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Effects of Black Soybean [*Glycine max* (L.) Merr.] Seed Coats and Its Anthocyanidins on Colonic Inflammation and Cell Proliferation *in Vitro* and *in Vivo*

JUNG-MI KIM,[†] JONG-SANG KIM,[‡] HOON YOO,[§] MYOUNG-GUN CHOUNG,[||] AND MI-KYUNG SUNG*,[†]

Department of Food and Nutrition, Sookmyung Women's University, Seoul 140-742, Korea, Department of Animal Science and Biotechnology, Kyungpook National University, Daegu 702-701, Korea, Department of Pharmacology and Dental Therapeutics, Chosun University, Gwangju 501-759, Korea, and Department of Pharmacognocy Material Development, Kangwon National University, Samcheok 245-711, Korea

Anti-inflammatory and antiproliferative activities of anthocyanidins and anthocyanin-rich black soybean seed coats were studied in HT-29 human colon adenocarcinoma cells and carcinogen-treated F344 rats, respectively. Cyanidin and delphinidin significantly inhibited cell growth at concentrations of ≥ 1 μM. Anthocyanidins suppressed cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) mRNAs in TPA-stimulated HT-29 cells. Both yellow and black soybean seed coat supplementation (10%, w/w) did not significantly reduce the number of aberrant crypt foci (ACF), although a modest decrease in the number of ACF was observed in animals fed soybean seed coats. The colonic COX-2 mRNA level was suppressed in rats fed both soybean seed coat diet. The plasma prostaglandin E_2 (PGE_2) level was reduced only in rats fed black soybean seed coats. No difference was observed in either colonic iNOS mRNA or plasma nitric oxide level. These results indicate that anthocyanidins are possible anti-inflammatory agents; however, further studies are required to determine required intake levels *in vivo* to exert antitumor effect.

KEYWORDS: Colon cancer; inflammation; anthocyanidin; Black soybeans [*Glycine max* (L.) Merr.]

INTRODUCTION

Colorectal cancer is one of the most common types of cancer worldwide. Previous epidemiological and experimental studies have suggested that legume intake decreases the risk of colorectal cancer (*1*). A recent comprehensive report indicates, however, that there is no conclusive evidence showing that legumes reduce the risk of cancer, suggesting that more systematic studies are required (*2*).

Soybeans are one of the most frequently studied legumes for their anticancer activity. Among the components of soybeans that exert biological activities are isoflavones, saponins, phytic acid, fiber, and trypsin inhibitors (*3*). These components may contribute individually or synergistically to the aforementioned health benefit.

Black soybeans have been widely used as materials for traditional oriental medicines, unlike yellow soybeans, which have been used mostly as food materials. A distinctive difference between yellow and black soybeans lies in their anthocyanin contents because anthocyanins are responsible for most of the purple and dark-blue colors exhibited by plants.

A case control study (*4*) reported that black soybean consumption reduces the risk of breast cancer in Korean women. Daily intakes of black soybean were also associated with a reduced cardiovascular disease risk (*5*). A possible explanation for the disease risk reduction by black soybean intake is the fact that black soybeans contain antioxidants, such as anthocyanins, proanthocyanidins, and flovonoids. Anthocyanins are glycoside forms of anthocyanidins, which possess four or more phenolic groups in their aglycon moieties. Because of their phenolic structures, anthocyanins exert a strong radical-scavenging activity and thereby behave as natural antioxidants.

Recent studies have provided evidence that inflammation is associated with carcinogenesis (*6*). Chronic inflammation of the intestinal tract, typified by ulcerative colitis (UC), is a risk factor for colon cancer (*7*). Patients with persistent UC have a 5−7-fold higher risk of developing colon cancer compared to those who do not have UC (*8*). It has been widely accepted that regular

* To whom correspondence should be addressed: Department of Food and Nutrition, Sookmyung Women's University, 52 Hyochang-won-gil, Yongsan-gu, Seoul 140-742, Korea. Telephone and Fax: +82-2-710-9395. E-mail: mksung@sm.ac.kr.

[†] Sookmyung Women's University.

[‡] Kyungpook National University.

[§] Chosun University.

[||] Kangwon National University.

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nonsteroidal anti-inflammatory drug users have a lowered risk of developing colorectal cancer because inducible cyclooxygenase-2 is inhibited to synthesize pro-inflammatory PGE_2 (*9*). The increase in PGE_2 is observed in cancerous tissues, and it induces the signals for abnormal cell proliferation (*10*).

