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Dose-response assessment of the anti-cancer efficacy of soy isoflavones in dimethylhydrazine-treated rats fed 6% fructooligosaccharide*

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Abstract

We investigated the combinatorial effects of different doses of dietary soy isoflavones (SI) and fructooligosaccharide (FOS) in a rat model of colon cancer. We hypothesized that increased bioavailability of SI metabolites due to dietary FOS may increase production of bioactive equol and affect colon carcinogenesis in a dose-dependent manner. Sprague-Dawley male rats were injected with 12-dimethylhydrazine (DMH) and were provided experimental diets that contained 0, 10, 50, 150, or 500 mg SI per kg of diet and 6% FOS for 12 weeks. The number of aberrant crypt foci (ACF) and the expression of cyclooxygenase-2 (COX-2) in colonic tissues were significantly decreased in the 6% FOS-fed groups compared to the control group. Gut transit time and fecal pH were significantly lower, and fecal concentrations of bifidobacteria were increased with 6% FOS. However, dietary SI supplementation in combination with 6% dietary FOS did not affect ACF formation or COX-2 expression. Plasma equol concentrations were dose-dependently increased by supplementation of SI up to 500 mg/kg of diet. In conclusion, SI supplementation up to 500 mg/kg of diet appeared to have no additive beneficial effects in rats with chemically-induced colon cancer that were fed 6% FOS, although plasma equol was dose-dependently increased.

Key Words: Aberrant crypt foci, cyclooxygenase-2, fructooligosaccharide, soy isoflavone, colon cancer

Introduction

Diet and behavior underlie the etiology of various types of cancer. Specifically, colon and rectal cancer occurrence is ten-fold higher in developed countries than in non-developed countries (WHO, 2003), and diet-related factors may account for up to 80% of this regional difference in cancer occurrence (Cummings & Bingham, 1998). Consumption of foods containing soy isoflavones (SI) such as genistein and daidzein has been weakly associated with reduced colon cancer risk (Cotterchio *et al.*, 2006; Messina & Bennink, 1998). Dietary SI and estrone were shown to reduce colon tumorigenesis in azoxymethane-treated ovariectomized mice (Guo *et al.*, 2004); however, the bioavailability of such isoflavones is influenced by their chemical form in foods, their hydrophobicity and susceptibility to degradation, the microbial flora of the consumer, and the food matrix (Birt *et al.*, 2001). In particular, equol, a product derived from intestinal bacterial metabolism (Atkinson *et al.*, 2004; Setchell *et al.*, 2002), displays more estrogenic potency than its precursor isoflavone, daidzein (Atkinson *et al.*, 2005). Nevertheless, the inter-relationships and effects of SI and colonic microflora on colon cancer are unclear.

Evidence from a wide range of sources supports the notion that colonic microflora are involved in the etiology of colon cancer (Lim *et al.*, 2005; Onoue *et al.*, 1997). Dietary fructooligosaccharide (FOS) improves the gut ecosystem by increasing bifidobacteria and dose-dependently shortens gut transit time in 1,2-dimethylhydrazine (DMH)-treated rats (Sung *et al.*, 2006). Specifically, FOS alters the bioavailability of daidzein and genistein (Uehara *et al.*, 2001) and inhibits the formation of aberrant crypt foci (ACF) (Pierre *et al.*, 1997). In a previous study, we observed that FOS dose-dependently suppressed ACF formation and that supplementation of the diet with SI (1,000 mg/kg) resulted in an additional suppressive effect on colon carcinogenesis (Sung & Choi, 2008).

Therefore, we hypothesized that production of SI metabolites such as equol in the presence of FOS may increase the relative proportion of bioactive isoflavones and alter the expression of colon cancer biomarkers. Here we assessed the dose-response effectiveness of SI fed concomitantly with 6% FOS on ACF formation, cyclooxygenase-2 (COX-2) expression in colonic tissues, gut ecosystem, and plasma equol concentration in a rat model of colon cancer.

