Research Article

Cancer Prevention Research

St. John's Wort Attenuates Colorectal Carcinogenesis in Mice through Suppression of Inflammatory Signaling

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Abstract

Despite widespread use as well as epidemiologic indications, there have been no investigations into the effect of St. John's wort (SJW) extract on colorectal carcinogenesis *in vivo*. This study reports a systematic evaluation of the impact of dietary supplementation of SJW extract on azoxymethane-induced colorectal carcinogenesis in mice. Mice were fed with either AIN-93G (control) diet or SJW extract–supplemented diet (SJW diet) prior to azoxymethane treatment. SJW diet was found to significantly improve the overall survival of azoxymethane-treated mice. Pretreatment with the SJW diet significantly reduced body weight loss as well as decrease of serum albumin and cholesterol levels associated with azoxymethane-induced colorectal tumorigenesis. SJW diet–fed mice showed a significant decrease in tumor multiplicity along with a decrease in incidence of large tumors and a trend toward decreased total tumor volume in a dose-dependent manner. A short-term study, which examined the effect of SJW prior to rectal bleeding, also showed decrease in colorectal polyps in SJW diet–fed mice. Nuclear factor kappa B (NF-κB) and extracellular signal–regulated kinase (ERK1/2) pathways were attenuated by SJW administration. SJW extract resulted in early and continuous attenuation of these pathways in the colon epithelium of SJW diet–fed mice under both short-term and long-term treatment regimens. In conclusion, this study demonstrated the chemopreventive potential of SJW extract against colorectal cancer through attenuation of proinflammatory processes. *Cancer Prev Res; 8(9); 786–95. ©2015 AACR.*

Introduction

Colorectal cancer is the fourth leading cause of cancer-related mortality worldwide (1). The identification of effective preventive measures can significantly contribute to reduce the incidence and mortality. Apart from genetic predispositions, several lifestyle–related factors, such as red meat consumption, smoking, and obesity, were found to increase the risk of developing colorectal cancer. With rapid urbanization, especially in the developing world, with a setting of sedentary lifestyle and change in food habits, such as increased consumption of processed meat, the incidence of colorectal cancer may increase significantly (2). Development of viable preventive strategies for diverse population is an integral part of cancer prevention.

Dietary supplements and alternative medicines continue to be an integral part of both preventive and therapeutic practices all over the world, particularly in developing nations. Many of these practices are culturally integrated into the lifestyle as customs and have been found to have beneficial effects. Promoting such practices would contribute to formulation of acceptable and effective preventive strategies against colorectal cancer. However, there is a requirement for careful and thorough evaluation to determine the short-term and long-term physiologic impacts of these supplements. Unfortunately, there has been a general lack of such studies on dietary supplements.

St. John's wort (SJW) is a widely used dietary supplement available over the counter. Components of SJW extract, such as hypericin and hyperforin, are widely studied and found to attenuate neurotransmitters receptors (3). Hyperforin triggers apoptosis of lung and the C-26 colon cancer cell line (4). Consumption of SJW extract was associated with reduced risk of developing colorectal cancer (5). However, the underlying mechanism of protection has not been determined. This is particularly important given the fact that SJW constituents and/or extracts were shown to increase the metabolism and compromise the efficacy of drugs including anticancer agents (6–9). In this study, the prophylactic effect of SJW extract on colorectal carcinogenesis as well as the overall physiology was examined using an azoxymethane (AOM)-induced colorectal carcinogenesis model in 129S6/SvEvTac mice.

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

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Materials and Methods

Chemicals

Azoxymethane was supplied by Syncon (10). Hyperforin and hypericin were purchased from Sigma-Aldrich. Flowering tops of SJW were collected, dried, extracted, and purified by Euromed Inc. and was provided as powder for research purposes. The composition of the powdered extract (Supplementary Table S1) met European formulation standards for the supplement certified for human use. The extract was stored at 4°C and shipped in refrigerated condition in light-resistant containers.

Oligonucleotides

Primers for qPCR analysis were designed using qPrimerDepot (11) and synthesized from Integrated DNA Technologies.

Diet preparation

Powdered SJW extract was added to purified diet (AIN-93G; Con group) to produce either 2.5% (SJW(L) group) or 5% (SJW group) SJW diet (see Supplementary Table S2 for details). All diets were prepared by Dyets Inc. under dimmed yellow light and dried in a cool and dark room. SJW diets were prepared fresh every 2 weeks and all diets were stored at 4°C.

Evaluation of stability and integrity of SJW constituents in the diet

SJW extract powder and the SJW diet were extracted with methanol and analyzed using an Acquity UPLC (ultra-performance liquid chromatography) C_{18} column (1.7 μm) connected to a XEVO G2 ESI-QTOFMS (Waters Corporation; Supplementary Data). Constituents were identified using accurate mass as well as characteristic fragments in MS/MS experiments (Supplementary Data). The hyperforin and hypericin peaks were confirmed by comparison of retention time and fragmentation pattern against authentic standards. The SJW diet was extracted after 0, 4, 8, and 20 days of storage following preparation. The relative amounts of constituents in the diet were measured from areas under the peak of the respective extracted ion chromatogram.