Reactive oxygen species possibly act as mitogens to stimulate COX-2 expression via nuclear factor κB (NFκB) activation (*11*), therefore inducing inflammatory responses. Activated NFkB is also shown to overexpress inducible nitric oxide synthase (iNOS), and the increased production of nitric oxide stimulates cyclooxygenase-2 (COX-2) expression, thereby repeating the vicious cycle (*12*). Therefore, antioxidants may effectively reduce inflammatory responses and alleviate the related pathological conditions by scavenging reactive oxygen species.

The aim of this study was to investigate the anti-inflammatory and antiproliferative activities of anthocyanins and anthocyanin-rich black soybean seed coat in colon carcinogenesis.

MATERIALS AND METHODS

Materials. The human colon adenocarcinoma cell line HT-29 cells were obtained from Korea Cell Line Bank (KCLB, Seoul, Korea). The cell culture reagents were purchased from GIBCO BRL (Grand Island, NY). The cyanidin, delphinidin, and pelargonidin chloride were purchased from Indofine Chemical Co., Inc. (Hillsborough, NJ). The soybeans [*Glycine max* (L.) Merr.], harvested in 2005, were provided by National Agricultural Federation Cooperative (NAFC, Yeocheon, Chungbuk, Korea). The azoxymethane (AOM) and 0.2% methylene blue solution were purchased from Sigma Chemical Co. (St. Louis, MO). The [methyl-^{3}H]thymidine was purchased from Amersham Biosciences (Buckinghamshire, U.K.). All of the other chemicals were of reagent grade.

Determination of Anthocyanin Content in Seed Coats. Anthocyaninin content in black soybean seed coats was determined as described previously (*13*). Briefly, seeds were cleaned in distilled water and subsequently dried at 105 °C for 2 h. The seed coats were separated, and anthocyanin pigments in seed coats were extracted with a solution containing 1% (v/v) HCl and 40% (v/v) CH_3CH_2OH. The extract was purified by Shepadex LH-20 and Lichroprep RP-18 open-column chromatography as stated previously (*13*). The purified anthocyanins were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Tosoh ODS-120T column (150 × 4.6 mm i.d., Japan).

Cell Culture. The cells were cultured in Dulbecco's modified Eagle's medium, which contained 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 mg/mL). They were maintained at 37 °C in a humidified atmosphere of 5% CO_2. To determine cell proliferation, HT-29 cells were seeded on a 96-well plate (1×10^3 well^{-1}). On the following day, the medium was exchanged with a fresh medium containing a final concentration of 1, 10, or 50 μM anthycyanin and was incubated for 24 h at 37 °C. To measure mRNA levels of COX-2 and iNOS, cells (2×10^6 mL^{-1}) were seeded in 100 × 20 mm plate. On the following day, the medium was changed with fresh medium containing a final concentration of 1, 10, or 50 μM of cyanidin, delphinidin, or pelargonidin in each well and incubated for 72 h at 37 °C in the presence of 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

Cell Proliferation. The cell proliferation assay was performed using a SPA [^{3}H]thymidine uptake assay kit from Amersham Biosciences, according to the instructions of the manufacturer. Cells treated with anthocynin were pulsed with 2 μCi well^{-1} [methyl-^{3}H]thymidine for 4 h. To each well, 75 μL of the bead/lysis buffer mix and a 25 μL enhancer were added and the radioactivity was determined using a scintillation counter (Packard Instrument Co., Meriden, CT).

Animals and the Experimental Procedure. Male F344 rats (4 weeks old) were purchased from Shizuoka SLC Co., Shizuoka, Japan. All of the animals were then housed in wire cages (three rats/cage), with free access to drinking water and basal-diet CE-2 (CLEA Japan, Inc., Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), lighting (12 h light/dark cycle), and temperature (23 ± 2 °C).

Table 1. Composition of the Experimental Diets[a] (g/100 g)

ingredients	control	black soybean	yellow soybean
casein	24.0	23.1	23.1
corn starch	10.0	10.0	10.0
sucrose	32.0	31.42	31.42
corn oil	20.0	20.0	20.0
cellulose	8.0	0	0
D,L-methionine	0.36	0.36	0.36
mineral mix[b]	4.2	3.8	3.8
vitamin mix[c]	1.2	1.1	1.1
choline chloride	0.24	0.22	0.22
black soybean coat	0	10	0
yellow soybean coat	0	0	10