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Table 1. Experimental diet compositions (g/kg diet)

Ingredients	Normal	Control	SI-0 FOS	SI-10 FOS	SI-50 FOS	SI-150 FOS	SI-500 FOS
Cornstarch	509.5	509.5	509.5	509.45	509.25	508.75	507
Casein	200	200	200	200	200	200	200
Sucrose[1)]	120	120	60 (10.30)	60 (10.30)	60 (10.30)	60 (10.30)	60 (10.30)
Soybean oil	70	70	70	70	70	70	70
Fiber	50	50	50	50	50	50	50
Mineral mix	35	35	35	35	35	35	35
Vitamin mix	10	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Soy isoflavones[2)]	0	0	0	0.05	0.25	0.75	2.5
Fructooligosaccharide[3)]	0	0	60 (146.3)	60 (146.3)	60 (146.3)	60 (146.3)	60 (146.3)
Total	1,000	1,000	1,000	1,000	1,000	1,000	1,000

[1)] Sucrose and glucose contained in the fructooligosaccharide (FOS) syrup account for the difference (actual amount used shown in parentheses).
[2)] Amount of soy isoflavone powder containing 20.23% aglycone forms (daidzein 8.18%, glycitein 9.85%, and genistein 2.20%).
[3)] Fructooligosaccharide syrup (actual amount used shown in parentheses) was composed of 41% fructooligosaccharide, 25% water, glucose, and sucrose.

Methods and Materials

Animals and diets

Four week-old male Sprague Dawley rats (n = 70) were procured from Daehan Biolink Co. (Chungbuk, Korea) and were acclimated to the facility for 1 week. Water and standard rodent chow (Samyang Feed Co., Gangwon, Korea) were provided *ad libitum*. Rats (n=60) were injected subcutaneously twice per week for 4 weeks with DMH (20 mg/kg body weight) in ethylene-diaminetetraacetic acid (EDTA, 1 mM, pH 6.7) to induce colon cancer. Ten rats were injected with the equivalent volume of EDTA solution for use as normal rats. The DMH-injected rats were divided into six groups of ten rats each and were fed different experimental diets (Table 1) for 12 weeks.

The AIN93-based experimental diets (American Institute of Nutrition, 1993) contained 0, 10, 50, 150, or 500 mg of SI per kg of chow and were given concomitantly with 6% FOS. The ingredients for the AIN93 diets were purchased from Harlan Teklad (Madison, WI, USA). The FOS (CJ Co., Seoul, Korea) had 3-4 degrees of polymerization and consisted of 37% GF_2, 16% GF_3, and 2% GF_4 on a dry weight basis. The soy isoflavones (Shindongbang Co., Seoul, Korea) contained 20.23% aglycones (8.18% daidzein, 9.85% glycitein, and 2.20% genistein).

The rats were weighed twice per week. The protocols and use of rats were approved by the Animal Care and Use Committee of Daegu University (Gyeongsan, Korea).

Measurement of fecal pH and gut transit time

Fresh feces collected at week 12 of the experimental diet were diluted four times with distilled water, and a pH electrode (ORION model 420A; Thermo Electron Co., Waltham, MA, USA) that was completely immersed in the sample solution was used to measure the pH. The gut transit time was determined by measuring the time of the first appearance of carmine red in the feces following the addition of 0.5% carmine red to the diet.

Measurement of fecal microflora

At week 12 of the experimental diet, fresh feces were collected, placed in sterilized phosphate buffer, homogenized, and diluted. The fecal strains of bifidobacteria, lactobacilli, and bacteroides were cultured using *Bifidobacterium*-selective (BS) agar, Man Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany), and vancomycin-added (VA) medium (Ji *et al.*, 1994), respectively. The culture dishes were incubated at 37℃ under anaerobic conditions using Anaerocult A (Merck) for 72 h. Colonies characteristic of each bacterial group were visually counted, and the results were expressed as $\log_{10}$ colony-forming units (CFU) per gram of wet weight. Total anaerobes and aerobes were cultured in glucose-liver-blood (BL) agar under anaerobic and aerobic conditions, respectively, at 37℃ for 72 h, and the number of colonies formed was recorded.

Measurement of plasma concentration of equol

Plasma equol glucuronides and sulfates were hydrolyzed, and equol was extracted for time-resolved fluorescence (TR-FIA; Brouwers *et al.*, 2003) using an equol kit (Labmaster Ltd, Turku, Finland). All reagents and samples were brought to room temperature (20-25℃) before use. All reagents were purchased from Wallac Oy (Turku, Finland), and the TR-FIA for equol was performed as follows. An aliquot of 200 μL of acetate buffer (0.1 M, pH 5.0, containing 0.2 U/mL of β-glucuronidase and 2 U/mL of sulfates) was added to tubes containing 200 μL of plasma. After mixing, the samples were incubated overnight at 37℃. The following day, the free equol was extracted with 1.5 mL diethyl ether by careful mixing of the two phases for 3 minutes. After thawing, the water phase was frozen in a dry ice-ethanol mixture, and the ether phase was re-extracted with ether in a disposable glass tube; the ether phases were combined and evaporated to dryness at 45℃ in a water bath. Assay buffer (200 μL) was added to the tubes, and, after careful mixing, 20 μL of the solution corresponding to 20 μL of the original plasma sample were taken for TR-FIA. Its equol concentration was determined by a Victor 1420 multi-label counter with software version 2.0 for fluorescence measurements (Perkin Elmer Life and Analytical Sciences, Inc., Finland).