Mice and treatments

In order to evaluate the effect of SJW exposure on body weight gain, food intake and liver enzyme levels, 24, 4-week-old male 129S6/SvEvTac mice were purchased from Taconic Farms, acclimated to the NIH animal facility for 1 week after arrival, and, then, randomly divided into three groups ($n = 8$) receiving either control or 2.5% SJW diet or 5% SJW diet for 3 weeks. For the long-term observation study, another 46, 4-week-old mice were similarly acclimated and, then, randomly divided into five study groups to examine the effect of SJW diet (Fig. 1A) on colorectal carcinogenesis. Three groups of mice were fed either control (Con/AOM, $n = 12$), 2.5% SJW (SJW(L)/AOM, $n = 8$), or 5% SJW diet (SJW/AOM, $n = 10$) for 2 weeks and then administered intraperitoneal (i.p.) injections with azoxymethane (10 mg/kg body weight) weekly for 6 weeks. Two more groups of mice on control diet (Con/Sal, $n = 8$) or SJW diet (SJW/Sal, $n = 8$) were injected (i.p.) with saline weekly. Mice continued to receive their respective diet throughout the rest of the study. Body weights and general conditions of the mice were monitored weekly. Rectal bleeding was monitored monthly and scored on a relative scale of 0 (no bleeding) to 10 (severe bleeding). Mice were killed at 21 weeks after the last azoxymethane injection or upon deterioration of health as indicated by loss of >20% body weight, dehydration, profuse rectal bleeding, and/or prolapse in accordance with the guideline for humane use of animals for research. Another 60 mice were randomly divided into four groups in order to determine the effect of SJW diet at early stages of azoxymethane-induced tumorigenesis (Fig. 1B). Two groups of mice were fed either control (Con/AOM, $n = 18$) or the 5% SJW diet (SJW/AOM, $n = 18$) for 2 weeks and then injected with azoxymethane (10 mg/kg body weight; i.p.) weekly for 6 weeks. Two other groups of mice on control diet (Con/Sal, $n = 12$) or SJW diet (SJW/Sal, $n = 12$) were injected weekly with saline. Nine mice from each of the azoxymethane-treated groups and 6 mice from each of the saline-treated groups were euthanized at 2 weeks after the last azoxymethane injection. The remaining mice were killed at 4 weeks after the last azoxymethane injection. Colons from mice were flushed with saline, longitudinally opened, and examined under optical microscope for polyps or tumors. The length and width of tumors were measured, and volume of tumors calculated using a method described earlier (12). Colon epithelial tissue layer was carefully scraped and stored at −80°C for RNA extraction. Colons from other mice were preserved in 10% formalin for histology. All studies were reviewed and approved by the NCI Animal Care and Use Committee.

Colon histology

Colon tissue was formalin-fixed, cut in a 4-μm thickness, and stained with hematoxylin and eosin to check azoxymethane-induced inflammation. The samples were examined with an optical microscope (Olympus BX4).

Biochemical parameters

Serum albumin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), bile acid, blood urea nitrogen (BUN), and cholesterol were measured by loading 100-μL serum onto VETSCAN mammalian liver profile disks using a VETSCAN VS2 analyzer (Abaxys).

Quantitative polymerase chain reaction

Total RNA was extracted from colon scrapes using the RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized from 1 μg of total RNA using SuperScript II Reverse Transcriptase Kit (Invitrogen), and used for both microarray and qPCR analysis. Primers for qPCR were designed with qPrimerDepot. SYBR green PCR master mix (Applied Biosystems) was used to carry out qPCR in an ABI Prism 7900HT Sequence Detection System and changes in gene expression were quantified with the comparative $\Delta\Delta C_t$ method and normalized to 18S ribosomal RNA.

Microarray

Complementary DNA was dye-coupled and hybridized to Agilent 44K mouse 60-mer oligonucleotide microarrays (Agilent Technologies). Samples from saline-treated and azoxymethane-treated mice on control as well as SJW diet were independently hybridized and processed. Microarray data were processed and analyzed using Genespring GX 11.5.1 software (Agilent Technologies). The relative gene expression data were analyzed using orthogonal projection to latent structures discriminant analysis (OPLS-DA) using SIMCA-P12+ (Umetrics) software. Changes in gene expression due to the SJW diet in saline- and azoxymethane-treated mice were subjected to Ingenuity Pathway Analysis (Ingenuity Systems) to identify pathways of interest. Microarray data (GSE56571) were deposited in the Gene Expression Omnibus site and can be directly accessed (13).

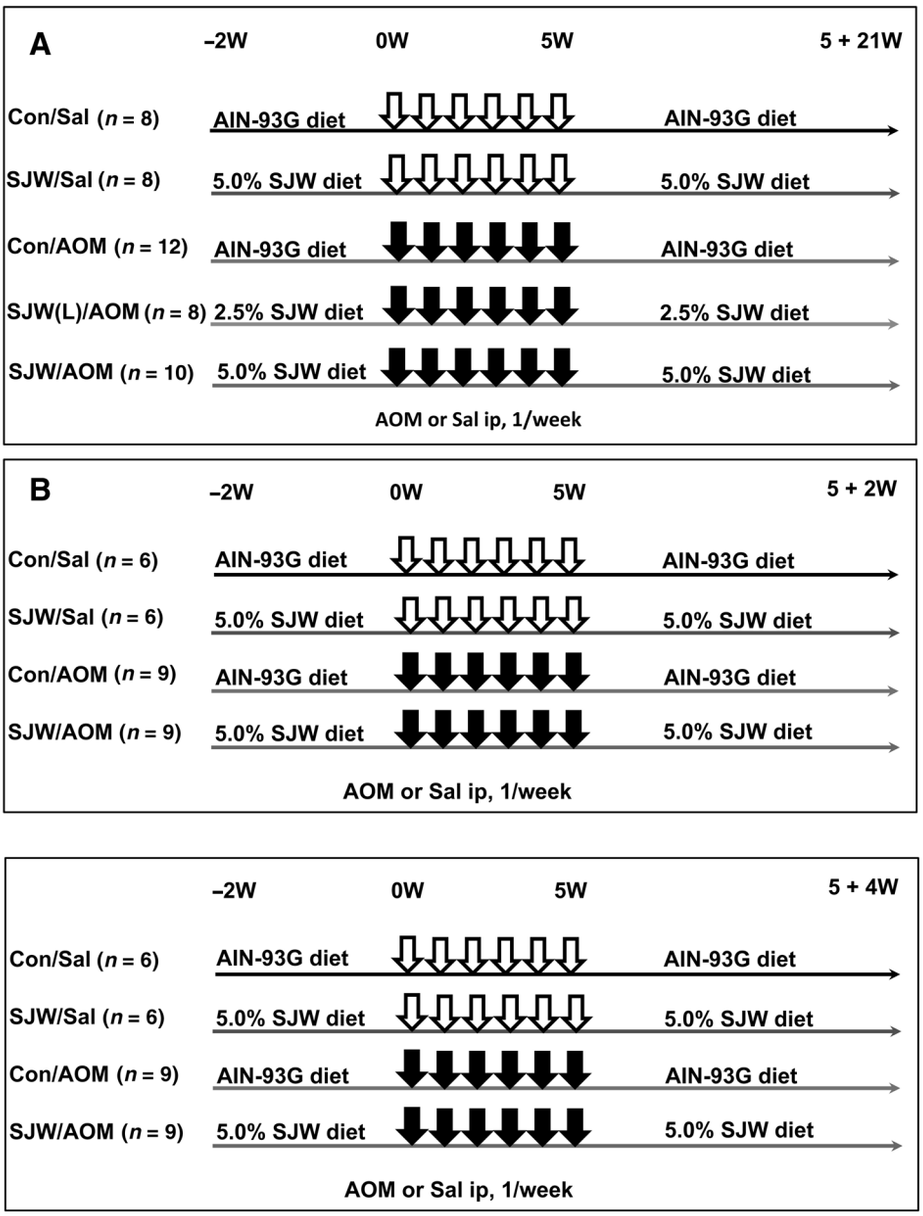

Figure 1.
Schematic representation of the animal study design. A, long-term study (21-week after azoxymethane follow-up). B, short-term study (2- and 4-week after azoxymethane follow-up). Solid arrows indicate azoxymethane injection (10 mg/kg body weight, weekly i.p.) and empty arrows indicate saline i.p. injection.