[a] A modified AIN-76 diet; J. Nutr. 1980, 110, 1726. [b] Mineral mixture (g/kg): calcium phosphate dibasic, 500.00 g; sodium chloride, 74.00 g; potassium citrate monobasic, 220.00 g; potassium sulfate, 5200 g; magnesium oxide, 24.00 g; manganous carbonate, 3.50 g; ferric citrate, 6.00 g; zinc carbonate, 1.60; cupric carbonate, 0.30 g; potassium iodate, 0.01 g; sodium selenite, 0.01 g; chrominum potassium sulfate $12H_20$, 0.55 g. [c] Vitamin mixture (g/kg): riboflavin, 0.60 g; thiamin-HCl; 0.60 g; pyridoxine-HCl, 0.70 g; niacin, 3.00 g; calcium pantothenate, 1.60 g; folic acid, 0.20 g; biotin, 0.02 g; vitamin B_{12} (0.1%), 1.00 g; vitamin D_3 (400 000 IU/g), 0.25 g; vitamin E acetate (500 IU/g), 10.00 g; menadione sodium bisulfite, 0.08 g.

The animals were randomly assigned to three groups (12 rats/group) following 1 week acclimatization period. Groups I, II, and III were assigned to the modified AIN 76A control diet (American Institute of Nutrition, 1980), which contained 8% cellulose (w/w), 10% black soybean seed coat, and 10% yellow soybean seed coat, respectively. These three experimental diets contained identical amounts of total dietary fiber. The black and yellow soybean seed coat samples were collected by heating soybeans at 105 °C for 2 h and then removing the seed coats from each of them. The carbohydrate, fat, protein, and fiber contents of each experimental diet were adjusted to have equivalent energy density. The basal diet contained high fat (20%) to mimic the high-risk diet. The compositions of the experimental diets are shown in **Table 1**. All of the groups received two subcutaneous AOM injections (15 mg/kg body weight) at 7 and 8 weeks of age. All of the rats were fed these diets for 11 weeks. At the end of the experiment period, the animals were anesthetized with ether after food was withheld from them for 12 h. Seven rats from each group were used for the evaluation of the frequencies of precancerous lesions in the colon. The colonic mucosas of five rats from each group were collected and kept frozen at −80 °C for mRNA analysis. Blood samples were obtained by heart puncture for biochemical analysis. The protocol and use of the rats were approved by the Animal Care and Use Committee of the Department of Food and Nutrition of Sookmyung Women's University (Seoul, Korea).

Aberrant Crypt Foci (ACF) Count. The colons were cut open longitudinally to expose their luminal surfaces, and they were flushed with a physiological saline solution. Once opened, the colons were placed on a strip of filter paper along with another strip that was used to cover their exposed luminal surfaces. They were then placed on a tray containing 10% buffered formalin (Sigma Chemicals, St. Louis, MO) for fixation overnight. The proximal and distal portions of the colons were separated, and each half was cut into 3 cm long segments. These segments were placed on a Petri dish and were stained with a 0.2% methylene blue solution (Sigma Chemicals, St. Louis, MO). Each segment was examined, and the total numbers of ACFs and crypts per focus were counted.

RNA Extraction and Semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The extraction of the total RNA from HT-29 cells and the colonic mucosa of animals was carried out using a Trizol reagent. Single-stranded cDNAs were generated from the total RNAs, using a superscript first-strand synthesis system (Invitrogen, Carlsbad, CA), and these were amplified using a Maxim RT-PCR PreMix kit (iNtRON Biotechnology, Inc., Korea). The PCR oligonucleotide sequences for HT-29 mRNA that were used were as follows: forward 5′-CAGCCCACCAACTTACAATG and reverse 3′-TACACCTCTCCACCGATGAC for COX-2, forward 5′-ATGTC-

CGAAGCAAACATCAC and reverse 3′-TAATGTCCAGGAAGTAGGTG for iNOS, and forward 5′-TGTGATGGTGGGAATGGGTCAG and reverse 3′-TTTGATGTCACGCACGATTTCC for β-actin. The conditions of the PCR for the target genes were 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min for COX-2 and 94 °C for 30 s, 42 °C for 30 s, and 72 °C for 1 min for iNOS. The termination cycle included a prolonged extension at 72 °C for 5 min. The PCR oligonucleotide sequences for colonic mucosa that were used were as follows: forward 5-CAGCCCACCAACTTACAATG-3′ and reverse 5′-TACACCTCTCCACC GATGAC-3′ for COX-2 and forward 5-CGGATATCTCTTGCAAG TCCAAA-3′ and reverse 5′-AAGTATGTGTCTGCAGATATG-3′ for iNOS. The conditions of PCR for the target genes were 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min for COX-2 and 94 °C for 30 s, 042 °C for 30 s, and 72 °C for 1 min for iNOS. The termination cycle included a prolonged extension at 72 °C for 5 min. The PCR products were confirmed through 1.5% agarose gel electrophoresis and were visualized through UV transillumination (Bio-Rad Laboratories, Inc., Hercules, CA). All of the signals were normalized to the mRNA levels of the housekeeping gene β-actin and were expressed as ratios.