Quantification of ACF

Colons were removed, flushed with ice-cold saline, and slit open from the anus to the cecum on a cold plate. The distal

5-cm portion was excised, fixed in 10% buffered formalin solution for 24 h, and stained in 0.2% methylene blue solution for 10-15 min. The colons were placed mucosal side up on a microscopic slide and viewed using a microscope at 40× magnification. The numbers of ACF and aberrant crypts observed per focus were recorded (Bird, 1995).

COX-2 protein measurements

The remaining portion of the colonic mucosa was scraped with a glass slide, frozen in liquid nitrogen, and stored at -70℃. The frozen mucosa was homogenized in lysis buffer [10 mM Tris (pH 7.4) buffer containing 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10 mg/mL phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, 10 mg/mL leupeptin, 5 mM phenanthroline, and 28 mM benzamidine-HCl] at 4℃ as described previously (Cho *et al.*, 2005). After 30 minutes, the cell lysates were centrifuged for 30 minutes at 8,000 × *g* at 4℃. The protein concentration of the supernatant was measured using BCA Protein Assay Reagent (Bio-Rad, Hercules, CA, USA). The proteins were separated by 10% SDS-PAGE and blotted electrophoretically onto nitrocellulose membranes (Bio-Rad). The membranes were washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) that contained 0.05% Tween 20 (TBS-T) and blocked overnight at 4℃ in TBS-T buffer that contained 5% non-fat dried milk; they were subsequently incubated with rabbit anti-rat COX-2 polyclonal antibody (Abcam Ltd., Cambridge, UK) at room temperature for 2 h. After washing, the membranes were treated with goat polyclonal antibody to rabbit immunoglobulin G H & L (Cy5-conjugated; Abcam). The COX-2 protein band densities were measured using a Fluorescence Imaging Analysis System (Typhoon 9200; Amersham Biosciences, Piscataway, NJ, USA), and were expressed in densitometry units. Immunoblot analysis using rabbit polyclonal anti-β-actin (Abcam) was performed as a control for protein loading.

Statistical analysis

The results were expressed as means ± standard error of the mean (SEM). The SPSS release 15.0 software package (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. Differences of means between the groups that were significant at $P < 0.05$ using one-way analysis of variance (ANOVA) were tested by Duncan's multiple range tests at $P < 0.05$.

Results

Food intake and body weight gain

Weight gains were lower and food intakes and food efficiency ratios tended to be lower in FOS-fed groups than in the control group (Table 2). Average intakes of SI based on body weight and the amount consumed per day were calculated from the

Table 2. Effects of variable doses of soy isoflavones (SI) and of 6% fructooligosaccharide (FOS) on weight gain, food intake, and food efficiency ratio (FER)

Group	Initial weight (g)	Final weight (g)	Weight gain (g/day)	Food intake* (g/day)	FER* (g/100g)
Normal	361.60 ± 3.99	500.60 ± 14.07	1.59 ± 0.167^{b}	23.224 ± 0.632^{a}	6.979 ± 1.008^{b}
Control	339.10 ± 8.31	517.70 ± 10.78	2.04 ± 0.088^{a}	22.496 ± 0.376^{ab}	9.102 ± 0.422^{a}
SI 0-FOS	339.10 ± 8.40	479.80 ± 18.38	1.60 ± 0.124^{b}	21.338 ± 0.865^{ab}	7.476 ± 0.690^{ab}
SI 10-FOS	339.40 ± 8.54	478.70 ± 12.71	1.59 ± 0.054^{b}	21.169 ± 0.864^{b}	7.556 ± 0.406^{ab}
SI 50-FOS	339.60 ± 8.42	503.20 ± 10.34	1.87 ± 0.076^{ab}	21.460 ± 0.294^{ab}	8.719 ± 0.494^{ab}
SI 150-FOS	341.10 ± 8.28	489.70 ± 9.76	1.70 ± 0.851^{b}	20.911 ± 0.477^{b}	8.130 ± 0.332^{ab}
SI 500-FOS	340.30 ± 8.06	496.70 ± 16.50	1.78 ± 0.105^{ab}	20.847 ± 0.465^{b}	8.503 ± 0.516^{ab}

DMH-treated rats were fed an experimental diet that contained 0, 10, 50, 150, or 500 mg SI per kg of diet with 6% FOS for 12 weeks. Normal and control rats were fed AIN-93 diet.
Values are mean ± SEM for n=10; *n=5 (2 rats pooled).
Values in the same column with different superscripts are significantly different at $p < .05$.