Statistical analysis

Analysis of the difference between survival curves were compared using the log-rank (Mantel–Cox) test. One-way ANOVA with the Tukey correction for multiple comparisons and two-tailed Mann–Whitney test for comparison between two groups was performed using GraphPad Prism 6 for Windows (GraphPad Software, Inc.). The differences were considered significant when the *P* value was less than 0.05.

Results

SJW diet integrity and stability of constituents

UPLC-ESI-QTOFMS analysis of the methanol extract of SJW extract as well as SJW diet indicated the presence of a number of compounds that have earlier been reported in SJW extract (14). Notably, the chromatogram of the methanolic extract of 20-day-old SJW diet (Supplementary Fig. S1A) showed the presence of bioactive constituents such as hyperforin, hypericin, pseudohypericin, quercetin, and hyperoside. Extracted ion chromatograms for some of the representative compounds such as hyperforin (m/z = 535.378, ESI-), hypericin (m/z = 503.076, ESI-), and quercetin (m/z = 301.034, ESI-) shown in Supplementary Fig. S1B. Identities of hyperforin (Supplementary Fig. S2A) and hypericin (Supplementary Fig. S2B) were confirmed by comparing their retention times and fragmentation patterns with authentic standards. For other compounds, putative identities were determined by a match of characteristic fragmentation patterns with reported MS/MS spectra of respective compounds. For example, chromatograms of quercetin (Supplementary Fig. S3A), quercitrin (Supplementary Fig. S3B), and rutin (Supplementary Fig. S3C) showed major fragments that matched reported MS/MS spectra in the METLIN

database (15, 16). The stability of these constituents was examined by comparing areas under the curve of the extracted chromatograms of respective compounds upon storage. The area under the curves for hyperforin (Supplementary Fig. S4), hypericin (Supplementary Fig. S5), and quercetin (Supplementary Fig. S6), respectively, at day 0, 4, 8, and 20 days after diet preparation revealed no significant trends toward decreased abundance of these compounds in the SJW diet upon storage at 4°C over a period of 20 days.

General conditions and survival

All mice receiving control or 2.5% SJW or 5% SJW diet for 3 weeks were healthy and active, and there was no significant difference in food intake or body weight gain of mice on control, 2.5% SJW and 5% SJW diet (Supplementary Fig. S7A and S7B). Short-term studies (2 and 4 weeks after azoxymethane injection) also did not show any significant differences in body weight gain between mice on the control or 5% SJW diet (Supplementary Fig. S7C) irrespective of whether they received saline or azoxymethane injections (Fig. 1B). In the 3-week study, there was no significant difference in levels of ALT in any of control diet or 2.5% and 5% SJW diet mice (Supplementary Fig. S8A). ALP levels in 2.5% SJW mice were significantly lower than the control diet ($P < 0.01$); however, no significant difference was seen between 2.5% and 5% SJW diet (Supplementary Fig. S8B). Similarly, none of the saline-treated mice on either control (Con/Sal) or SJW diet (SJW/Sal) showed any adverse health effects and survived through the duration of the long-term chemoprevention study (Fig. 1A). Analysis of the Kaplan–Meier survival curves showed that there was no difference between survival of the SJW/AOM groups and the Con/Sal or SJW/Sal groups (Fig. 2A). On the other hand, 50%