Measurement of Plasma PGE_2 and Total Nitric Oxide (NO). The PGE_2 levels in the plasma were measured using commercially available ELISA kits (R&D, Ann Arbor, MI). This assay is based on the competitive binding technique, in which the PGE_2 that is present in a sample competes with a fixed amount of horseradish peroxidase (HRP) labeled PGE_2 for the sites on a mouse monoclonal antibody. The absorbance was determined at 450 nm, with the wavelength correction set at 540 nm. The nitric oxide levels were measured using a commercially available competitive enzyme immunoassay kit (R&D Systems, Ann Arbor, MI). This assay determines the nitric oxide concentrations based on the enzymatic conversion of nitrate to nitrite via nitrate reductase. The absorbance was measured at 540 nm using a spectrophotometer plate reader.

Statistic Analysis. All of the data were expressed as mean ± standard deviation (SD). The differences between the means were analyzed using Student's t test, with $p < 0.05$ considered significant.

RESULTS

Cell Proliferation. The cellular uptake of [^{3}H]thymidine was measured to determine the effect of anthocyanidins on colon cancer cell proliferation. Cyanidin at a 1 μM concentration inhibited the proliferation of HT-29 cells by 19% at 48 h and by 16% at 72 h (**Figure 1**). Cell proliferation was suppressed by 41−63% at a 50 μM concentration. Delphinidin exhibited the highest growth inhibitory effect, showing 22−32% inhibition at a 1 μM concentration and 30−66% inhibition at 50 μM. Pelargonidin showed significant cell growth inhibitory effects only at a 50 μM concentration, with 72 h of incubation.

COX-2 mRNA Expression. The COX-2 mRNA expression of the HT-29 cells that had been treated with TPA was significantly increased compared to that of the untreated cells (**Figure 2**). Cyanidin suppressed the TPA-induced increase in COX-2 expression by 42.8 and 44.7% at 1 and 50 μM concentrations, respectively. Delphinidin reduced the expression by 14.4−26.3% at all of the concentrations that were used. Pelargonidin, however, showed a significant inhibitory effect only at a 50 μM concentration.

iNOS mRNA Expression. The HT-29 cells that had been treated with TPA showed a significantly increased expression of iNOS mRNA compared to the untreated cells (**Figure 3**). The TPA-induced increase in iNOS expression was significantly lowered in the cells treated with 1 and 10 μM concentrations of cyanidin by 18.1 and 14.6%, respectively. Delphinidin at 1, 10, and 50 μM concentrations inhibited iNOS expression by 22.2, 19.1, and 20.8%, respectively. Pelargonidin at a 10 μM concentration suppressed the expression by 9.6%.

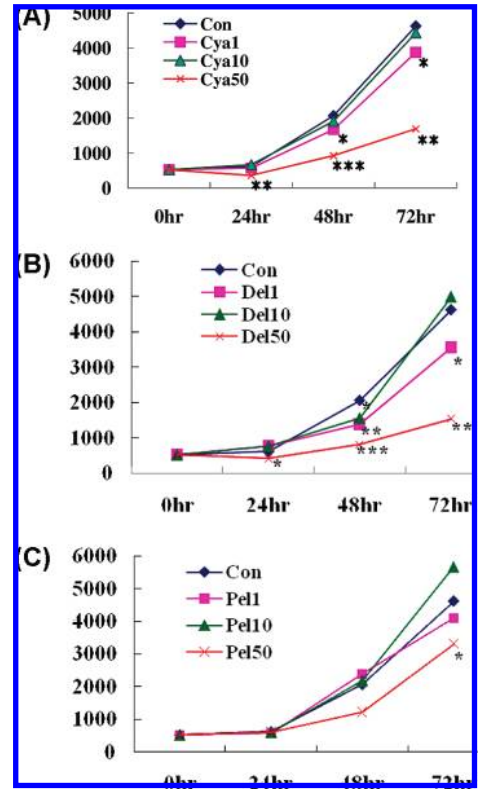

Figure 1. Effects of anthocyanidins on the proliferation of the human colon adenocarcinoma cell line HT-29 cells. (A) HT-29 cells were treated with cyanidin (A), delphinidin (B), and pelargonidin (C) for 24, 48, or 72 h (1, 10, or 50 μM concentration), followed by a 4 h pulse with 2 μCi well^{-1} [methyl-^{3}H]thymidine. The data are expressed as mean ± SD of $n = 3$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Anthocyanin Content in Black Soybean Seed Coats. The anthocyanin contents in seed coats of black soybean were determined on the basis of HPLC peak area with monitoring at 530 nm. Cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside content were 0.38, 0.16, and 0.05 mg/g black soybean seed coat, respectively.