Table 3. Effects of soy isoflavones (SI) and fructooligosaccharide (FOS) on fecal microflora of DMH-treated rats

Group	Bifidobacteria	Lactobacilli	Bacteroides	Total anaerobes	Total aerobes
			$\log_{10}$ CFU/g		
Normal	5.798 ± 0.199^{b}	6.513 ± 0.161^{b}	6.766 ± 0.150^{b}	6.864 ± 0.123^{b}	6.962 ± 0.170
Control	5.918 ± 0.157^{b}	6.560 ± 0.181^{b}	6.955 ± 0.269^{b}	6.913 ± 0.224^{b}	7.052 ± 0.219
Control-FOS	7.574 ± 0.175^{a}	7.106 ± 0.254^{ab}	7.916 ± 0.218^{a}	8.122 ± 0.201^{a}	7.147 ± 0.307
SI 10-FOS	7.473 ± 0.286^{a}	7.603 ± 0.342^{a}	8.458 ± 0.167^{a}	8.355 ± 0.195^{a}	6.867 ± 0.485
SI 50-FOS	7.145 ± 0.246^{a}	7.423 ± 0.297^{a}	8.081 ± 0.285^{a}	8.015 ± 0.221^{a}	7.419 ± 0.265
SI 150-FOS	7.036 ± 0.219^{a}	7.628 ± 0.283^{a}	8.247 ± 0.150^{a}	8.498 ± 0.134^{a}	7.352 ± 0.351
SI 500-FOS	7.344 ± 0.238^{a}	7.724 ± 0.220^{a}	8.293 ± 0.153^{a}	8.222 ± 0.179^{a}	6.927 ± 0.344

DMH-treated rats were fed an experimental diet that contained 0, 10, 50, 150, or 500 mg/kg of diet SI with 6% FOS for 12 weeks. Normal and control rats were fed AIN-93 diet.
Values are mean ± SEM for n=10.
Values in the same column with different superscripts are significantly different at $p < .05$.

contents of SI in the experimental diet and from food intake. The intakes of total SI/kg body weight/day were 0.562, 2.821, 8.319, and 27.679 mg from diets containing 10, 50, 150, and 500 mg SI/kg of diet, respectively. The daily intake of SI per animal corresponded to 0.212, 1.073, 3.137, and 10.424 mg, respectively. Nevertheless, there were no significant differences among groups with increasing doses of SI (Table 2).

Fecal pH, fecal microflora, and gut transit time

After 12 weeks of feeding, fecal pH was significantly lower in rats fed 6% FOS diets than in rats from normal and control groups. Gut transit time was significantly reduced with FOS supplementation, except that containing 500 mg of SI, compared to the control diet (Fig. 1). However, SI supplementation did not affect fecal pH or gut transit time.

The dosage of SI did not affect the fecal microflora (Table 3). Fecal concentrations of bifidobacteria, lactobacilli, bacteroides, and total anaerobes were significantly increased by FOS in diets, except lactobacilli concentration of the control-FOS group which was not significantly different from that of the control group. However, total aerobe concentrations were not significantly different among the groups.