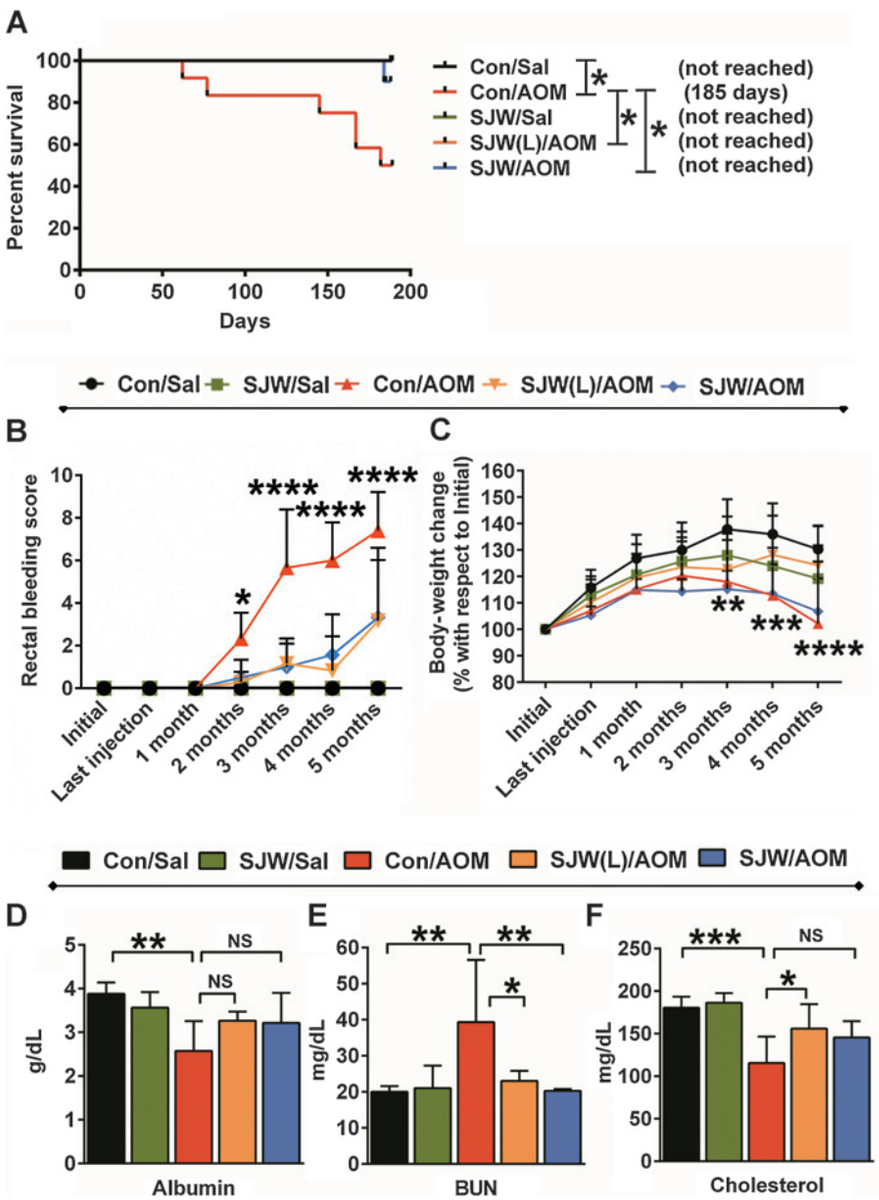

Figure 2.
Effect of SJW diet on overall survival, rectal bleeding, and biochemical parameters in azoxymethane-treated mice. A, comparison of the Kaplan-Meier survival curves for saline-treated mice on control (Con/Sal; black line) or 5% SJW diet (SJW/Sal; green line), azoxymethane-treated mice on control diet (Con/AOM; red line), 2.5% (SJW(L)/AOM, orange line) or 5% SJW diet (SJW/AOM, blue line). The median survival for each group is mentioned on the right side. *P* values for difference in survival were calculated using the Mantel–Cox (log-rank) test. B, rectal bleeding score of mice under study on a relative scale of 0 to 10. C, body weight gain of mice during the course of the study with respect to initial body weight. Change in (D) serum albumin, (E) BUN, and (F) cholesterol levels in mice. The statistical analysis for B–F was analyzed using one-way ANOVA with the Tukey correction for multiple testing. Color codes are same as those used for A. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$, respectively. NS, not significant.

(6 out of 12) of the azoxymethane-treated mice on control diet (Con/AOM) were euthanized either due to a drastic decrease in body weight, low ambulatory activity, rectal bleeding, and/or prolapse before the study ended. Survival analysis showed a significant decrease (HR, undefined; 95% CI, 1.3–33.3; $P < 0.02$) in survival of the Con/AOM group (median survival, 185 days) compared with the Con/Sal groups (median survival not reached; Fig. 2A). Azoxymethane-treated mice on the 2.5% SJW diet (SJW(L)/AOM; median survival not reached) survived throughout the study and only 10% of the azoxymethane-treated mice on the 5% SJW diet (SJW/AOM; median survival not reached) were euthanized before the end of the study. Further analysis showed significant improvement in overall survival of the SJW/AOM group (HR, 0.204; 95% CI, 0.045–0.918; $P < 0.03$) and SJW(L)/AOM group (HR, 0.150; 95% CI, 0.029–0.7676; $P < 0.02$) compared with the Con/AOM group, while there was no significant difference between survival of SJW(L)/AOM and SJW/AOM groups (Fig. 2A). Consistent with these observations, mice in the Con/AOM group showed a significant and progressive increase in rectal bleeding starting from 2 months ($P < 0.001$) after the last azoxymethane injection (Fig. 2B) and the increase in rectal bleeding was significantly higher at 3 ($P < 0.0001$), 4 ($P < 0.0001$), and 5 months ($P < 0.0001$) compared with Con/Sal group. Although a few mice in SJW(L)/AOM and SJW/AOM groups showed rectal bleeding at 2 months after the last azoxymethane injection, the increase was significantly lower than the Con/AOM group ($P < 0.01$ and $P < 0.05$ for SJW(L)/AOM and SJW/AOM, respectively). At 3, 4, and 5 months (post-azoxymethane), mice in SJW(L)/AOM and SJW/AOM groups showed a further increase in rectal bleeding. However, their rectal bleeding scores were still significantly lower than their counterparts in the Con/AOM group ($P < 0.0001$ and $P < 0.0001$, at 3 months, $P < 0.0001$ and $P < 0.0001$ at 4 months and $P < 0.0001$ and $P < 0.0001$ at 5 months for SJW(L)/AOM and SJW/AOM groups, respectively; Fig. 2B). Although the body weight gains of mice in the SJW/Sal group appeared to be slightly lower than those in Con/Sal group in the long-term study, these differences were never statistically significant (Fig. 2C). The body weight gain of mice in Con/AOM group was 14% ($P < 0.005$), 17% ($P < 0.005$), and 22% ($P < 0.0001$) lower than mice in Con/Sal group, respectively, at 3, 4, and 5 months after last azoxymethane injection (Fig. 2C). On the other hand, mice in SJW(L)/AOM) or SJW/AOM groups showed no significant decrease in body weight gain compared with the SJW/Sal group throughout the duration of study (Fig. 2C).

Effect on nutritional status and liver function

There was no significant difference between serum albumin concentrations of mice from Con/Sal (3.9 mg/dL) or SJW/Sal (3.6 mg/dL) group (Fig. 2D). However, mice from the Con/AOM group showed a 30% ($P < 0.005$) decrease in serum albumin concentration (2.7 mg/dL) compared with the Con/Sal group (Fig. 2D). In fact, the albumin level in SJW/AOM and SJW(L)/AOM group did not show any significant difference from the Con/AOM group. Mice in the Con/Sal and SJW/Sal group also showed no significant difference in BUN (Fig. 2E), cholesterol (Fig. 2F), ALT, ALP (Supplementary Fig. S9A and S9B), and bile acid (Supplementary Fig. S9C) levels in serum. However, the BUN level (Fig. 2E) of mice in the Con/AOM group (39.3 mg/dL) was 97% higher ($P < 0.01$) compared with that in the Con/Sal group (20.0 mg/dL). Mice from SJW(L)/AOM (23 mg/dL) and SJW/AOM (20 mg/dL) groups showed no significant increase in BUN levels compared with the SJW/Sal group and these levels were also significantly lower ($P < 0.02$, $P < 0.005$ for SJW(L)/AOM and SJW/AOM, respectively) compared with that the Con/AOM group. The serum cholesterol levels (Fig. 2F) of the Con/AOM group (107 mg/dL) were 31% lower ($P < 0.001$) compared with the Con/Sal group (155 mg/dL). The serum cholesterol level of mice from the SJW/AOM (145 mg/dL) was also 22% ($P < 0.05$) lower than the SJW/Sal group (186 mg/dL). However, the serum cholesterol levels in SJW(L)/AOM ($P < 0.05$) group were higher than that in Con/AOM group. The bile acid levels were also notably higher ($P < 0.01$) in the Con/AOM group (11.3 μmol/L) compared with the Con/Sal group (2.2 μmol/L). Furthermore, bile acid levels in the SJW(L)/AOM (11 μmol/L) and SJW/AOM group (9.3 μmol/L) were also higher ($P < 0.02$) than that in the SJW/Sal group (2.2 μmol/L; Supplementary Fig. S9C).