Food Intake, Body Weight, Colon Length, and Liver Weight. There was no significant difference in daily food intake and body weight gain in all of the groups (**Figure 4**). Neither were there statistically significant differences in liver weight and relative liver weight between the control and the soybean seed coat supplemented groups. The lengths of the colons of the rats in the three experimental groups were not different from each other.

Incidences of ACF. The rats that had been fed the control diets showed a higher number of total aberrant crypts and total aberrant crypt foci compared to the rats that had been fed the 10% black soybean seed coat diet or the 10% yellow soybean seed coat diet, without a statistical significance (**Table 2**). No difference was shown either in the number of ACFs with ≥4 ACs.

Expression of COX-2 and iNOS mRNA in Colonic Mucosa. The COX-2 mRNA expression in both the 10% black soybean seed coat and the 10% yellow soybean seed coat supplemented groups was suppressed by 26.2 and 25.6%, respectively (**Figure 5**A). No significant difference was found, however, between the three experimental groups in terms of iNOS mRNA expression (**Figure 5**B).

Plasma PGE_2 and Nitric Oxide Level. The plasma PGE_2 level at week 12 is shown in **Table 3**. The rats that had been fed 10% black soybean seed coat showed a significantly lower

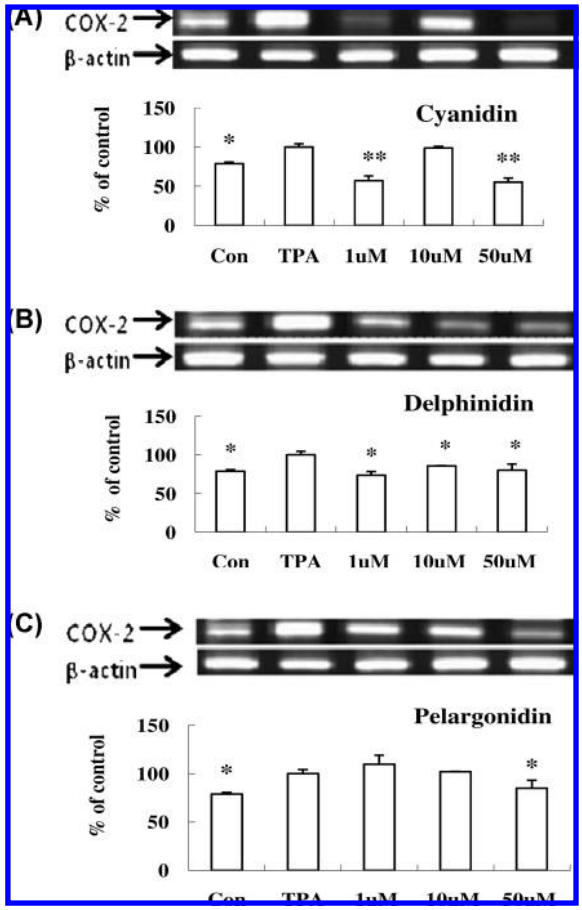

Figure 2. Effects of anthocyanidins with TPA-induced COX-2 mRNA expression in HT-29 colon adenocarcinoma cells. HT-29 cells (2×10^6 cells/mL) were cultured for 72 h with cyanidin (A), delphinidin (B), and pelargonidin (C). The data are expressed as mean ± SD of $n = 3$ (∗, $p < 0.05$; ∗∗, $p < 0.01$).