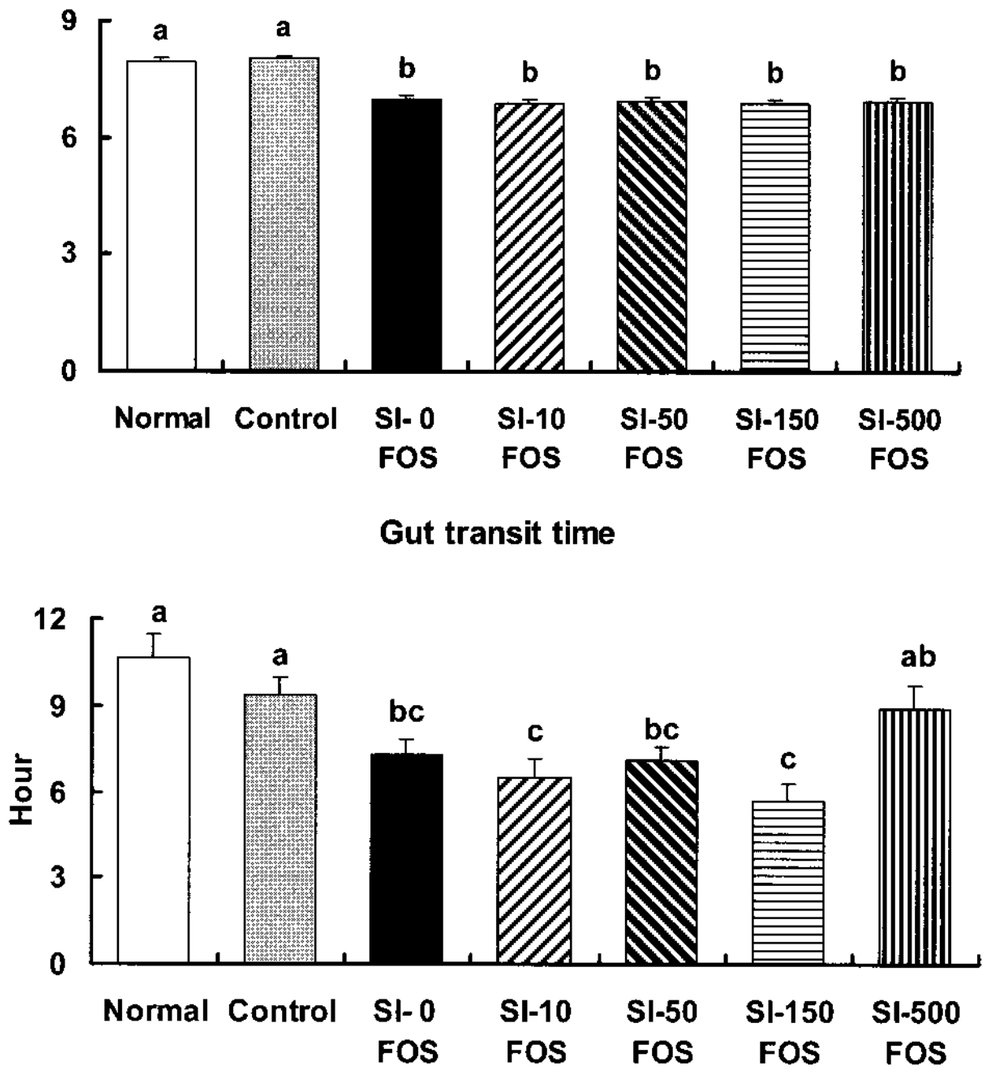

Fig. 1. Effects of different doses of soy isoflavones (SI) on fecal pH and gut transit time in DMH-treated rats fed a diet containing fructooligosaccharide (FOS). Rats were fed experimental diets that contained 0, 10, 50, 150, or 500 mg SI per kg diet and 6% FOS for 12 weeks. Data are mean ± SE values (n = 10). [a,b,c]Means with different letters are significantly different from each other by Duncan's multiple range test at $p < .05$.