Effect of SJW on colorectal tumorigenesis

All mice in the Con/AOM and SJW(L)/AOM groups as well as all except 1 mouse in the SJW/AOM group developed colorectal tumors. The average number of tumors in the SJW(L)/AOM group were slightly lower ($P < 0.02$) than in the Con/AOM group (Fig. 3A). However, the average number of tumors in the SJW/AOM group was significantly lower than that of the Con/AOM ($P < 0.0001$) as well as SJW(L)/AOM group ($P < 0.01$). Consistent with this observation, although inflammatory cell infiltration could not be prevented completely, the non-tumor mucosa from the mice on the SJW diet appeared to have milder azoxymethane-induced inflammation compared with mice on control diet (Supplementary Fig. S10). It was also noted that mice in the SJW/AOM group developed significantly lower number of large tumors (diameter > 2 mm) compared with those on Con/AOM ($P < 0.0005$) as well as SJW(L)/AOM group ($P < 0.02$; Fig. 3B). Although it did not reach statistical significance, the combined volume of all tumors in mice from SJW/AOM group was lower than those from Con/AOM group (Fig. 3C). These data indicated that SJW diet attenuates colorectal tumorigenesis. In order to examine whether this attenuation takes effect during the early stages of tumorigenesis, two short-term studies were conducted using 5% SJW diet, which showed a significant chemopreventive effect, with a follow-up time of 2 and 4 weeks after the last azoxymethane injection (Fig. 1B). The average number of polyps in the Con/AOM group at 2 (Fig. 3D) and 4 weeks (Fig. 3E) were found to be 9 and 11, respectively. On the other hand, the average number of polyps in the SJW/AOM group went down from 7 at 2 weeks (Fig. 3D) to 5 at 4 weeks (Fig. 3E). The average tumor number in the SJW/AOM group was significantly lower than that in the Con/AOM group at both 2 weeks ($P < 0.01$) as well as at 4 weeks ($P < 0.0001$). In addition, the histologic analysis of non-tumorous mucosa (Supplementary Fig. S11) revealed that while the colon from the SJW/AOM group was similar to saline-treated mice, those from Con/AOM group showed mild infiltration of inflammatory cells at 2 weeks after the last azoxymethane injection.

Microarray analysis

Microarray analysis revealed changes in expression of several genes associated with SJW diet in the colon of azoxymethane-treated mice. Supervised OPLS-DA analysis was used to identify genes that contributed significantly ($p(corr)[1] > 0.8$, upregulated or $p(corr)[1] < -0.8$, downregulated) to the overall change

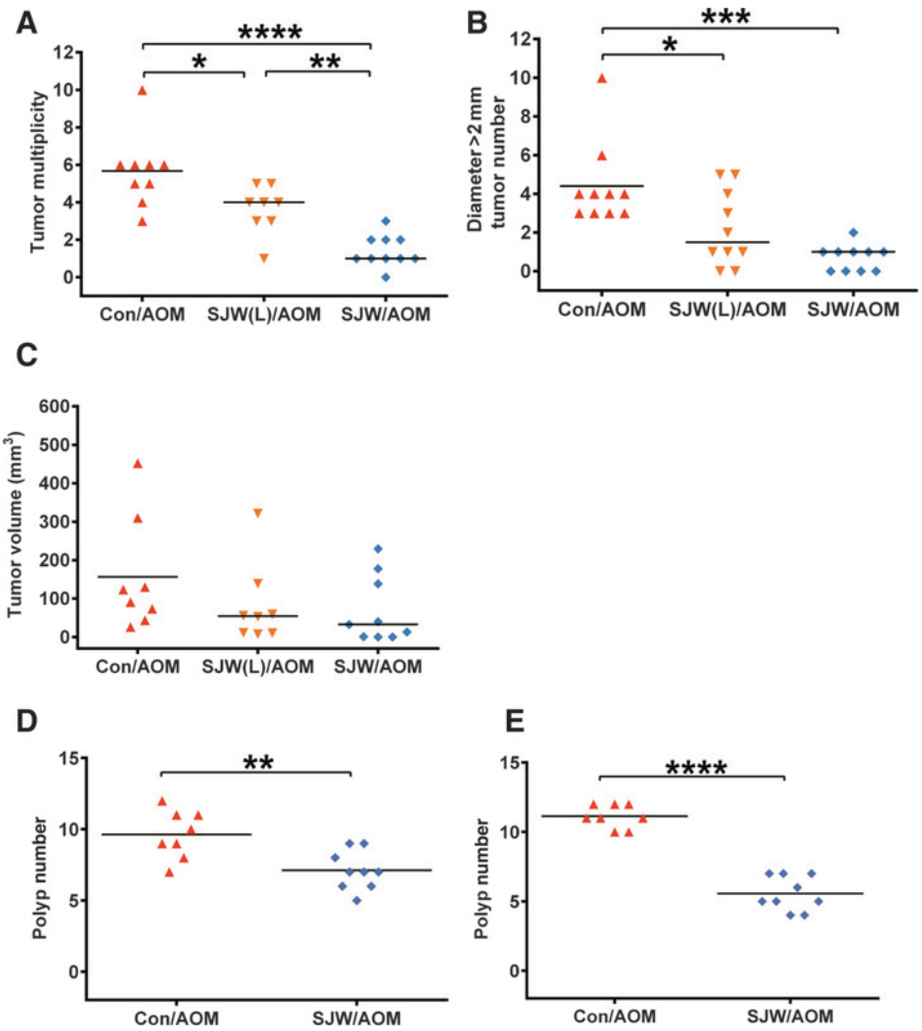

Figure 3.
Effect of SJW diet on azoxymethane-induced colorectal tumorigenesis. Scatter plots for (A) total number of tumors (multiplicity), (B) number of tumors with diameter > 2 mm and (C) total tumor volume in azoxymethane-treated mice on control diet (red) or 2.5% (orange) or 5% (blue) SJW- diet. Scatter plots for number of polyps found in the colon of azoxymethane-treated mice on control (red box) or 5% SJW diet (blue triangle) after (D) 2 week or (E) 4 week of last azoxymethane injection. Statistical significance was calculated using one-way ANOVA with the Tukey correction for multiple testing for A, B, and C. The two-tailed Mann–Whitney test was performed for D and E. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$, respectively.

in gene-expression signature (Supplementary spreadsheet). The Ingenuity Pathway Analysis using these genes revealed that SJW diet resulted in attenuation of nuclear factor kappa B (NF-κB; Fig. 4A) and extracellular signal–related kinase 1/2 (ERK1/2; Fig. 4B) signaling pathways in the colon of azoxymethane-treated mice. Heatmap analysis revealed the relative expression levels of genes related to these pathways (Fig. 4C).

