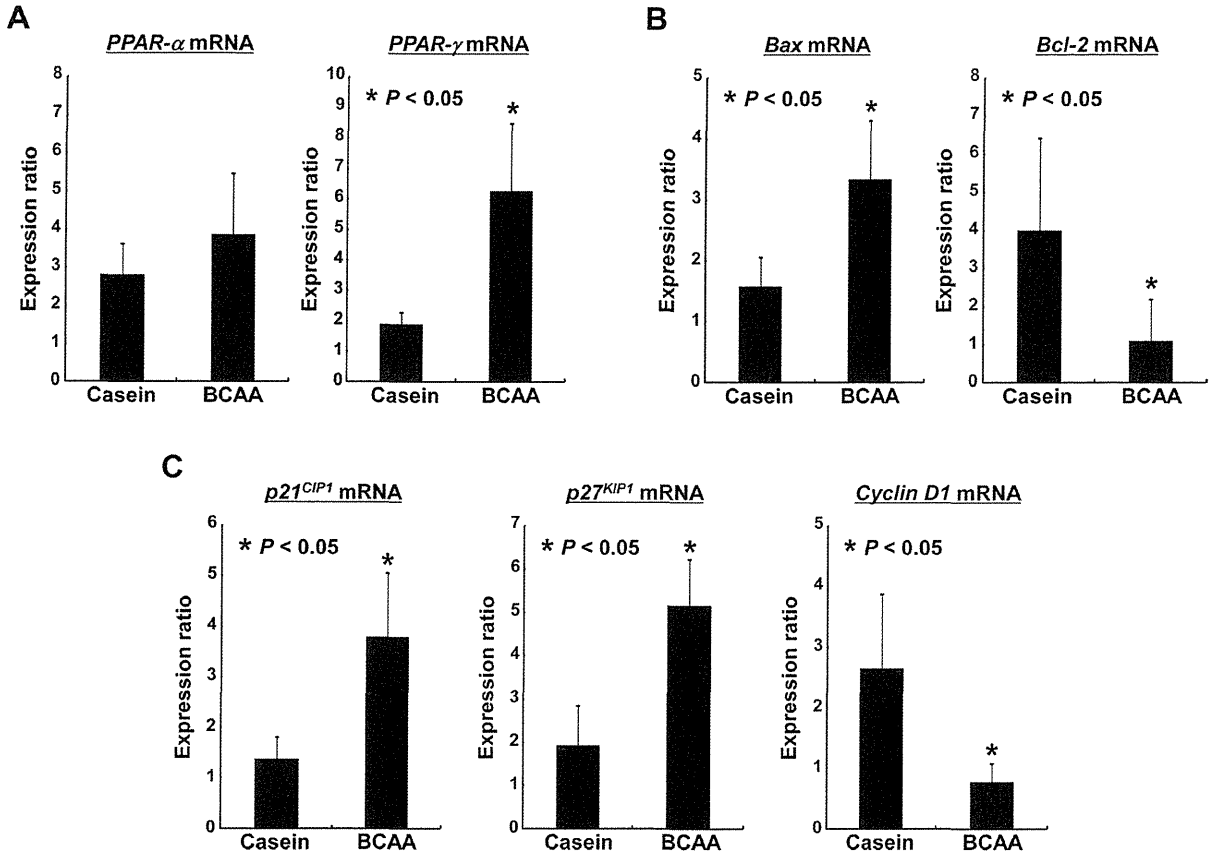

Fig. 5. Effect of BCAA supplementation on the expression of PPAR-α, PPAR-γ, Bax, Bcl-2, $p21^{CIP1}$, $p27^{KIP1}$ and cyclin D1 mRNA in the livers of the *db/db* mice. The expression levels of (A) PPAR-α and PPAR-γ, (B) Bax and Bcl-2 and (C) $p21^{CIP1}$, $p27^{KIP1}$ and cyclin D1 mRNA in the liver of the casein-supplemented and the BCAA-supplemented mice were examined using quantitative real-time RT–PCR with specific primers. The values are expressed as the mean ± SEM. *$P < 0.05$ versus the casein-supplemented group.

Moreover, BCAA supplementation increased the expression of PPAR-α mRNA, which can be a key regulator of inflammatory signaling (23,24), in the WAT of the *db/db* mice. Furthermore, the expression levels of PPAR-γ, a master regulator of adipocyte differentiation, and its downstream adiponectin mRNA, which also possesses the ability to suppress proinflammatory signaling (24,25), in the WAT were both significantly upregulated by BCAA supplementation ($P < 0.05$, Figure 4C).

Effects of BCAA supplementation on the expression levels of PPAR-α, PPAR-γ, Bax, Bcl-2, $p21^{CIP1}$, $p27^{KIP1}$ and cyclin D1 mRNA in the livers of the db/db mice

Recent studies have revealed the activation of PPAR-γ to exert a beneficial effect against HCC by inducing apoptosis and cell-cycle arrest (32,33). Therefore, in addition to the WAT (Figure 4C), whether BCAA supplementation also increases the expression levels of PPAR-γ in the liver was next examined. The expression levels of PPAR-γ mRNA in the liver were found to be significantly increased by BCAA supplementation ($P < 0.05$), whereas this agent did not alter the levels of PPAR-α mRNA (Figure 5A). Supplementation with BCAA increased the levels of Bax mRNA, which accelerates apoptosis, and decreased the levels of Bcl-2 mRNA, an anti-apoptotic member of the Bcl-2 family, in the livers of the experimental mice (Figure 5B, $P < 0.05$). There were also significant increases in the expression levels of $p21^{CIP1}$ and $p27^{KIP1}$ mRNA and decreases in the levels of cyclin D1 mRNA in the livers of the mice supplemented with BCAA (Figure 5C, $P < 0.05$).

Discussion

Obesity, which is implicated in the development of NAFLD and NASH, has been shown to increase the risk of developing HCC (1–5). The present study, a NAFLD mice model in which obesity and severe steatosis were developed, clearly indicates that dietary supplementation with BCAA effectively prevents the spontaneous development of liver preneoplastic lesions in *db/db* mice through the inhibition of cell proliferation. These findings are consistent with our recent report that BCAA supplementation suppresses the chemically induced liver tumorigenesis in obese mice by improving insulin resistance (17). We considered that the results of the present study showed an equivalent significance to those of the previous experiment (17) because NAFLD that has not yet progressed to NASH can induce hepatocyte proliferation and hepatic hyperplasia, both of which initiate the hepatic neoplastic process in obesity (34). Therefore, targeting NAFLD, a hyperproliferative field, through intervention using specific agents such as BCAA supplementation might be an effective strategy for preventing obesity-related liver carcinogenesis.

In various obesity-related metabolic disorders, substantial evidence has shown that chronic inflammation caused by obesity contributes to the progression of NAFLD to NASH and finally to HCC (2,6–10). Hepatic steatosis, which is a source of inflammation, also promotes the development of HCC (8,9). Therefore, reduction of lipid accumulation and attenuation of chronic inflammation in the liver achieved by BCAA supplementation play a critical role in the suppression of the spontaneous development of hepatic neoplastic lesions in obese mice. The inhibition of the expression of IL-6 and TNF-α by BCAA supplementation is particularly important in the suppression of the

spontaneous development of hepatic neoplastic lesions because increases in these proinflammatory cytokines, which are accompanied by lipid accumulation in the liver, are critically involved in obesity-related liver carcinogenesis (2,6–10). The preventive effects of obesity-related liver tumorigenesis by targeting IL-6 and TNF-α expression and liver steatosis are also demonstrated in other rodent studies (28,35,36). In addition, the alleviation of hepatic steatosis with BCAA supplementation, which might be associated with the effects of improving insulin resistance (13), is consistent with previous reports (17,20).

In addition to the benefits observed in the liver, the present study also showed that BCAA supplementation significantly attenuates chronic inflammation in the WAT of *db/db* mice. Macrophage infiltration into WAT, which is accompanied by IL-6 and TNF-α production, is an early contributing event for the development of chronic low-grade systemic inflammation (21,22). MCP-1 plays a crucial role in the recruitment of macrophages into obese adipose tissue (30,31). MCP-1 is also capable of inducing steatosis in hepatocytes, indicating that secretion of this chemokine by adipose tissue may induce steatosis not only by recruiting macrophages but also by acting directly on hepatocytes (37). In addition, upregulation of IL-6, TNF-α and MCP-1 in WAT is critically involved in the induction of systemic insulin resistance (21,22), which is a key factor for accelerating obesity-related liver carcinogenesis (2,6–10). Therefore, the inhibition of enhanced adipose tissue inflammation, that is increased macrophage infiltration and IL-6, TNF-α and MCP-1 expression, by BCAA supplementation is important in preventing the development of steatosis and subsequent liver tumorigenesis in obese mice.