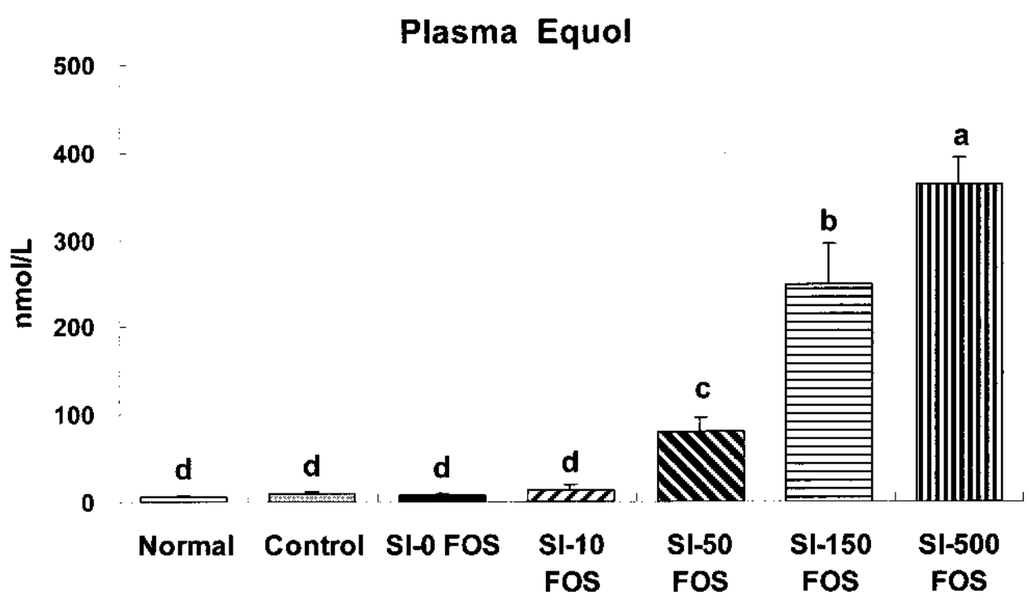

Fig. 2. Effects of different doses of soy isoflavones (SI) and fructooligosaccharide (FOS) on plasma equol concentrations in DMH-treated rats. Rats were fed experimental diets that contained 0, 10, 50, 150, or 500 mg SI per kg diet and 6% FOS for 12 weeks. Data are mean ± SE values (n = 10). [a,b,c]Means with different letters are significantly different from each other by Duncan's multiple range test at $p < .05$.

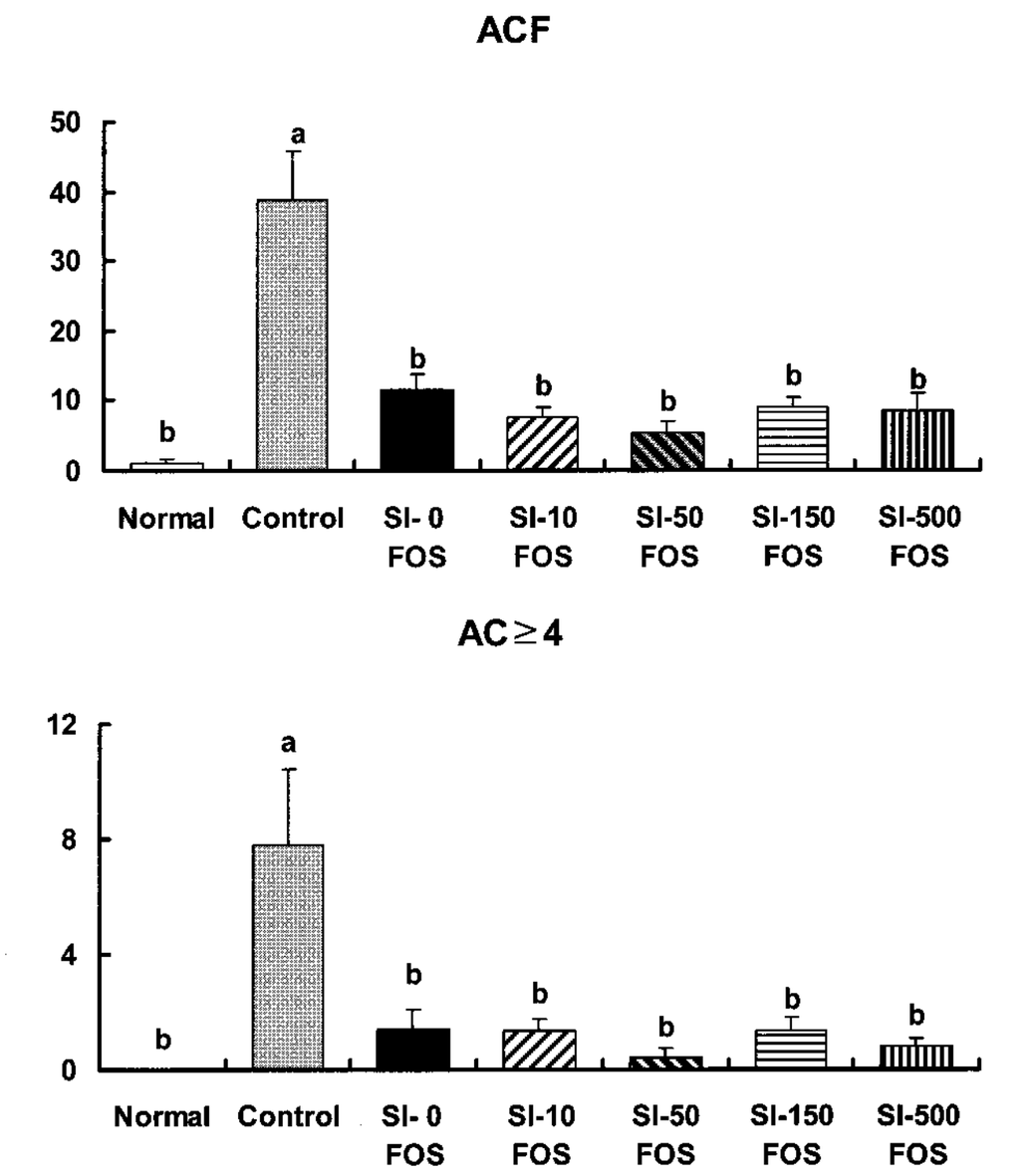

Fig. 3. Effects of different doses of soy isoflavones (SI) and fructooligosaccharide (FOS) on numbers of (top) ACF and (bottom) ACF with four or more ACs per focus in the colons of DMH-treated rats. Rats were fed experimental diets that contained 0, 10, 50, 150, or 500 mg SI per kg diet and 6% FOS for 12 weeks. Data are mean ± SE values (n = 10). [a,b]Means with different letters are significantly different from each other by Duncan's multiple range test at $p < .05$.

