Chemoprevention of Azoxymethane Induced Colon Cancer in Rats by Feeding Orange Juice, Soy, Wheat Bran and Flaxseed

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ABSTRACT

Epidemiologic studies consistently demonstrate an inverse relationship between risk for colon cancer and consumption of fruits and vegetables. Wheat bran, flax and soy contain dietary fiber and phytochemicals, such as lignans and isoflavones, that may inhibit colon carcinogenesis. Orange juice contains hesperidin, a flavanone glucoside that protects against colon carcinogenesis. This study determined if feeding orange juice, wheat bran, soy and flaxseed (combined diet) would inhibit azoxymethane (AOM) induced colon cancer. Cancer was initiated in male Fisher 344 rats by injecting 15 mg AOM/kg of weight at 22 and 29 days of age. One week after the second AOM injection, rats (N = 30) in the combined diet group received dry diet containing wheat bran (4%), soy with ethanol soluble phyto-chemicals(13%) and flaxseed (8%) and orange juice replaced drinking water. The control group remained on the control diet and received distilled water to drink. The rats were killed 28 weeks later, and colon tissues and tumors were removed for histologic analysis. Feeding the combined diet significantly reduced tumor incidence (p < 0.05), however tumor multiplicity was not changed (p > 0.05, 0.9 tumors/rat fed the combined diet vs 1.2 for controls). Also, tumor burden was only marginally reduced in rats fed the combined diet vs control rats (65 vs 210 mg of tumor/rats, respectively). The reduction in tumor incidence was associated with a decreased labeling index and proliferation zone in normal appearing colon mucosa. Therefore, this study shows that phytochemicals in wheat bran, soy, flax and orange juice reduce colon carcinogenesis, presumably by decreasing cell proliferation and enhancing cell differentiation.

KEY WORDS: chemoprevention, phytochemicals, cell proliferation, colon cancer, rats.

INTRODUCTION

Colon cancer is the only cancer that occurs with similar frequency in women and men and is the third (women) and fourth or (men) cause of cancer deaths in the word.[1] Epidemiologic studies[2,3] demonstrate that there is an inverse relationship between the consumption of fruits and vegetable and risk for colon cancer. Diet is a major factor in the etiology of colon cancer.[4] Dietary soy, wheat bran, flaxseed, and citrus products inhibit colon cancer.

Dietary soy is considered protective against the development of prostate, breast and colon cancer.[5,6] Soy contains a variety of anti-cancer phytochemicals such as isoflavones, phytates, phytosterols, protease inhibitors and saponins.[6] The anti colon cancer activity in specific soy products is associated with ethanol soluble soy phytochemicals, not with soy protein.[7] Since soy phytochemicals are associated with many health-related and clinical benefits, soy and soy products have drawn much attention as promising chemopreventive agents.

Wheat bran is an effective dietary fiber with anti-cancer affects in the colon.[8] Proposed mechanisms for colon cancer inhibition by fiber is through: 1) dilution of carcinogens, procarcinogens, and tumor promoters; 2) enhanced transit through colon; 3) fermentation of fiber to butyric acid which can promote differentiation and apoptosis of colon tumor cells and inhibit their growth[9]; and 4) caffeic and ferulic acid components in insoluble fibers.[10]

Flaxseed is the richest dietary source of lignans.[11] Plant lignans are metabolized by bacteria in the colon to produce the mammalian lignans, enterolactone and enterodiol.[12,13] Flaxseed ingestion inhibits aberrant crypt foci formation in the colon, presumably due to the anti-carcinogenic lignans in flaxseed.[14] Certain flavanoids, limonoids, and cumarins present in citrus fruits have anti-cancer activity.[15]

Many chemopreventive phytochemicals in foods are present as glycosides that are not absorbed in the small intestine but pass to the colon. Colonic bacteria hydrolyze the glycosidic bonds to liberate aglycones that can be absorbed. Thus, the luminal surface of the colon mucosa is exposed to relatively high concentrations of potential anti-cancer phytochemicals. We hypothesized that combining

wheat bran, flax, soy and orange juice (combined diet) would provide a mixture of anti-cancer phytochemicals that would strongly protect against colon cancer. This study tested the hypothesis that feeding the combined diet would provide sufficient quantities of chemopreventive phytochemicals to inhibit AOM induced colon cancer. In addition, colon epithelium proliferation characteristics were determined to further understand the mechanism of chemoprevention by phytochemicals in combined diet.