The present study demonstrated that adipocyte size in BCAA-supplemented mice is much smaller than that in control mice. This finding might be associated with the effects of BCAA on the induction of PPAR-α and PPAR-γ in WAT because activation of these nuclear receptors significantly prevents adipocyte hypertrophy (24,38). An increase in the number of small adipocytes induces adiponectin and its receptors, which downregulates the production of IL-6 and TNF-α, thereby reducing obesity-related inflammation in adipose tissue (24,25). A lack of adiponectin enhances the progression of hepatic steatosis and tumor formation in a mice model of NASH (39), whereas this adipokine alleviates hepatic steatosis by decreasing TNF-α production (40). Moreover, the induction of adiponectin plays a role in the suppression of chemically induced liver tumorigenesis in obese mice (28). Therefore, in the present study, the effects of BCAA on the upregulation of PPAR-α, PPAR-γ and adiponectin achieved by inhibiting adipocyte hypertrophy may contribute to preventing obesity-related liver tumorigenesis.

In addition to the WAT, the present study also showed the first evidence that BCAA supplementation increases the mRNA level of PPAR-γ, but not that of PPAR-α, in the livers of obese mice. The precise mechanisms underlying the upregulation of the expression of PPAR-γ in the liver by BCAA have not yet been clarified. However, these findings are significant when considering the prevention of liver carcinogenesis because PPAR-γ is regarded to be an antitumorigenic factor in HCC, whereas the role of PPAR-α in HCC development is contradictory (32,33,41). The overexpression of PPAR-γ suppresses the growth of HCC cells by reducing cell proliferation and inducing apoptosis (32). The activation of PPAR-γ by its ligand also inhibits the proliferation of HCC cells by upregulating the $p21^{CIP1}$ and $p27^{KIP1}$ expression, which thus leads to the G_1 arrest of the cell cycle (33). These reports (32,33), together with the results of the present study showing that BCAA supplementation increases the expression of PPAR-γ, Bax, $p21^{CIP1}$ and $p27^{KIP1}$ mRNA and decreases the expression of Bcl-2 and cyclin D1 mRNA, suggest that the induction of apoptosis and regulation of cell-cycle progression induced by BCAA via the upregulation of PPAR-γ in the liver may also help to inhibit the development of FCA.

Finally, it should be noted again that improved insulin resistance achieved from BCAA supplementation, which has been demonstrated in several basic and clinical studies (13,16), is critical to suppress the development of neoplasms in both the liver and the colon of obese mice (17,18). Because chronic inflammation occurring in WAT plays a role in systemic insulin resistance (30,31), BCAA supplementation might prevent the spontaneous development of hepatic preneoplastic lesions via the attenuation of adipocyte inflammation and the subsequent improvement of insulin resistance. These findings suggest that in addition to the liver, as shown in the present and previous studies (17,42), WAT might be a critical target for BCAA to exert chemopreventive properties in obesity-related liver carcinogenesis.

In conclusion, supplementation with BCAA may be an effective strategy for the chemoprevention of HCC, especially in obese patients who are at an increased risk of developing HCC. The results of the present study further strengthen our hypothesis that targeting obesity-induced pathologic conditions, such as chronic inflammation, might be effective for preventing liver carcinogenesis in obese individuals (11).

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Research Article

Dietary Crocin Inhibits Colitis and Colitis-Associated Colorectal Carcinogenesis in Male ICR Mice

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A natural carotenoid crocin is contained in saffron and gardenia flowers (crocuses and gardenias) and is used as a food colorant. This study reports the potential inhibitory effects of crocin against inflammation-associated mouse colon carcinogenesis and chemically induced colitis in male ICR mice. In the first experiment, dietary crocin significantly inhibited the development of colonic adenocarcinomas induced by azoxymethane (AOM) and dextran sodium sulfate (DSS) in mice by week 18. Crocin feeding also suppressed the proliferation and immunohistochemical expression of nuclear factor- (NF-) κB but increased the NF-E2-related factor 2 (Nrf2) expression, in adenocarcinoma cells. In the second experiment, dietary feeding with crocin for 4 weeks was able to inhibit DSS-induced colitis and decrease the mRNA expression of tumor necrosis factor α, interleukin- (IL-) 1β, IL-6, interferon γ, NF-κB, cyclooxygenase-2, and inducible nitric oxide synthase in the colorectal mucosa and increased the Nrf2 mRNA expression. Our results suggest that dietary crocin suppresses chemically induced colitis and colitis-related colon carcinogenesis in mice, at least partly by inhibiting inflammation and the mRNA expression of certain proinflammatory cytokines and inducible inflammatory enzymes. Therefore, crocin is a candidate for the prevention of colitis and inflammation-associated colon carcinogenesis.

1. Introduction

A perennial stemless herb *Crocus sativus* L. (Iridaceae), commonly known as saffron, is widely cultivated worldwide, especially in Iran, India, Greece, Morocco, Spain and China. Saffron has several biological activities and is used in folk medicine [1, 2]. The major biologically active ingredient in saffron is known to be crocin, which is an ester glycoside of crocetin.

The other typical components are picrocrocin and safranal, which are related to the flavor of the herb [1–4]. Pharmacological studies have reported that saffron extracts and/or the active constituents have properties that improve learning and memory [5, 6], as well as anticonvulsant [7], antidepressant [8], antiinflammatory [9, 10], and antitumor effects [1, 2]. Free radical scavenging, antioxidant activity, and the promotion of the diffusion of oxygen in different tissues were also reported for saffron extracts or their bioactive constituents [11–13]. Other biological effects of saffron and its constituents include the induction of apoptosis [14, 15], antihyperlipidemic effects [16], immuno modulation [17], and anti-neurodegenerative effects [18–20]. Our previous

studies on saffron and/or crocetin glycosides indicated the prevention of skin tumor promotion in mice [21] and the decrease in the proliferation of human colorectal cancer (CRC) cells [22].

With regard to the effects of saffron and its active ingredients on carcinogenesis, many *in vitro* studies have demonstrated that extracts of saffron and certain components of the herb are able to inhibit the growth of several types of human cancer cells [14, 23–25], including CRC cells, as we reported in a previous study [22]. However, there have so far been few *in vivo* studies conducted to demonstrate the anticancer effects of saffron and its constituents [26–30].

Patients with ulcerative colitis (UC) and Crohn's disease, two major types of inflammatory bowel disease (IBD), are at high risk of developing CRC [31–33]. Unlike sporadic CRC, the CRC in UC patients arises from focal or multifocal dysplastic crypts that are present in areas of inflammation [32]. Growing evidence supports a significant role for several cytokines produced by epithelial and immune cells, in the pathogenesis of IBD-related CRC [34]. To investigate the pathobiology of IBD-related CRC, we developed a colitis-associated and two-stage mouse CRC model [35]. Using this model that mimics human CRC in the inflamed colon [35, 36], we have reported several synthetic and natural compounds which effectively suppressed colitis-associated colon carcinogenesis [37–40].

Nuclear factor (NF-) κB is a well-established regulator of genes encoding cytokines, cytokine receptors, and cell adhesion molecules that drive immune and inflammatory responses [41]. Recently, NF-κB activation has also been connected with multiple aspects of oncogenesis and NF-κB is one of the potential targets of anticancer agents [42]. NF-κB regulates the expression of several genes, such as cyclooxygenase- (COX-) 2, inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF-) α, interleukin (IL-) 1β, cell surface adhesion molecules, and antiapoptotic proteins, which are involved in tumor initiation, promotion, and metastasis [43]. Therefore, NF-κB has become one of the most important targets for cancer chemoprevention [44]. Since crocetin has been reported to inhibit chemically-induced colitis in mice by down regulation of NF-κB [10], crocin, a glycoside of crocetin (see Figure 1), may also inhibit colitis and colitis-associated CRC by affecting inflammatory phenomena. The production of interferon (IFN-) γ has been reported in human IBD and IBD-related CRC [45] and experimental UC-associated CRC [46]. A protective role for NF-E2-related factor 2 (Nrf2) against the toxicity of xenobiotics has been suggested [47, 48], making it one of the targets for cancer chemoprevention [47–50]. Therefore, these factors can be used to assess the effects of molecules against inflammation and cancer.

The aim of this study was to investigate the possible inhibitory effects of crocin isolated from saffron against colitis-associated colon carcinogenesis using an AOM/DSS mouse model. This study contained two different experiments. In the first experiment, we evaluated the effects of three different concentrations (50, 100, and 200 ppm) of crocin in the diet on colitis-associated colorectal carcinogenesis in mice. In addition, the immunohistochemical expression of NF-κB and Nrf2 [49–51] in adenocarcinoma cells was examined. The second experiment was conducted to determine the effects of these concentrations of crocin on DSS-induced colitis and the mRNA expression of NF-κB, IFN-γ, TNF-α, IL-1β, IL-6, COX-2, iNOS, and Nrf2 in mice, since the elevated mRNA expression of pro-inflammatory cytokines and inducible inflammatory enzymes caused by inflammatory stimuli plays a significant role in carcinogenesis [38].