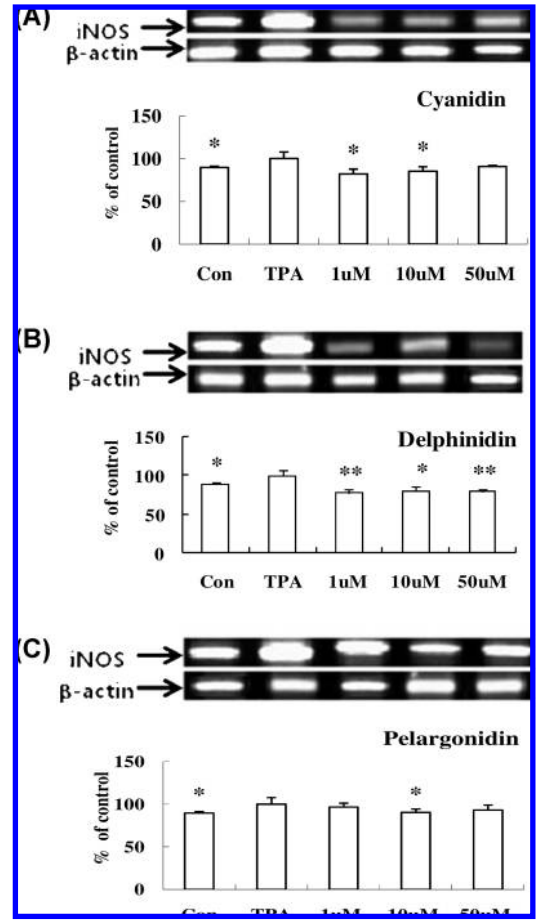

Figure 3. Effects of anthocyanidins with TPA-induced iNOS mRNA expression on HT-29 colon adenocarcinoma cells. HT-29 cells (2×10^6 cells/mL) were cultured for 72 h with cyanidin (A), delphinidin (B), and pelargonidin (C). The data are expressed as mean ± SD of $n = 3$ (∗, $p < 0.05$; ∗∗, $p < 0.01$).

PGE_2 level than the control rats. No such effect was found, however, with 10% yellow soybean seed coat. No difference was observed in the plasma level of total NO at week 12 (**Table 3**).

DISCUSSION

Anthocyanins are present in a wide variety of fruits and vegetables, especially in berries. The major anthocyanins found in higher plants are cyanidin, delphinidin, malvidin, pelargonidin, and petunidine, with an approximate distribution of 50, 12, 12, 7, and 7%, respectively (*14*). Black soybeans contain anthocyanins in the epidermis palisade layer of their seed coat. The anthocyanin content of black soybean seed coat ranges from 1.6 to 20.2 mg/g, depending upon the seed variety (*13*). Our study showed total anthocyanin content of 0.593 mg/g.

Anthocyanins have been cited as powerful antioxidants and possible anticarcinogens. Cyanidin glycoside and anthocyanin extracts from different plant sources inhibited cancer cell proliferation, possibly through their apoptotic (*15, 16*) and/or cell cycle arrest (*17, 18*) activities. In this study, the antiproliferative activities of major anthocyanins were compared by directly measuring the DNA synthesis of the cancer cells. Both cyanidin and delphinidin exerted significant growth inhibitory effects at ≤1 μM concentrations, while pelargonidin was effective at 50 μM. Previous studies have reported that anthocyanins inhibited cell survival at various concentrations (*16, 19*). Most of the studies showed effective inhibition of cell survival

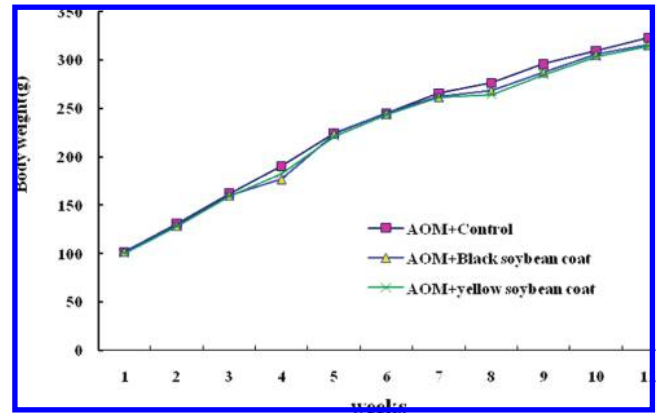

Figure 4. Body weight gain of the animals.

at concentrations between 50 and 200 μM, depending upon the types of cell lines that were used. In this study, 1−50 μM concentrations of anthocyanins were used because a less than 100 μM concentration did not have cytotoxic effects on the HT-29 colon cancer cells, indicating that anthocyanins at these levels may be used to suppress colon cancer cell growth without cytotoxicity. The difference in cancer chemopreventive activity between the anthocyanins can be explained by the presence of a free hydroxyl group at the 3 position in the B ring (*20*), and both cyanidin and delphinidin possess a free OH at the 3′ position, unlike pelargonidin, which does not have one at the same position. Another possible explanation is the number of

Table 2. Incidence of Aberrant Crypt Foci[a]

treatment	number of ACs[b]	number of ACF/colon	number of ACF with 4 ACs
control	153.25 ± 16.34	148.0 ±31.0	10.0 ± 2.5
10% black soybean	139.75 ± 6.65	115.2 ±31.1	10.8 ± 2.4
10% yellow soybean	145.40 ± 30.07	119.2 ±31.1	9.0 ± 3.2

[a] Values are means ± SD ($n = 7$). [b] AC, aberrant crypt; ACF, aberrant crypt foci.