Plasma equol concentration

Plasma equol concentration was increased by SI in a dose-dependent manner up to 500 mg SI/kg diet (Fig. 2). The increase of plasma equol concentration at the dose of 10 mg SI/kg diet was insignificant compared to the control group. Concentrations of plasma equol were increased significantly with diets containing ≥50 mg SI/kg diet.

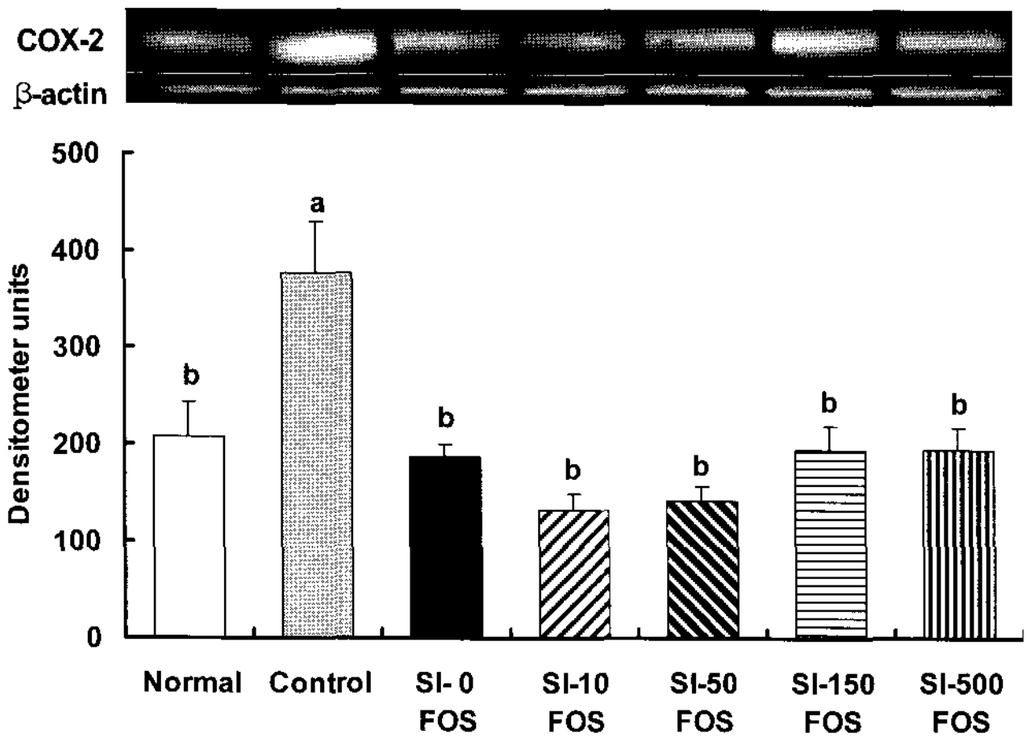

Fig. 4. Effects of different doses of soy isoflavones (SI) and fructooligosaccharide (FOS) on COX-2 protein levels in colonic tissues of DMH-treated rats. Rats were fed experimental diets that contained 0, 10, 50, 150, or 500 mg SI per kg diet and 6% FOS for 12 weeks. Data are mean ± SE values (n = 10). [a,b]Means with different letters are significantly different from each other by Duncan's multiple range test at $p < .05$.

ACF and COX-2 protein in colonic tissues

The numbers of ACF and the numbers of ACF containing four or more crypts per focus were decreased significantly in rats fed FOS, regardless of the dose of SI, compared to control rats that were not fed FOS. SI at all doses did not affect the number of ACF in rats (Fig. 3).

The expression level of COX-2 in the colonic mucosa was increased significantly in the control rats compared to the normal rats. Expression of COX-2 was significantly decreased with the addition of 6% FOS to the diet. However, the COX-2 expression level was not significantly affected by the addition of SI to the diet (Fig. 4).