2. Materials and Methods

2.1. Animals, Chemicals, and Diet. Male Crj: CD-1 (ICR) mice (Charles River Japan, Inc., Tokyo) aged 4 weeks were used in these studies. They were maintained in the BioGate Inst., Co., Ltd., (Yamagata City, Gifu 501-2123, Japan), according to the Institutional Animal Care Guideline. All animals were housed in plastic cages (4 or 5 mice/cage) with free access to drinking water and a basal diet, CE-2 (CLEA Japan Inc., Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), light (12/12 h high/dark cycle), and temperature (23 ± 2°C). After arrival, animals were quarantined for the first seven days and randomized by body weights into the experimental and control groups. AOM and DSS (with a molecular weight of 36,000–50,000, Cat. no. 160110) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and MP Biomedicals, LLC (Aurora, OH, USA), respectively. DSS was dissolved in water at a concentration of 1.5% (w/v) to induce colitis. The experiments and study designs were approved by the Institutional Committee. All handling and procedures were carried out in accordance with the appropriate Institutional Animal Care Guidelines.

2.2. Purification of Crocin. Crocin (purity ≥ 96% by HPLC) was purified from the stigmas of *C. sativus* as reported previously [52]. Briefly, the air- and shade-dried saffron (500 g) was extracted with 50% EtOH (2.0 l × 3 times) at 40°C under sonication. The combined extracts were concentrated to produce a dark-brown syrup (280 g). A part of the obtained crude extract (105 g) was suspended in water (500 mL), then partitioned with CH_2Cl_2 (500 mL × 3), and the water layer was subjected to a Diaion HP-20 column elution with a stepwise gradient of MeOH-H_2O (25, 50, 75, and 100% MeOH; v/v) to afford four fractions (fr. 1.1–1.4). Fr. 1.3 (12.5 g) was subjected to a reversed-phase column with MeOH-H_2O (3 : 4, v/v) to give five fractions (fr. 4.1–4.4). Fr. 4.2 (3.2 g) was then repeatedly separated over a reversed-phase column with MeOH-H_2O (1 : 1, v/v) to yield crocin (2100 mg). The structure of crocin was confirmed by NMR and mass spectrometry.

2.3. Experiment 1 (18-Week Study). A total of 100 male ICR mice were divided into five experimental and control groups (Figure 2(a)). Mice in groups 1–4 were given a single intraperitoneal injection of AOM (10 mg/kg body weight). Starting one week after the injection, they received 1.5% DSS in their drinking water for seven days. Subsequently, groups 1 (n = 20), 2 (n = 20), 3 (n = 20), and 4 (n = 20)

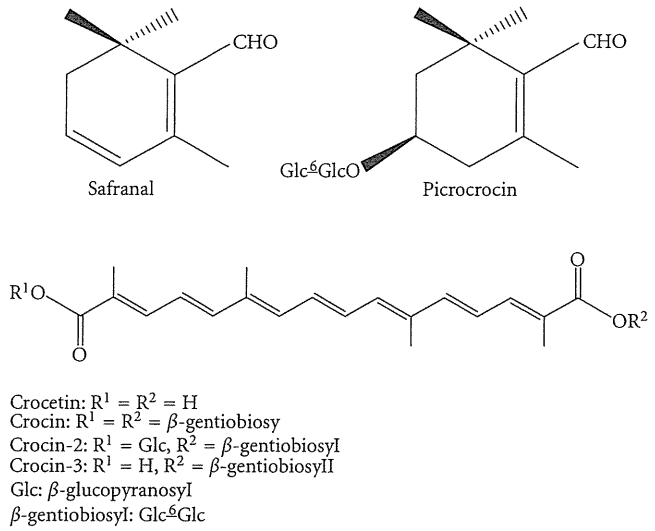


FIGURE 1: Structures of the principle constituents (crocetin, crocetin-diglycoside, crocetin-triglycoside, crocin, picrocrocin, and safranal) of saffron.

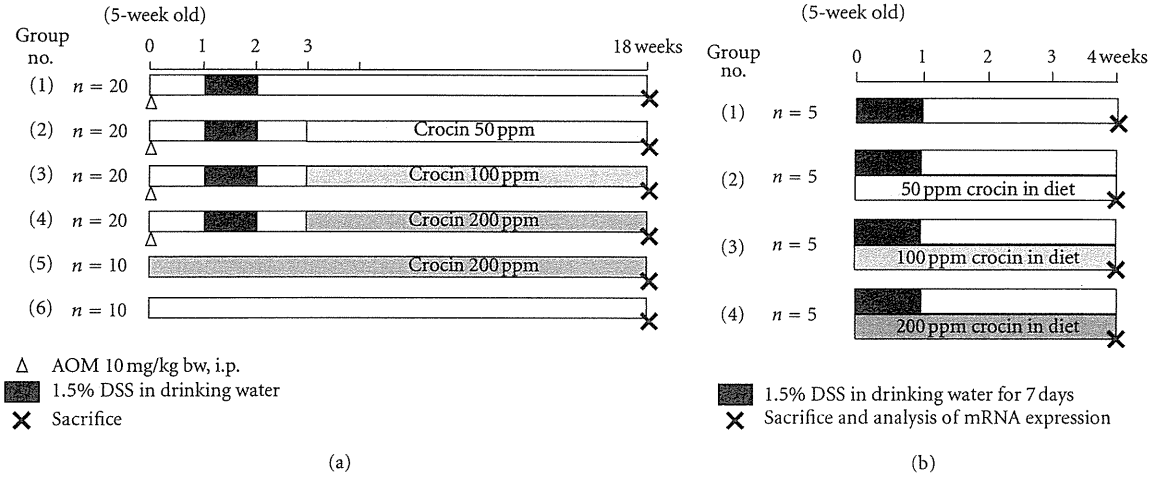


FIGURE 2: Experimental protocols for (a) Experiment 1 (18-week study) and (b) Experiment 2 (four-week study).

received diets containing 0, 50, 100, and 200 ppm crocin for 15 weeks, respectively, starting one week after cessation of DSS exposure. Group 5 (n = 10) not treated with AOM or DSS and was fed the 200 ppm crocin-containing diet for 18 weeks. Group 6 (n = 10) was served as an untreated control. All animals were sacrificed at week 18 by an overdose of ether to determine the effects of crocin on colon tumorigenesis. At the time of sacrifice, complete necropsies were done on all mice. The entire body, liver, and kidneys were weighted, and then liver and kidneys were fixed in 10% buffered formalin for 24 h. After macroscopic inspection, tissues from the large bowel, liver, and kidneys were processed for histopathological examination by conventional methods. The histopathological examination was performed on paraffin-embedded sections after hematoxylin and eosin (H&E) staining.

2.4. Experiment 2 (Four-Week Study). A total of 20 male ICR mice were divided into four experimental and control groups and subjected to a four-week experiment (Figure 2(b)). Mice in groups 1 through 4 were fed the experimental diets containing 0, 50, 100, and 200 ppm crocin, respectively, for four weeks. During the first week of the experiment, all groups were given 1.5% DSS in their drinking water. All animals were sacrificed at week four and their large bowels were flushed with saline and then excised. After measurement of their length (from the ileocecal junction to the anal verge), they were cut open longitudinally along the main axis, and gently washed with saline to remove feces. The large bowel was macroscopically inspected for the presence of pathological lesions, including ulcerations and cuts and fixed in 10% buffered formalin for 24 h. A histopathological examination was performed on paraffin-embedded sections

from the large bowel after H&E staining to determine the inflammation score of the colonic mucosa.

2.5. Scoring Inflammation in the Colorectum. The inflammation in the large bowel was scored on the H&E-stained sections. Large intestinal inflammation was graded according to the morphological criteria described in our previous study [53]: grade 0, normal appearance; grade 1, shortening and loss of the basal 1/3 of the actual crypts with mild inflammation in the mucosa; grade 2, loss of the basal 2/3 of the crypts with moderate inflammation in the mucosa; grade 3, loss of all of the crypts with severe inflammation in the mucosa and submucosa, while retaining the surface epithelium; grade 4, the presence of mucosal ulcer with severe inflammation (infiltration of neutrophils, lymphocytes, and plasma cells) in the mucosa, submucosa, muscularis propria, and/or subserosa. The scoring was performed on the entire colon with or without proliferative lesions and was expressed as a mean score/mouse.