Effect SJW on inflammatory signatures

In order to elucidate the molecular basis of the prophylactic effect of SJW, changes in gene expression signatures related to NF-κB and ERK1/2 pathways in the colon epithelium of mice from Con/AOM and SJW/AOM groups were further confirmed using qPCR. Results showed that expression of tumor necrosis factor α (*Tnf*), which is elevated in the colon epithelium of azoxymethane-treated mice on control diet, was significantly attenuated in their counterparts on the SJW diet (Fig. 5). In addition, expression of other genes related to the NFκB pathway, such as inducible nitric oxide synthase (*Nos2*), matrix metalloproteinase 7 (*Mmp7*), and matrix metalloproteinase 9 (*Mmp9*), was also significantly attenuated on SJW diet (Fig. 5). Although not statistically significant, expression of the proinflammatory cytokine interleukin 1b (*Il1b*), which is influenced by NF-κB signaling, showed a trend toward downregulation. In addition, bone morphogenetic protein 7 (*Bmp7*) that is related to the ERK1/2 signaling pathway was attenuated by SJW diet (Fig. 5). In order to examine whether attenuation of these pathways by SJW diet takes place early during the tumorigenesis process and contributes to the observed decrease in polyp numbers, the expression of these genes was examined in the colon epithelium of mice from short-term study. The results were strikingly similar to that found in the long-term study. All of these genes (*Tnf*, *Il1b*, *Nos2*, *Mmp7*, *Mmp9*, and *Bmp7*) were upregulated in colon of the Con/AOM group compared with the Con/Sal group. However, the SJW diet significantly attenuated expression of these genes associated with NF-κB and ERK1/2 signaling pathways in azoxymethane-treated mice. It was noted that the relative expression levels with respect to that in Con/Sal, of *Tnf*, *Il1b*, *Nos2*, *Mmp7*, *Mmp9*, and *Bmp7* mRNAs, in the Con/AOM group increased from 2 weeks (Fig. 6A) to 4 weeks (Fig. 6B). Interestingly, the relative expression level of these genes in the SJW/AOM group decreased from 2 to 4

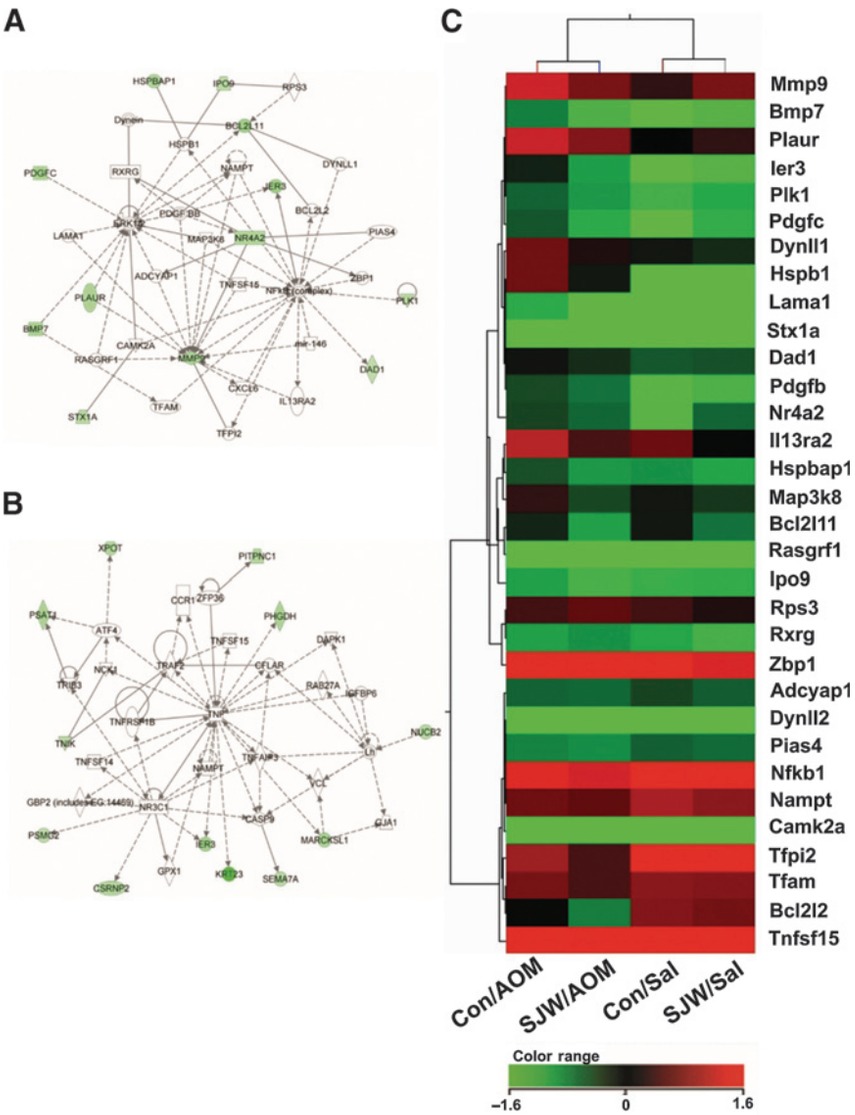

Figure 4.
Pathways found to be downregulated in the colon epithelium of azoxymethane-treated mice due to SJW diet. Microarray-based analysis of gene expression and the Ingenuity Pathway Analysis revealed downregulation of (A) NF-κB (A) and, ERK1/2 signaling (B). C, heatmap showing relative expression level of genes related to NF-κB and ERK1/2 signaling pathways in normal colon epithelium from saline-treated mice on control diet (Con/Sal) or 5% SJW diet (SJW/Sal) and non-tumor colon epithelium from azoxymethane-treated mice on control diet (Con/AOM) or 5% SJW diet (SJW/AOM).

weeks. These results demonstrate that SJW has chemopreventive effect against azoxymethane-induced colorectal carcinoma likely due to attenuation of inflammatory signaling.