Discussion

We assessed whether dietary SI, given with 6% FOS, would affect the development of DMH-induced early lesions of colon cancer in rats. The FOS-supplemented diet lowered ACF formation as well as COX-2 expression in colonic tissues, and these results are in agreement with those of our previous study (Sung & Chioi, 2008). ACF are lesions in which more than two aberrant crypts are stuck together, and ACF with four or more AC per focus are reliable predictors of malignancy (Bird & Good, 2000; Onoue *et al.*, 1997). Overexpression of COX-2 has also been observed in colon tumors (Sheehan *et al.* 1999); in animal studies, blocking COX-2 chemically or by mutation causes a marked decrease in colon cancer development (Jacoby *et al.*, 2000; Oshima *et al.*, 1996). Suppression of colon cancer biomarkers by 6% FOS in diets most likely occurred due to an improvement in the gut ecosystem. The FOS diet increased the proportion of bifidobacteria relative to total anaerobes, shortened gut transit time, and lowered gut pH. However, SI, even at high doses, did not affect the gut ecosystem or suppress biomarkers of colon cancer in rats.

Nielsen and Williamson (2007) summarized data from 16 studies on factors affecting the bioavailability of SI. According to their reports, the bioavailabilities of genistein and daidzein are increased by a rapid gut transit time and by low fecal digestion rates and are decreased by a fiber-rich diet; moreover, the summarized data suggest a reasonable correlation between the dose of SI and Cmax. In our study, feeding SI for 12 weeks resulted in a dose-dependent increase in the plasma equol concentration. The dose of 500 mg SI/kg diet corresponded to 27.68 mg SI/ kg body weight. There are few reports dealing with association of dose of SI, plasma equol concentration, and health benefit of increased SI intake. According to Mathey *et al.* (2004), plasma equol concentrations increased dose-dependently with SI up to 80 mg/kg body weight in ovariectomized rats fed experimental diets for 90 days. Furthermore, SI exhibited a bone-sparing effect at 20 mg/kg body weight/day, and addition of FOS to the diet significantly increased SI protection of the skeleton. Other work by Khalil *et al.* (2005) showed that 600 mg and 1200 mg SI/kg diet resulted in a bone-sparing effect in orchidectomized aged male rats that were fed the experimental diets for 180 days. Collectively, these studies demonstrate that fairly high levels of SI (i.e., ≥20 mg/kg body weight) are required for beneficial effects on bone. Of these isoflavones, daidzein which is a direct precursor of equol appears to be more efficient for the prevention of bone loss than genistein as demonstrated by studies in castrated rats (Picherit *et al.*, 2000).

The average intake of total SI/kg body weight/day estimated from the diet containing 500 mg SI/kg was 27.679 mg (11.192 mg daidzein and 2.955 mg genistein) in this study. The level is below NOAEL (no observed adverse effect level) of genistein, 50 mg/kg body weight/day estimated by McClain *et al.* (2006) who carried out hazard analyses for acute, subchronic and chronic safety of genistein in rats.

Several epidemiological studies suggest that isoflavones in soy may prevent the development of colon cancer in humans (Adlercreutz, 1995; Birt *et al.*, 2001; Cotterchio *et al.*, 2006). Consistently, our previous study showed an independent suppressive effect of SI (1,000 mg/kg of diet) on colon cancer biomarkers, and these effects of SI were also synergic with FOS. In contrast, Sørensen *et al.* (1998) reported that high amounts of SI (475 mg of total isoflavones per kg diet) in a Western-type high risk diet did not protect against intestinal tumor development in a relevant animal model such as *Min* mice. Furthermore, animal studies in general have not demonstrated an inhibitory effect by soy foods and isoflavones on the development of chemically-induced colorectal cancer (Messina & Bennink, 1998). In a recent study by Daly *et al.* (2007), dietary SI (2 g/kg diet) did not provide protection against the development of preneoplastic colonic ACF in female rats. However, Guo *et al.*, (2004) reported that estrone and SI have been associated with

lower incidences of colon tumors.

Prebiotics such as FOS increase the intestinal bioavailability and affect the metabolism of isoflavones in rats (Uehara *et al.*, 2001), and increase microbial production of equol in mice (Ohta *et al.*, 2002). In our study, even relatively large doses of SI (up to 500 mg/kg diet) that increased plasma equol did not offer additional protection to that provided by FOS against colon carcinogenesis. These results indicate that equol production from SI is not directly related to an inhibitory effect on colon cancer. It is plausible, however, that a favorable ecosystem of hosts that was maintained by the presence of 6% dietary FOS may negate the suppressive effect of SI on biomarkers of colon cancer. Further studies are required to delineate a potential role for SI themselves in colorectal carcinogenesis.

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