2.6. Immunohistochemistry of Minichromosome Maintenance Protein 2 (MCM2), NF-κB, and Nrf2 in Adenocarcinomas. We used 4 μm thick paraffin-embedded sections from the colons of the mice in all groups from both experiments for the immunohistochemical analysis using the labeled streptavidin biotin method with an LSAB Kit (DAKO Japan, Kyoto, Japan) and with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene, and rehydrated through graded ethanol solutions at room temperature. A Tris-HCL buffer (0.05 M, pH 7.6) was used to prepare solutions to rinse slides between the various steps. Incubations were performed in a humidified chamber. The sections were treated for 40 min at room temperature with 2% bovine serum albumin and incubated overnight at 4°C with primary antibodies. The primary antibodies used were anti-MCM2 rabbit monoclonal antibody (no. 3619, anti-MCM2 (D7611)XP, 1 : 400 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-NF-κB p50 (H-119) rabbit polyclonal antibody (sc-7178, 1 : 500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and anti-Nrf2 rabbit polyclonal antibody (ab31163, 1 : 500 dilution; Abcam, Inc. Cambridge, MA, USA). These antibodies were applied to the sections according to the manufacturer's protocol. The horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3′-diaminobenzidine for 5 min. As the final step, the sections were weakly counterstained with Mayer's hematoxylin (Merck, Tokyo, Japan). For each case, the negative controls were examined first in the serial sections without the primary antibodies.

In Experiment 1, an immunohistochemical analysis was done in five mice each from groups 1 through 4. The immunoreactivity against the antibodies was assessed in the colonic adenocarcinomas (>3 mm in diameter) that developed in these groups using a microscope (Olympus BX41, Olympus Optical Co., Tokyo, Japan). The intensity and localization of the immunoreactivity against the primary antibodies were determined by a pathologist (T. Tanaka) who was unaware of the treatment group to which the slide belonged. The number of nuclei with positive reactivity for MCM2 was counted in a total of 3 × 100 cells in three different areas of the colonic cancer and expressed as a percentage (mean ± SD). The immunoreactivity against the NF-κB and Nrf2 antibodies in the adenocarcinoma cells was evaluated and graded between 0 and 5; grade 0, <15% of cells showing positive reactivity; grade 1, 16~30% of cells showing positive reactivity; grade 2, 31~45% of cells showing positive reactivity; grade 3, 46~60% of cells showing positive reactivity; grade 4, 61~75% of cells showing positive reactivity; grade 5, 76%+ of cells showing positive reactivity.

2.7. Total RNA Extraction and Quantitative Real-Time PCR. Total RNA was extracted from the colonic mucosa using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The cDNA was then synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan Ltd., Tokyo, Japan). A quantitative real-time PCR analysis of individual cDNA was performed with an ABI Prism 7500 instrument (Applied Biosystems Japan Ltd., Tokyo, Japan) using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd., Tokyo, Japan; IFN-γ, Mm00801778_m1Mm00801778_m1; NF-κB, Mm00476361_m1: TNF-α, Mm00443258-m1; IL-1β, Mm00434228_m1; IL-6, Mm00446190-mL; COX-2 (Ptgs2), Mm00478374-mL; iNOS (Nos2), Mm00440485-mL; β-actin: Mm00607939-sl). The sense and antisense primers for Nrf2 mRNA were 5′-TTGGCAGAGACATTCCCAT-3′ and 5′-GCTGCCACCGTCACTGGG-3′, respectively. The PCR cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression level of each gene was normalized to the β-actin expression level using the standard curve method. Each assay was performed in triplicate and the average was calculated.

2.8. Statistical Analysis. Measurements of multiplicity of colonic lesions and scores of histology and immunohistochemistry were statistically analyzed using either the Tukey or Bonferroni multiple comparison posttest. The incidences of colonic lesions between the groups were compared by Fisher's exact probability test. The statistical analysis of mRNA expression was performed by the Kruskal-Wallis test. Differences were considered to be statistically significant at $P < 0.05$.

3. Results

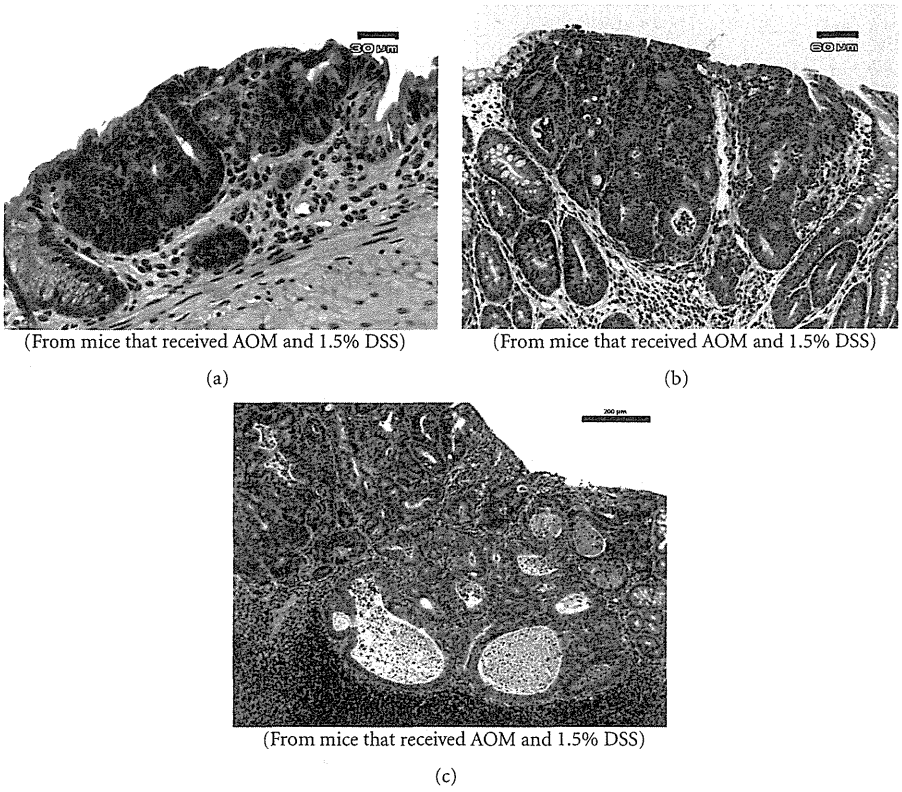
3.1. Experiment 1 (18-Week Study)

3.1.1. General Observations. Feeding the mice with the three different crocin-containing diets did not produce any observable clinical toxicity. This was confirmed by histopathological examinations of the liver and kidneys of the mice (data not shown). The mean weights of the whole body, and liver (g/100 g body weight) and the colon length in all groups at week 18 did not differ significantly among the groups (Table 1).

Table 1: Body and liver weights and the length of large bowel of mice at wk 18.

Group no.	Treatment	No. of mice examined	Body wt (BW, g)	Liver wt (g)	Relative liver wt (g/100 g BW)	Length of large bowel (cm)
1	AOM/DSS	20	44 ± 5[a]	2.26 ± 0.37	5.19 ± 0.72	12.6 ± 1.4
2	AOM/DSS/50 ppm crocin	20	47 ± 6	2.31 ± 0.46	4.90 ± 0.58	12.7 ± 1.0
3	AOM/DSS/100 ppm crocin	20	47 ± 4	2.35 ± 0.39	5.00 ± 0.88	12.8 ± 1.2
4	AOM/DSS/200 ppm crocin	20	45 ± 4	2.20 ± 0.31	4.95 ± 0.74	12.8 ± 1.5
5	200 ppm crocin	10	42 ± 5	1.99 ± 0.31	4.74 ± 0.66	12.1 ± 1.4
6	None	10	42 ± 4	1.95 ± 0.18	4.70 ± 0.27	12.6 ± 0.9

[a]Mean ± SD.



(From mice that received AOM and 1.5% DSS) (From mice that received AOM and 1.5% DSS)

(a) (b)

(From mice that received AOM and 1.5% DSS)

(c)

Figure 3: Representative histopathology of colonic proliferative lesions that developed in mice that received AOM and 1.5% DSS (Experiment 1). (a) Dysplastic crypts, high grade (bar, 30 μm); (b) tubular adenomas (bar, 60 μm); (c) tubular adenocarcinoma (bar, 200 μm). H&E stain.

3.1.2. Incidence and Multiplicity of Severe Inflammation with Mucosal Ulcers and High-Grade Dysplastic Crypts. AOM and/or DSS treatment resulted in the occurrence of veracious colorectal lesions, such as colitis with mucosal ulcers, dysplastic crypts (high grade, Figure 3(a)), tubular adenoma (Figure 3(b)), and tubular adenocarcinoma (Figure 3(c)). The incidences and multiplicity of severe colorectal inflammation with mucosal ulcers, the inflammation score, and the presence of dysplasia at week 18 are shown in Figure 4. The incidence of severe inflammation with mucosal ulcers (Figure 4(a), $P < 0.05$ or $P < 0.01$) significantly decreased after feeding the mice with all three concentrations of crocin compared with group 1 (AOM + DSS). Similarly, the inflammation score (Figure 4(b), $P < 0.05$ or $P < 0.01$) decreased after crocin treatment at the higher concentrations (100 and 200 ppm). The incidence of high-grade dysplastic crypts (Figure 4(c), $P < 0.05$ or $P < 0.01$) significantly decreased by feeding the mice with all three concentrations of crocin compared with group 1 (AOM + DSS). The multiplicity of high-grade dysplastic crypts (Figure 4(d), $P < 0.01$) also decreased by crocin treatment at the higher concentrations (100 and 200 ppm).