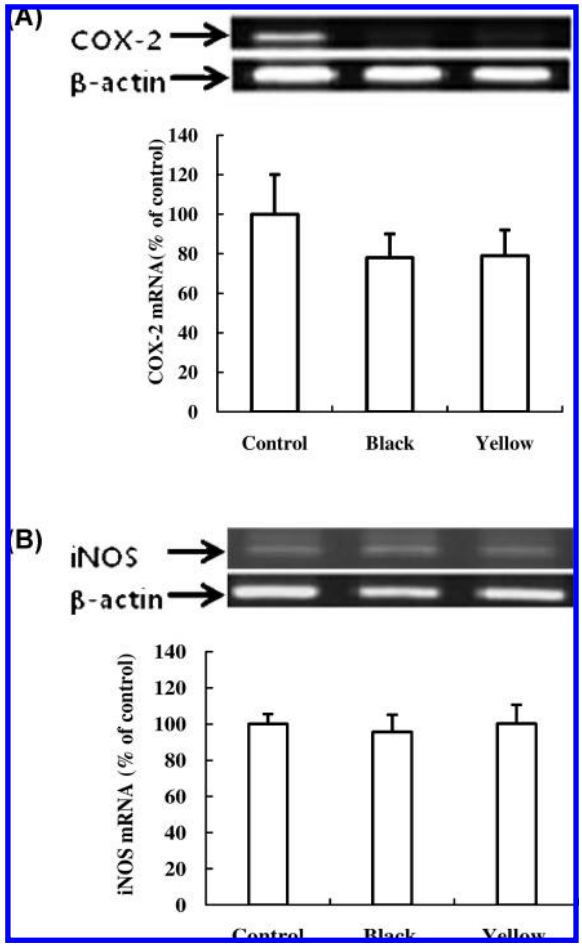

Figure 5. Effects of dietary black or yellow soybean seed coat on the colonic COX-2 (A) and iNOS mRNA (B) expressions in the rats that had been fed a high-fat diet. The data are expressed as mean ± SD of $n = 3$.

Table 3. Effect of Dietary Soybean Seed Coat Supplementation on Plasma PGE_2 and Nitric Oxide Concentration[a]

diet	PGE_2 (pg/mL)	nitric oxide (nmol/L)
control	2.20 ± 1.70 a	80.17 ± 20.63
10% black soybean	0.85 ± 0.97 b	71.76 ± 20.40
10% yellow soybean	2.62 ± 1.97 a	85.24 ± 21.18

[a] Values are means ± SD ($n = 5$). Means with letters (a and b) within a column are significantly different from each other at $p < 0.05$ as determined by Duncan's multiple range test.

hydroxyl groups in the B ring, because the numbers of total hydroxyl groups for delphinidin, cyanidin, and pelargonidin were 2, 1, and 0, respectively.

Bioavailability studies have indicated that the plasma concentration of total or individual anthocyanins is 1.4 nM with 500 mL of red wine consumption and 115 nM with black current concentrate consumption (*21*). Therefore, 1 μM anthocyanin contains less than 10 times the achievable plasma level of anthocyanins by fruit extract consumption.

Many human colon cancers exhibit increased expression of both COX-2 and iNOS. Both COX-2 and iNOS have been reported to be upregulated through the NFκB-mediated inflammatory process, and cellular oxidative stress is known to stimulate NFκB activation (*22*). COX-2 catalyzes the synthesis of prostaglandins, including PGE_2, which is shown to be elevated in cancer tissue compared to the adjacent normal tissue (*23*). Furthermore, nitric oxide, the product of iNOS, has been shown to upregulate COX-2 expression in many cell types (*24*). It has been suggested that iNOS expression may play an important role in dysplastic changes and that COX-2 expression is related to tumor growth (*25*). Therefore, COX-2 and iNOS may play an important role in colon carcinogenesis, whether tied to each other or independently. The results of this study show that anthocyanins, which are strong antioxidants, significantly reduced the expression of both COX-2 and iNOS, possibly by reducing the cellular oxidative stress, although there was no clear dose-dependent effect. Delphinidin significantly suppressed COX-2 and iNOS at all of the concentrations that were used, and this corresponds to its antiproliferative activity.