Discussion

Pretreatment with SJW diet was found to reduce tumor multiplicity, incidence of large tumors, and rectal bleeding as well as to restrict body weight loss and deterioration of nutritional status. Consequently, SJW was also found to increase the overall survival of azoxymethane-treated mice. Colorectal carcinogenesis is a multistep process that involves initiation, progression, and invasion. Azoxymethane is metabolized in the body to produce methyldiazonium and formaldehyde, which then cause DNA alkylation leading to mutations that initiate tumorigenesis (17). The fact that SJW extract was found to exert chemopreventive activity at early stages indicates that it affects early events during tumorigenesis. It was shown that both K-ras and β-catenin mutations are early events in azoxymethane-induced colorectal carcinogenesis (18). However, in this study, the Ingenuity Pathway Analysis indicated no significant trend of changes in the expression of gene cluster belonging to β-catenin pathway. K-ras mutations can activate MAPK and PI3K/Akt pathways that are involved in inflammatory processes via ERK1/2 and NF-κB signaling, respectively. Inflammation promotes growth, cell survival, and attenuates apoptosis to induce neoplastic transformation (19). Inflammatory signaling via NF-κB, ERK, and JAK-STAT were shown to be involved in human colorectal carcinogenesis (20–23). TNFα, a cytokine produced by macrophages recruited to sites of inflammation. Increased TNFα and activation of NF-κB signaling was implicated in the initiation of colorectal cancer during formation of aberrant crypt foci (ACF; ref. 24). Reduced TNFα indicates attenuation of inflammatory events in colons of mice on the SJW diet following azoxymethane treatment. Blocking TNFα was also shown to inhibit colitis-associated colorectal

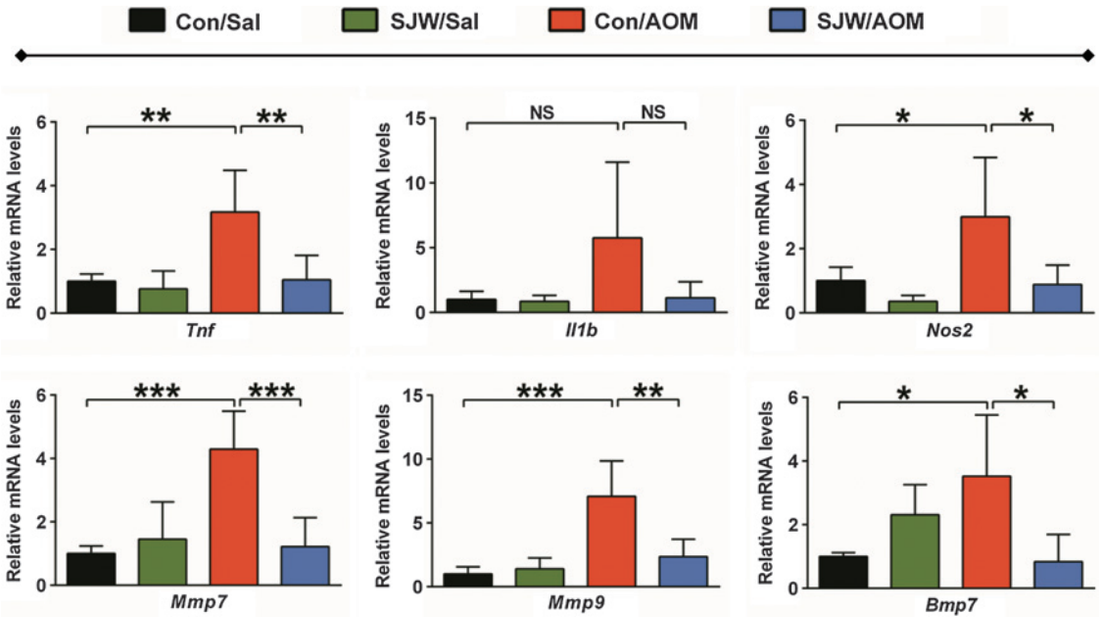

Figure 5.
qRT-PCR analysis of changes in the expression of representative genes related to NF-κB and ERK1/2 signaling pathway in colon epithelium due to SJW diet. All values are presented as mean + standard deviation. Statistical significance was calculated using one-way ANOVA with the Tukey correction for multiple testing *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$, respectively. NS, not significant.

cancer in mice (25). Thus, the prophylactic effect of SJW diet is consistent with decreased *Tnf* mRNA levels. In addition, expression of *Il1b* was attenuated early in colon of mice on SJW diet. This also reduced expression of two matrix metalloproteinases (*Mmp7* and *Mmp9*) mRNAs. Matrix metalloproteinases have roles in metastasis and invasion (26). *Mmp9* is a direct NF-κB target gene (27), whereas *Mmp7* expression was also shown to be dependent on NF-κB signaling (28). Overexpression of these genes in human colorectal cancer is associated with poor prognosis (29). In addition, these mice also showed reduced expression of *Nos2* that is overexpressed in some human colon tumors, particularly in metastatic tumors, and found to be highly correlated with vascular endothelial growth factor expression and angiogenesis (30). Thus, the changes in gene expression upon dietary supplementation of SJW extract, is consistent with attenuation of NF-κB signaling. NF-κB is a crucial regulator of the transcriptional activation of a number of genes involved in cell adhesion, immune and proinflammatory responses, apoptosis, differentiation, and growth (31). Many proto-oncogenes and carcinogens cause activation of NF-κB, whereas chemicals with known chemopreventive properties can suppress NF-κB activation (32). Similar to these earlier observations, SJW diet–induced attenuation of NF-κB signaling appears to suppress azoxymethane-induced colorectal tumorigenesis. In addition, this was accompanied by attenuation of the ERK1/2 signaling pathway. Earlier studies revealed that TNFα induced expression of *Mmp9* via regulation of NF-κB can be mediated by ERK1/2 (33). On the other hand, lipopolysaccharide stimulation of ERK1/2 was shown to increase TNFα production (34). The decrease in tumorigenesis in mice receiving SJW appeared to be due to attenuation of inflammatory signaling involving the NF-κB and ERK1/2 pathway activated by azoxymethane-induced K-ras mutation, an early event in colorectal carcinogenesis. Attenuation of these pathways takes place within 2 weeks of the last azoxymethane injection, before rectal bleeding or adenoma is observed, leading decreased polyps. Expression of these tumor-promoting genes increases during the initial stages in mice on control diet while SJW gradually attenuates their expression, consistent with reduced polyps.