3.1.3. Incidence and Multiplicity of Colorectal Adenomas and Adenocarcinomas. The incidence and multiplicity of colonic tumors at week 18 are shown in Figure 5. Group 1 (AOM + DSS) had colonic adenocarcinoma with an incidence of 90% and a multiplicity of 3.15 ± 1.87. Treatment with all three

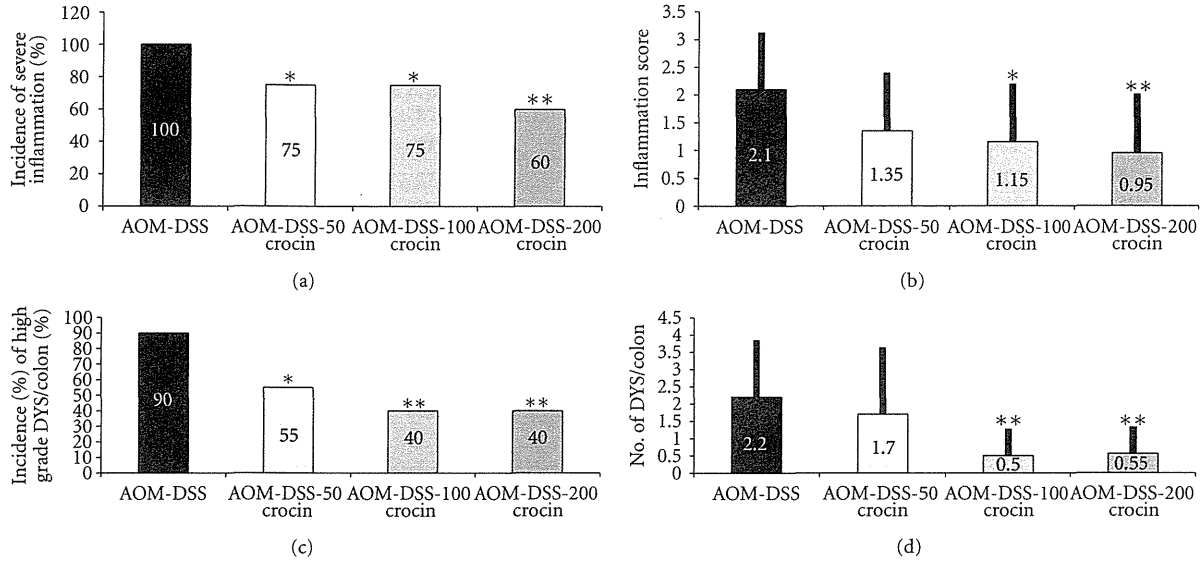


FIGURE 4: (a) The incidence of severe colorectal inflammation, (b) the inflammation score of colorectum, (c) the incidence of high-grade dysplastic crypts (DYS), and (d) the multiplicity (no./colon) of high-grade DYS. $*P < 0.05$, $**P < 0.01$ versus the the AOM + 1.5% DSS group.

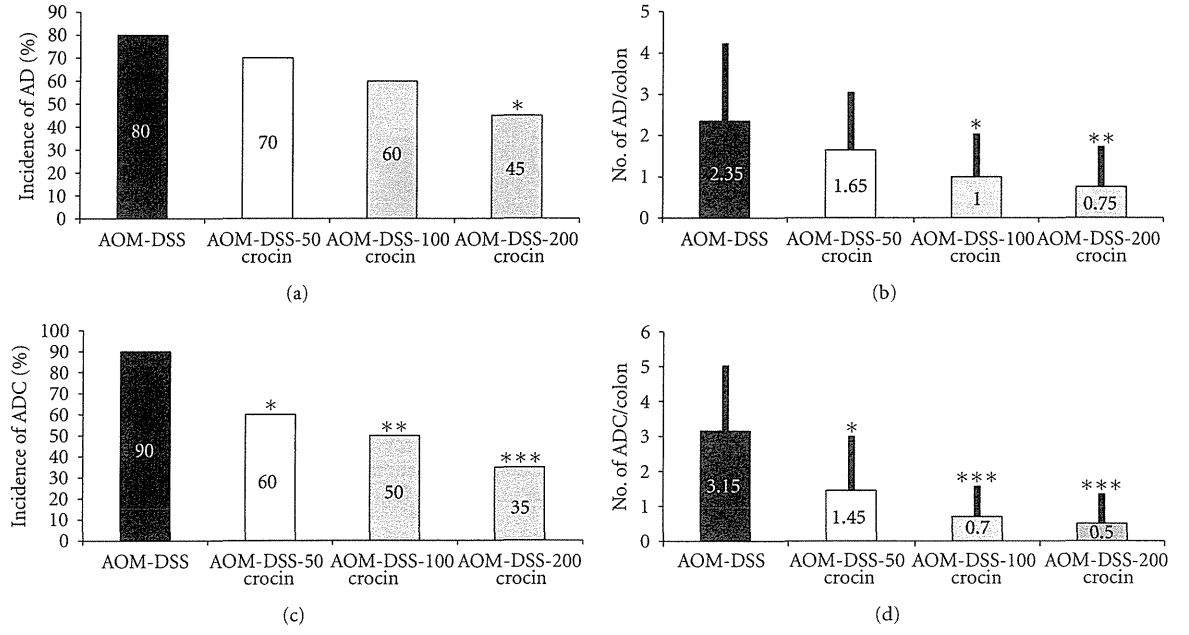


FIGURE 5: (a) The incidence of colorectal adenoma (AD), (b) multiplicity (no./colon) of colorectal AD, (c) incidence of colorectal adenocarcinoma (ADC), and (d) multiplicity (no./colon) of colorectal ADC. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus the AOM + 1.5% DSS group.

concentrations of crocin significantly reduced the incidence ($P < 0.05$ at 50 ppm, $P < 0.01$ at 100 ppm, and $P < 0.001$ at 200 ppm) and multiplicity of adenocarcinoma ($P < 0.05$ at 50 ppm, $P < 0.001$ at 100 ppm and 200 ppm). Dietary crocin also decreased the incidence of adenomas and the difference between groups 1 and 4 was statistically significant ($P < 0.05$). Dietary administration of crocin also decreased the multiplicities of colonic adenoma ($P < 0.05$ at 100 ppm and $P < 0.01$ at 200 ppm).

3.1.4. Cell Proliferation. We immunohistochemically analyzed the expression of MCM2 in colonic adenocarcinomas to determine the effects of crocin on the proliferation of cancer cells (Figures 6(a)–6(d)). As shown in the bar graph