In the present study, the effects of an anthocyanin-rich black soybean seed coat diet on *in vivo* carcinogenesis were compared to those of a yellow soybean seed coat diet in rats that had been treated with azoxymethane. A previous study reported that black soybean seed coat extract showed a stronger inhibitory effect on low-density lipoprotein (LDL) oxidation compared to yellow soybean seed coat extract, possibly because of the higher polyphenol content of the former (*26*). The results of the present study, however, indicate that both the black and yellow soybean seed coat supplemented diets significantly reduced the COX-2 expression. It was hypothesized that the black soybean seed coat supplemented diet reduces COX-2 expression to a greater extent than the yellow soybean seed coat supplemented diet because of its higher anthocyanin content. Anthocyanins, however, may not be the sole components that exerted the observed inhibitory effect because the yellow soybean seed coat fed animals showed the same level of suppression that was shown by the black soybean seed coat fed animals. Chemical composition studies indicated that the difference between the black soybean seed coat and yellow soybean seed coat lies not only in their anthocyanin content but also in the types of fatty acids and sugars that are present in them. The difference between black soybean seed coat and yellow soybean seed coat in terms of the non-nutrient phytochemicals that they contain other than anthocyanin has not been studied, and such a study may be required to understand the inhibitory effect of yellow soybean seed coat on COX-2 expression. Because, in the present study, the total fiber contents of the experimental diets were adjusted (i.e., the cellulose content of the control diet was increased) to abolish the effects of the fiber in seed coat, fiber does not seem to be the component that is responsible for the observed effects of the seed coat diets. Further comparative analytical studies on seed coat with or without dark colors may thus be necessary.

The plasma PGE_2 concentration, however, was significantly decreased only in the rats that had been fed a black soybean seed coat supplemented diet, indicating that the black soybean seed coat may be a useful antitumorigenic material because PGE_2 has been cited as an important regulator of colon carcinogenesis (*27*). The discrepancies in the COX-2 and PGE_2 results may be partly explained by the factors regulating PGE_2 production other than COX-2. A group of prostaglandins is synthesized by COX-2, while PGE_2 synthase specifically regulates PGE_2 synthesis (*28*). Therefore, the effects of black

soybean seed coat may not be solely derived from COX-2 inhibition. Recent findings (*29*) have suggested that PGE_2, a product of COX-2, stimulates the β-catenin/TCF/LEF pathway that positions PGE_2 as the upstream regulator of the inflammation, suggesting that the COX-2 expression may not be proportionally related to the PGE_2 release. Therefore, anthocyanins may exert signals to pathway(s) independently of the COX-2 expression. No differences in the colon tissue iNOS expression and plasma nitric oxide level were observed, as opposed to the *in vitro* results. Although nitric oxide is known to stimulate COX-2 expression, it can be assumed that many signaling molecules other than nitric oxide are involved in inflammatory responses *in vivo*.

The aberrant crypt foci formation results show that the total number of ACs or ACFs was lowest in the rats that had been fed a black soybean seed coat diet, followed by the rats that had been fed a yellow soybean seed coat diet. No statistical significance was found though. The total anthocyanin content in soybean seed coat was 0.593 mg/g, and the dietary supply was approximately 17.79 mg rat^{-1} day^{-1}, presuming that rats consume a 30 g diet. This level may be too low to exert the antiproliferative activity in this model. Other studies also reported that COX-2 expression may not be related to aberrant crypt foci and tumor formation (*30, 31*). Another inhibitor study (*32*) showed that COX-2 inhibitors work at the later stage of colon carcinogenesis and not at the initiation stage, where aberrant crypt foci formation occurs. Genetic differences in responding to COX-2 inhibition in colon cancer development have also been suggested (*33*).

This study clearly shows that anthocyanidins are possible anti-inflammatory agents and that they inhibit cancer cell proliferation *in vitro*. The *in vivo* results in this study, however, show a partial effect of the anthocyanin-containing soybean seed coat in suppressing inflammatory responses, possibly because of the low plasma level of anthocyanins. Further studies on the effective dose, other biologically active compounds in seed coats, and implications of each inflammatory marker in colon carcinogenesis are required.

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