Although this study demonstrates a prophylactic effect of SJW extract due to the attenuation of NF-κB and ERK1/2 signaling, the exact contribution of its constituent phytochemicals remains to be determined. Hyperforin, a prenylated phloroglucinol present in SJW extract, activate pregnane X receptor (PXR; ref. 35). Earlier studies revealed that hyperforin possesses anticancer activity against colon cancer cells *in vitro* (4). In addition, activation of PXR ameliorates DSS-induced colitis in mice through attenuation of expression of NF-κB target genes (36). These studies would suggest that the observed attenuation in NF-κB and consequent prophylactic effect is a result of PXR activation by hyperforin. However, others showed that PXR activation increase cell growth, invasion, and metastasis in human colon tumor cell lines as well as in a xenograft mouse model using primary colon cancer tissue (37). Thus, further investigation into the role of PXR in the chemopreventive effect SJW against colorectal cancer is needed.

Apart from the primary and metastatic sites, a systemic effect of carcinogenesis often leads to progressive body weight loss (cachexia), deterioration of quality of life (38–40). Maintenance of nutritional status, physiologic functions, and body weight can significantly improve the quality of life and overall survival. Cytokines are implicated in cachexia development (40, 41). Maintenance of body weight, serum albumin, and cholesterol levels in azoxymethane-treated mice receiving SJW extract indicate maintenance of nutritional status. Attenuation of NF-κB signaling pathway may result in the observed prevention of body weight

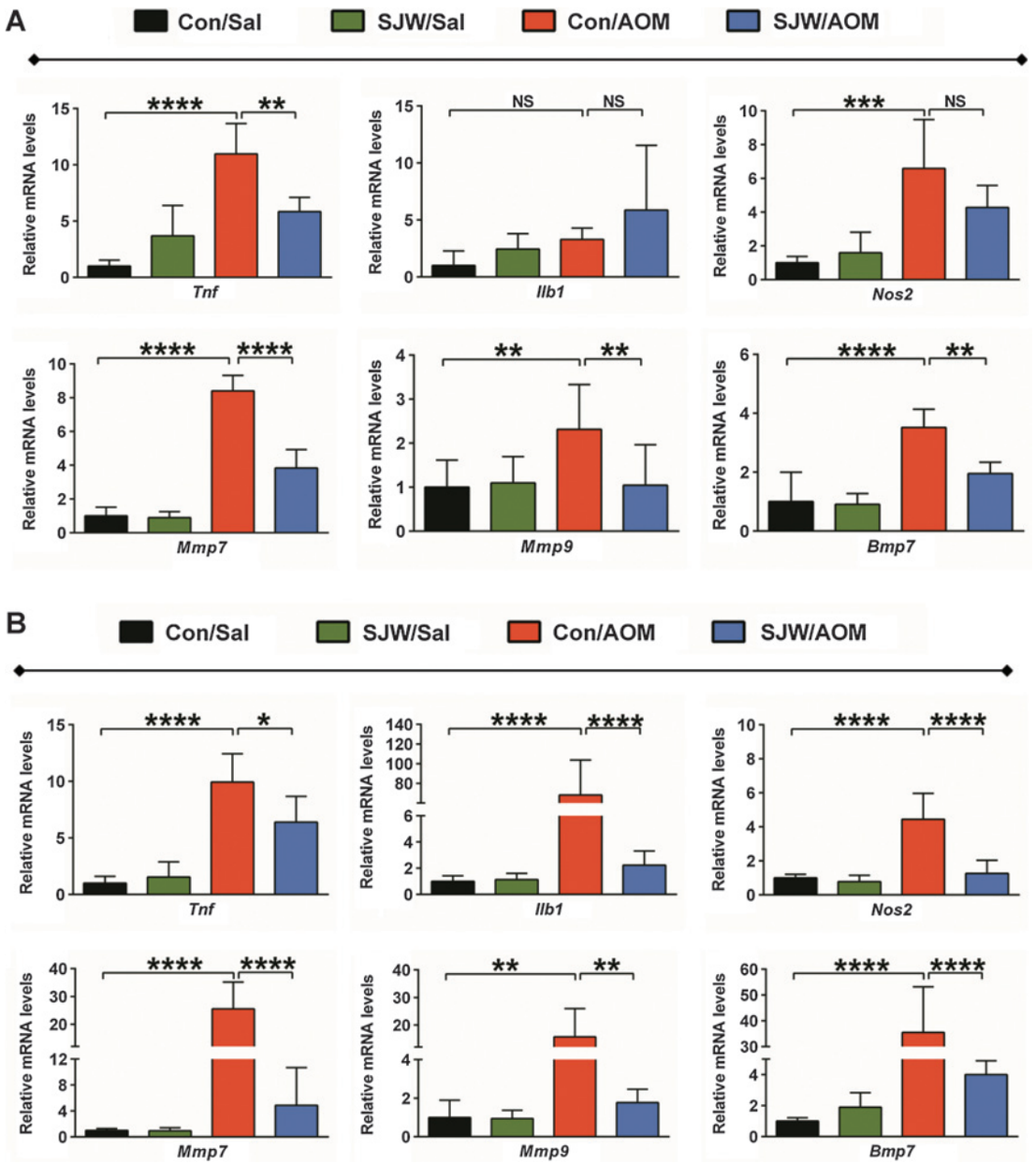

Figure 6.
qRT-PCR analysis of changes in the expression of representative genes related to NF-κB and ERK1/2 signaling pathway in colon epithelium due to SJW diet after just a 2 weeks (A) or 4 weeks (B) following the last azoxymethane injection: short-term study. Statistical significance was calculated using one-way ANOVA with the Tukey correction for multiple testing. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$, respectively. NS, not significant.

loss. These results suggest that long-term SJW use may be safe and attenuate colorectal carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.K. Manna, S. Golla, J.P. Golla, Y. Cai, K.W. Krausz, T. Matsubara
Writing, review, and/or revision of the manuscript: S.K. Manna, S. Golla, J.P. Golla, N. Tanaka, Y. Cai, F.J. Gonzalez
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.W. Krausz

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