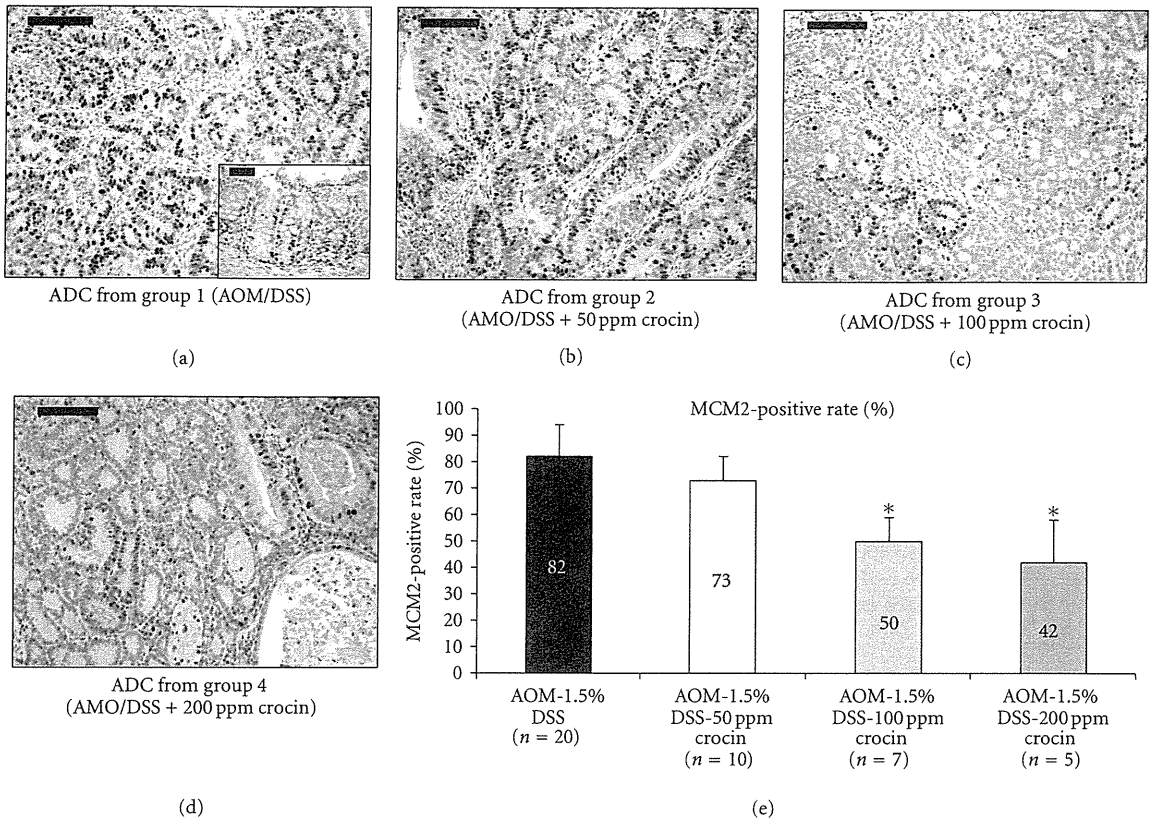


FIGURE 6: Immunohistochemical staining for MCM2 in an adenocarcinoma that developed in a mouse from (a) group 1 (AOM + 1.5% DSS), (b) group 2 (AOM + 1.5% DSS + 50 ppm crocin), (c) group 3 (AOM + 1.5% DSS + 100 ppm crocin), and group 4 (AOM + 1.5% DSS + 200 ppm crocin). The insert in (a) is normal colonic mucosa. Bars, 100 μm. The graph summarizes the data on the MCM2-positive rates of adenocarcinomas from groups 1 through 4 ($n = 5$ each). $^*P < 0.001$ versus the AOM + 1.5% DSS group.

in Figure 6(e), the mean MCM2-positive indices of colonic adenocarcinomas in groups 3 ($P < 0.001$) and 4 ($P < 0.001$) were significantly lower than that of group 1, thus indicating that crocin decreased the cancer cell proliferation.

3.1.5. Immunohistochemical Expression of NF-κB and Nrf2. Figure 7 shows the immunohistochemical expression of NF-κB (Figures 7(a) and 7(b)) and Nrf2 (Figures 7(c) and 7(d)) in the adenocarcinomas that developed in the colons of the mice from groups 1 and 4. When compared with group 1, the consumption of dietary crocin at 100 ppm ($P < 0.01$) and 200 ppm ($P < 0.01$) in the diet significantly suppressed the immunohistochemical score for NF-κB (Figure 7(a)), while significantly enhancing the expression of Nrf2 at 200 ppm crocin (Figure 7(b), $P < 0.05$). In groups 5 (200 ppm crocin alone) and 6 (untreated), the immunohistochemical expressions of NF-κB and Nrf2 in the colonic mucosa were very weak (data not shown).

3.2. Experiment 2 (Four-Week Study)

3.2.1. General Observations. Feeding with experimental diets containing three concentrations (50, 100, and 200 ppm) of crocin did not produce any clinical toxicity. All mice treated with DSS alone (group 1) had diarrhea with bleeding during the DSS treatment. However, fewer mice in groups 2 through 4 had such symptoms.

3.2.2. Inflammation Scores in the Large Bowel. Figure 8 shows the histopathology of the colonic mucosa and summarizes the scoring of colonic inflammation (Figure 9) at week 4. The colonic mucosa of the mice treated with crocin (200 ppm in the diet) alone showed almost normal histology (Figure 8(a)). DSS treatment caused severe colitis with mucosal ulcers (Figure 8(b)). However, the severity of colitis in the mice fed crocin at 100 ppm (Figure 8(c)) and 200 ppm (Figure 8(d)) decreased and regenerative crypt cells covered and healed the mucosal ulcers. As shown in Figure 9, the inflammation scores of the DSS + 50 ppm crocin ($P < 0.05$), DSS + 100 ppm crocin ($P < 0.01$), and DSS + 200 ppm crocin ($P < 0.001$) groups were significantly lower than those of the DSS alone group.

3.2.3. mRNA Expression Levels of Inducible Inflammatory Enzymes, Proinflammatory Cytokines, and Nrf2. Figures 9 and 10 show the relative mRNA expression levels of COX-2 (Figure 10(a)), iNOS (Figure 10(b)), IFN-γ (Figure 11(a)), TNF-α (Figure 11(b)), IL-1β (Figure 11(c)), IL-6 (Figure 11(d)), NF-κB (Figure 11(e)), and Nrf2 (Figure 11(f)), when the value of the DSS alone group was converted to 100

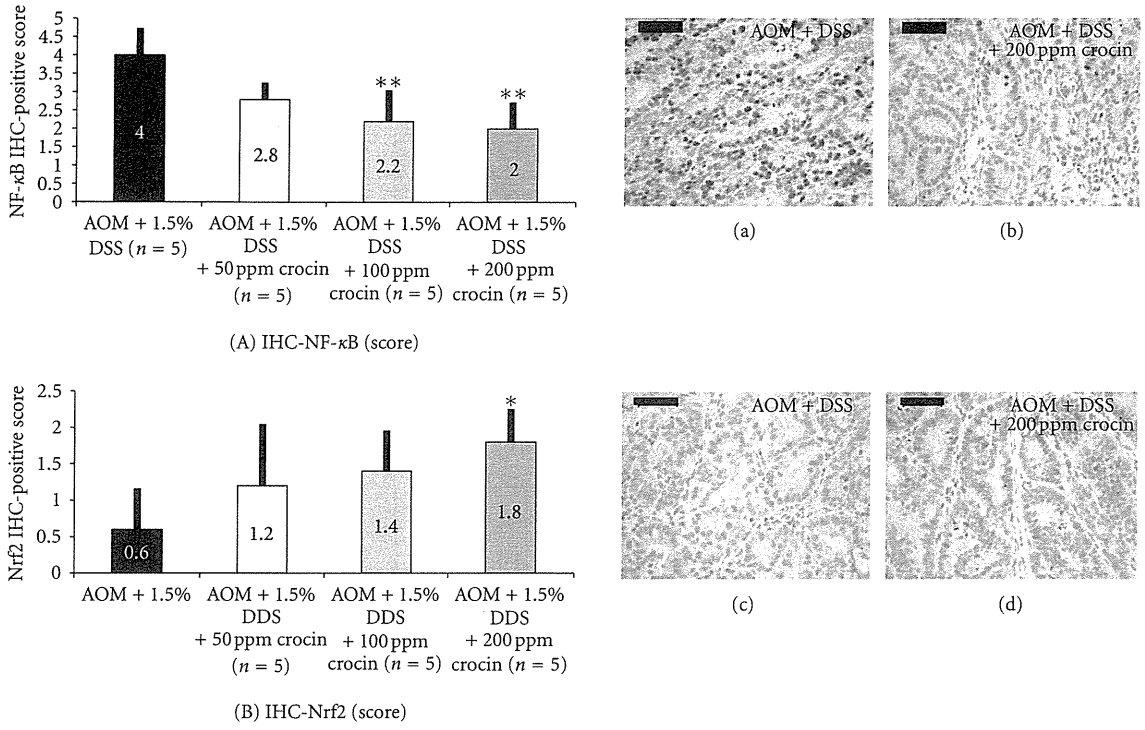


FIGURE 7: Immunohistochemical expression of (a) NF-κB and (b) Nrf2 in adenocarcinoma cells (Experiment 1). Both proteins were expressed in the nuclei of cancer cells. The scores of immunohistochemical expression of both proteins were changed by crocin treatment: crocin feeding lowered the score for NF-κB (A) and increased it for Nrf2 (B). $*P < 0.05$, $**P < 0.01$ versus the AOM + 1.5% DSS group.

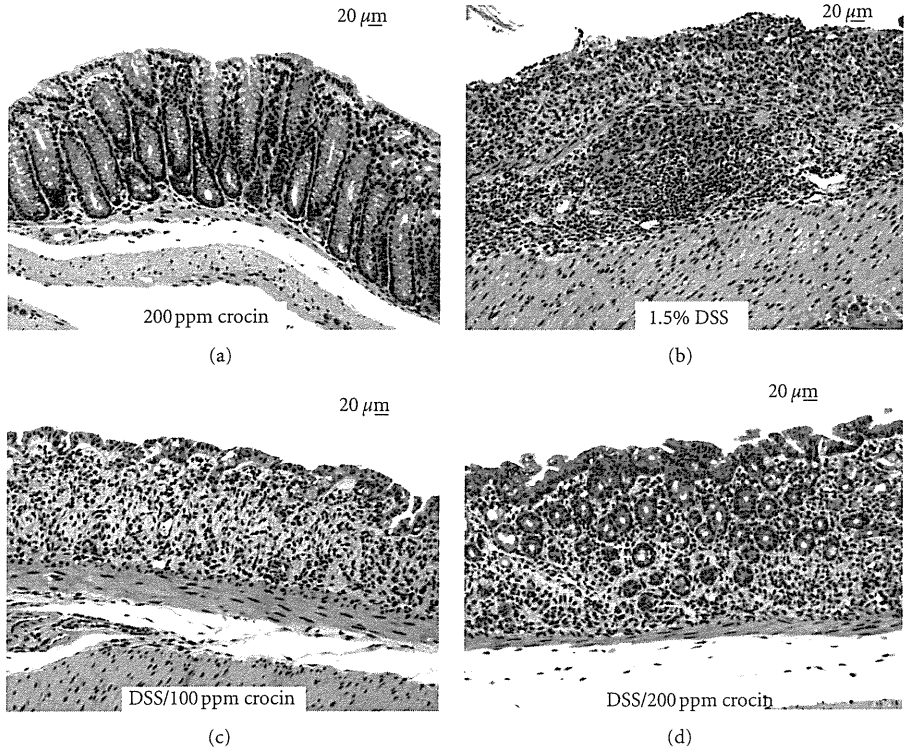


FIGURE 8: Representative histopathology of the colorectal mucosa (Experiment 2). When compared to (a) normal colorectal mucosa, 1.5% DSS treatment resulted in severe colitis with mucosal ulceration (b). In contrast, mucosal regeneration was observed in the colons of mice that were treated with crocin at (c) 100 ppm and (d) 200 ppm. Bars are 20 μm. H&E stain.

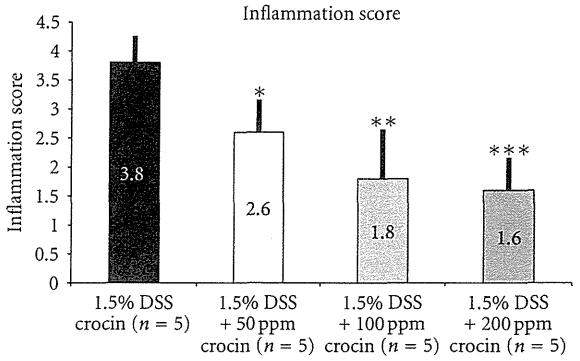


FIGURE 9: The inflammation scores in the colorectum of mice treated with DSS and or crocin (Experiment 2). Feeding with crocin at all three concentrations (50, 100, and 200 ppm) significantly decreased the inflammation score. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the AOM + 1.5% DSS group.

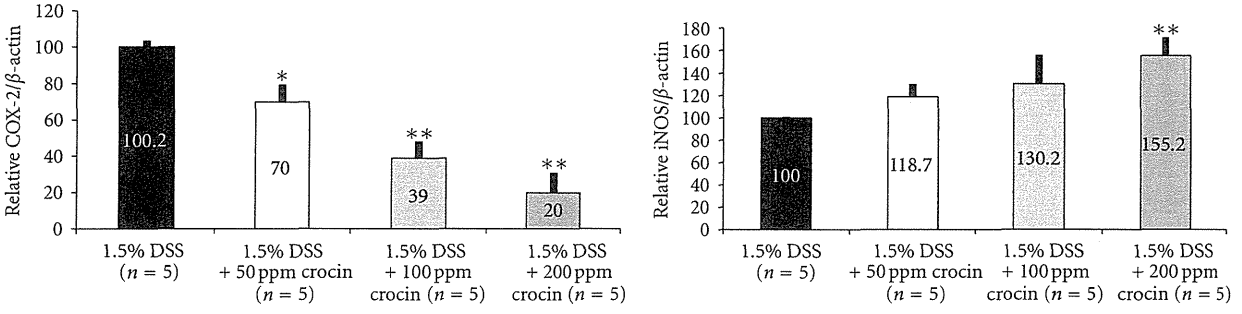


FIGURE 10: The mRNA expression levels of inducible inflammatory enzymes, (a) COX-2 and (b) iNOS, in the colorectum (Experiment 2) as determined by quantitative real-time RT-PCR. Crocin treatment significantly decreased the expression levels of COX-2 (50, 100, and 200 ppm) and iNOS (200 ppm), when compared with the AOM and DSS group. The expression was normalized to the β-actin mRNA expression. Samples were analyzed in triplicate. Data are the means ± SD from three independent assays ($n = 5$ from each group). The ordinates show the relative mRNA expression ($/\beta$-actin) versus the 1.5% DSS group. $^{*}P < 0.01$, $^{**}P < 0.001$ versus the 1.5% DSS group.

by an RT-PCR analysis. The expression levels of all of these genes except for Nrf2, in the mice treated with DSS were increased in comparison with the mice treated with 200 ppm crocin. Feeding with crocin at 200 ppm significantly decreased the mRNA expression of all of the molecules except for Nrf2. As to Nrf2 (Figure 11(f)), With regard to Nrf2, the mRNA expression level in the DSS alone group was the lowest, and the crocin treatment increased its expression.

4. Discussion

We demonstrated that dietary crocin, which is a water-soluble carotenoid used as a colorant, effectively suppressed colitis and colitis-related colorectal carcinogenesis in mice. The suppressive effects were at least partly due to the anti-inflammatory properties of the crocin (as indicated by the inhibition of several cytokines and inducible inflammatory enzymes). Crocin, isolated from saffron, was previously shown to inhibit the growth of several human cancer cell lines [1], including colorectal cancer cells [22]. Crocin also inhibited mouse skin [26, 28] and liver carcinogenesis [29], but there have so far been no reports of its effects on other tissues. This is the first report to show evidence that crocin inhibits colorectal carcinogenesis in rodents.

In general carotenoids possess potent cancer chemopreventive properties [54]. Major clinical trials using high-dose supplemental β-carotene were performed, because it is the carotenoid most frequently identified to have a protective activity against lung cancer, but it failed to demonstrate a sufficient protective effect [54]. These findings suggest that the use of carotenoids without the potential for conversion to vitamin A may provide protection and avoid this toxicity. In this study, we did not observe any clinical or histopathological toxicity of crocin. Therefore, crocin is of considerable interest because of its potent antiinflammatory, anticarcinogenesis, and antioxidant activities, which are distinctly different from those of β-carotene and other carotenoids [55, 56]. We also have confirmed that crocin has stronger antioxidant activity compared to α-tocopherol [57]. We have also recently reported the cancer chemopreventive ability of a marine carotenoid, astaxanthin, in an AOM/DSS model [40].

In this study, the dietary administration of three concentrations of crocin ameliorated AOM/DSS-induced colonic

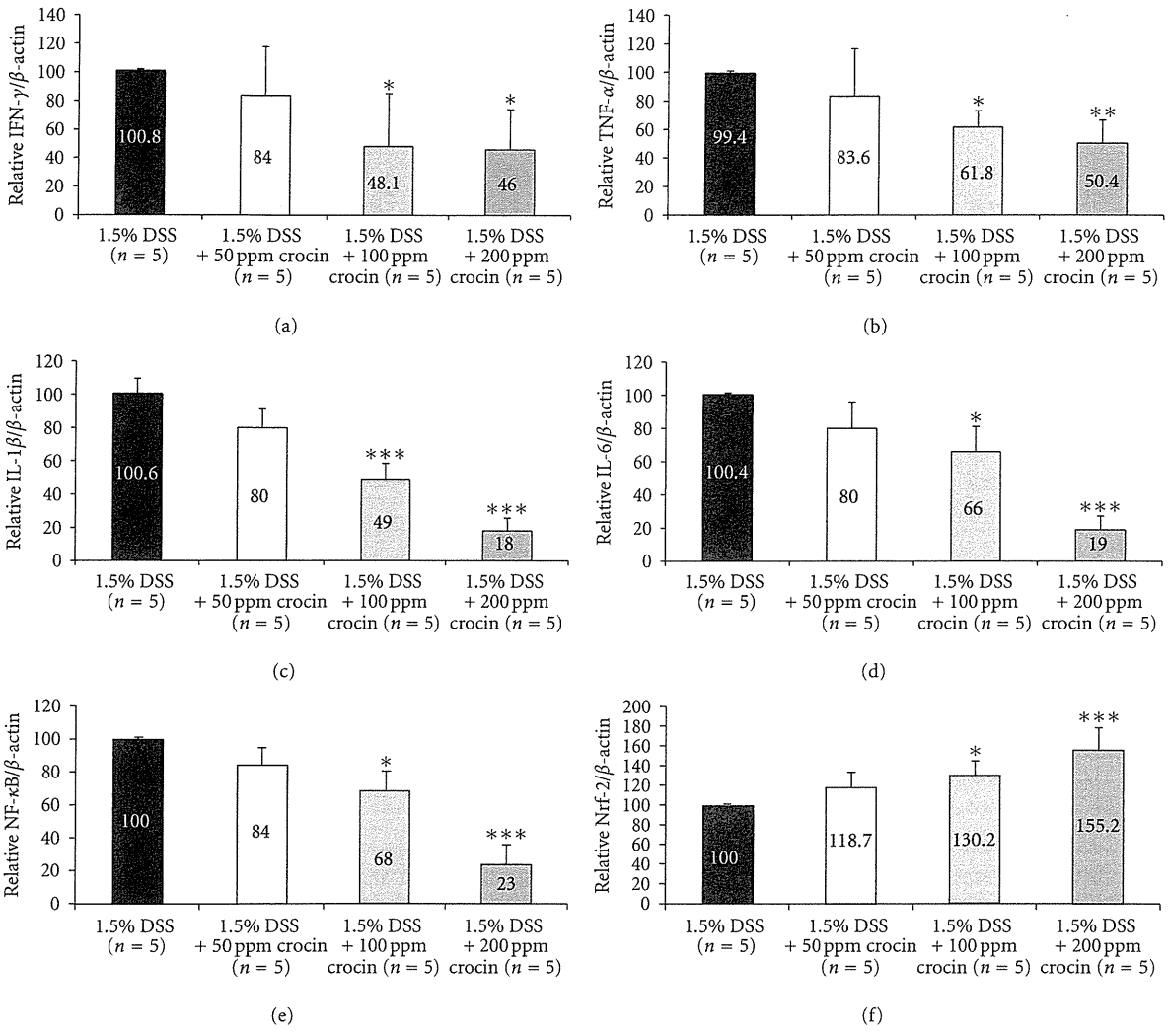


FIGURE 11: The mRNA expression levels of (a) IFN-γ, (b) TNF-α, (c) IL-1β, (d) IL-6, (e) NF-κB, and (f) Nrf2 in the colorectum (Experiment 2) as determined by quantitative real-time RT-PCR. Feeding with crocin significantly decreased the expression levels of IFN-γ (100 and 200 ppm), TNF-α (100 and 200 ppm), IL-1β (100 and 200 ppm), IL-6 (100 and 200 ppm), and NF-κB (100 and 200 ppm), compared with the AOM and DSS group. On the other hand, the mRNA expression of Nrf2 was significantly increased by the treatment with crocin (100 and 200 ppm). The expression was normalized to the β-actin mRNA expression. Samples were analyzed in triplicate. Data are the means $\pm$ SD from three independent assays ($n = 5$ from each treatment group). The ordinates are the relative mRNA expression levels (/β-actin) versus the 1.5% DSS group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the 1.5% DSS group.

proliferative lesions in mice. This suppression was prominent in mice treated with both 100 and 200 ppm crocin. Furthermore, the dietary crocin suppressed the proliferation activity in adenocarcinomas. These findings are in agreement with those of previous reports showing the antiproliferative effects of crocin [22, 58, 59]. Many studies have shown a variety of pharmacological effects of crocin [22, 58–61]. Among the mechanisms underlying its biological actions, the antioxidant activity was thought to be responsible for the various pharmacological effects of crocin [10, 12].

The antiinflammatory effects of crocin are suggested to be based on its antioxidant activity [10, 12]. In the current study, we further examined the effects of crocin on DSS-induced colitis in mice (Experiment 2). The dietary feeding of crocin significantly suppressed several inflammatory events and NF-κB expression in the colorectal mucosa of the mice that received DSS. Inflammatory genes, such as COX2, iNOS, TNF-α, and IL-1β, are the most common target genes participating in the activation of NF-κB and are associated with a number of chronic inflammatory diseases, including IBD and IBD-related colorectal carcinogenesis [37, 38, 45, 46, 62]. In the current study, we observed decreases in the mRNA expression levels of NF-κB, COX-2, iNOS, TNF-α, IL-1β, and IL-6 in the mice treated with DSS and crocin when compared to the mice given DSS alone. Our findings thus suggest that crocin suppressed the mouse colonic inflammation induced by DSS by modulating the NF-κB signaling pathway. The NF-κB signaling pathway also has

a major role in inflammation-associated carcinogenesis [63]. Therefore, NF-κB is a target for cancer chemoprevention [42, 44], and natural compounds that suppress NF-κB expression may be useful for cancer chemoprevention [64].

Although we mainly discussed the effect of crocin on the NF-κB pathway, the effects of dietary crocin on Nrf2 expression of adenocarcinoma cells and the inflamed colon are also of interest, because of the protective role of Nrf2 against the toxicity of xenobiotics [47, 48]. In this study, the immunohistochemical expression of Nrf2 was increased by crocin treatment (Experiment 1). In the inflamed colon exposed to DSS, crocin increased the mRNA expression of Nrf2. Although the exact mechanism(s) underlying the elevation of Nrf2 expression by crocin treatment need to be elucidated, this elevation might partly contribute to the inhibition of colitis and colitis-related colorectal carcinogenesis by feeding with crocin. Our findings also support that Nrf2 is one of the targets for cancer chemoprevention [47–50].

In this study, crocin inhibited DSS-induced colitis by directly affecting the absorption of DSS. It has been reported that intestinal microflora played important role in DSS-induced intestinal inflammation [65]. Since many plant extracts were reported to benefit intestinal microflora, it may be possible that alterations in intestinal microflora by crocin feeding contribute to the antiinflammatory effects of crocin in mice that received DSS. Further studies are necessary to assess the role of crocin in intestinal microflora in a colitis-associated colorectal carcinogenesis model which was used in this study.

Taken together, the results of the present study suggest that dietary crocin inhibits AOM/DSS-induced colitis-associated colon carcinogenesis and DSS-induced colitis in mice by suppressing the expression of cytokines including NF-κB. Our findings indicate that the NF-κB signaling pathway may also play an important role in colitis-associated colorectal carcinogenesis and is a potential target for colitis-related colorectal carcinogenesis. Our data also suggest that crocin is a potentially effective cancer chemopreventive agent that can be used to prevent the development of CRC in the inflamed colon. Importantly, crocin demonstrated negative results in bacterial tests for mutagenicity and did not produce any chromosome damage in mammalian cells in culture [30], thus suggesting that clinical trials of crocin may be possible.

Conflict of Interests

The authors declare no financial or commercial conflict of interests.

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INTRODUCTION

Introduction for inflammation and cancer

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How inflammation protects or injures tissues of the human body is quite an important issue, particularly in cancer development. Inflammation also affects the immune system or immune surveillance. We now know the importance of inflammation and the innate/adaptive immune system in tumor development, promotion, progression, and metastasis.

Inflammation has two faces in human tissues. The acute inflammatory response is the first system of alarm signals that are directed toward elimination of microbial invaders and wounded necrotic debris. Chronic inflammation has emerged as the pathophysiological basis of many chronic cardiovascular and neurodegenerative diseases that were not initially thought to be linked to an inflammatory response. It has now become apparent that inflammation is an important component of cancer development. This inflammation is now regarded as a "secret killer" of chronic diseases, such as cancer.

The concept of a relationship between inflammation and carcinogenesis is not new. Based on the presence of leukocytes within cancer tissue, the founder of cellular pathology, Dr. Rudolph Virchow, speculated that there was an association between inflammation and cancer development in 1863. In line with his notion, epidemiological data indicate that inflammation serves as a potential risk factor for the development of cancer in different tissues. It is generally accepted that up to 25 % of human malignancies are related to chronic inflammation with or without (viral, bacterial, or parasitic) infections. Evidence shows that inflammatory stimuli caused by inflammation increase the risk of cancer, promote tumor progression, and support metastatic spread. Thus, inflammatory cells and cytokines act as tumor promoters that affect cell survival, proliferation, invasion, angiogenesis, and resistance of chemotherapy.

The immune system always acts to prevent preneoplastic cells progressing to cancer. On the other hand, cancer cells possess immunosuppressive properties such that they escape immune surveillance. In addition, we know that environmental factors decrease the immune function, resulting in cancer development. These observations together indicate that analysis of the immune function could contribute to the early detection, prevention, and treatment of cancer.

Based on the types of inflammation associated with carcinogenesis, the tumor microenvironment can include cancer cells, innate immune cells (macrophages, neutrophils, mast cells, dendritic cells, natural killer cells, and myeloid-derived suppressor cells), adaptive immune cells (T and B lymphocytes), and stromal cells (fibroblasts, vessel endothelial cells, and other mesenchymal cells). To maintain the growth of cancer, these cells communicate with each other directly or indirectly by producing cytokines and chemokines in autocrine and/or paracrine manners.

This special issue entitled "Inflammation and Cancer" is mainly focused on this topic but also touches on the prevention of inflammation-related cancers. Several world-class experts from diverse fields and institutions have written review articles for this introductory special issue of the journal. Each of the reviews is presented as an introduction by experts who are involved in cutting-edge research in their area of expertise. Dr. Sven Brandau et al. describe the pro-tumor and anti-tumor functions of neutrophil granulocytes in tumorigenesis. They propose a model where homeostatic chronic recruitment and activation of neutrophils result in

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