MATERIALS AND METHODS

1. Animals and diets

Twenty one day-old Male Fischer 344 rats were obtained from Charles River Raleigh (Raleigh, NC). Upon receipt, the rats were housed three per plastic cage with sawdust bedding in an animal room maintained at $20-24\,^{\circ}C$ and $50-70\%$ relative humidity with a 12-hour light-dark cycle. The diets contained 16.7% fat, 18.9% protein, 8.1% total dietary fiber and 0.1% calcium (Table 1). The mineral and vitamin mixes, methionine, cystine, and choline were purchased from Dyets, Inc. (Bethlehem, PA). Other dietary ingredients were from local suppliers. The ratios of protein, minerals, and vitamins to energy were kept similar to the AIN-93G diet.[16] The control group was fed control diet and received distilled water to drink. The experimental group was fed a combined diet containing wheat bran (4%), soy with ethanol soluble phytochemicals (13%), and flaxseed (8%) (Table 1) and received single strength 'not from concentrate' orange juice (Tropicana Products, Inc., Bradenton, FL) to drink. Prototype breakfast cereals provided 64.9% of the diet. Additional ingredients were added to the ground cereals to provide essential nutritents for rats. The study was approved by the Michigan State University Committee on Animal Use and Care.

2. Experimental procedure

Colon cancer was induced by azoxymethane (AOM, Ash-Stevens, Inc., Detroit, MI). AOM (15 mg/kg of body weight) was subcutaneously injected between 8 and 10 a. m. on days 22 and 29 of age. All rats were fed control diet during the initiation period, i.e., from days 21 to 36 of age. On day 35, the rats were divided into two groups to ensure equal initial body weight. Starting on day 36, half of the rats (N = 30) were fed combined diet and given orange juice to drink while the other 30 rats were continued to receive control diet and water.

Table 1. Composition of diets[1]

Ingredients	Control diet	Combined diet
Cereal product	64.87[2]	64.87[2]
Whole wheat flour	8.31	8.31
Wheat starch	13.83	13.83
Oat flour	5.93	5.93
Corn flour	9.84	9.84
Sugar	12.89	12.89
Salt	0.70	0.70
Sodium bicarbonate	0.35	0.35
Citric acid	0.07	0.07
Full fat soy flour	0.00	7.00
Isolated soy protein	0.00	5.95
Soy concentrated	12.95	0.00
Additional Ingredients	35.13	35.13
Soy concentrate	6.62	6.47
Defattened flax flour	0.00	5.60
AACC wheat bran	0.00	2.80
Corn flour	5.63	0.00
Corn oil	10.53	10.62
Soy oil	4.85	3.56
Fiber	1.42	0.00
Mineral mix	3.89	3.89
Calcium carbonate	0.25	0.25
Vitamin mix	1.11	1.11
Methionine	0.22	0.22
Cystine	0.33	0.33
Choline bitartrate	0.28	0.28
BHT[3]	0.002	0.002
Total	100.00	100.00
Calculated composition[1]		
Protein	18.89	18.89
Dietary fiber	8.09	8.09
Total fat	16.67	16.67
Corn oil	11.67	11.67
Soy oil	5.00	5.00

[1]The amounts of protein, mineral mix and vitamin mix per 1000 kcal are similar to that prescribed for the AIN-93G diet (32). The mineral mix did not contain calcium.
[2]Percent
[3]Butylated hydroxytoluene
[4]Flours provided 1.14 and 1.05% fat

Seven months later at 35 wk of age, the rats were killed by over exposure to carbon dioxide followed by exsanguination. The colons were removed, cut longitudinally, rinsed with tap water to remove debris, pinned flat to cardboard and fixed in 10% neutral buffered formalin. Location of suspected neoplasms in the fixed colons were noted before the presumptive neoplasms were dissected and weighed. The abdominal cavities were examined visually for tumors and all suspected neoplasms were fixed in 10% neutral buffered formalin. A two cm long piece of normal appearing colon, approximately one-third of the colon length above the anus, was removed to determine cell proliferation by immunohistochemistry.[17]

3. Pathology and immunohistochemistry

Suspected neoplasms and normal colon were dehydrated and embedded with paraffin by routine histologic procedures. Three mm thick sections were cut from the neoplasms, stained with hematoxylin and eosin, and classified by a pathologist. The pathologist was blind to treatment.

Proliferating cells were detected in normal appearing colon mucosa by immunohistochemistry using a monoclonal mouse primary antibody to proliferating cell nuclear antigen (PCNA). Three mm thick sections were mounted on poly-L-lysine coated slides and placed in an oven at 58℃ for 2 hours. The sections were deparaffinized, hydrated and subjected to antigen retrievial by immersion in 10 mM citrate buffer (pH 6.0) for 20 min at 92 – 95℃ and rinsed in distilled water after the slides had cooled to room temperature. Endogenous peroxidase activity was blocked by placing the slides in a 0.3% hydrogen peroxide solution for 10 min. After rinsing in distilled water, the sections were incubated with primary antibody (PC-10, diluted 1 : 100 with 1% BSA in 10 mM Tris buffered saline, pH 7.4) overnight at 4℃, incubated for 45 min with biotinylated rabbit anti-mouse immunoglobulins (diluted 1 : 100), and incubated for 45 min with peroxidase-conjugated streptavidin (diluted 1 : 300). Peroxidase activity was detected by incubation with 3-amino-9-ethylcarbazole (AEC) for 15 min, lightly counter stained with Meyer's hematoxylin, and cover slipped using Faramount mounting medium. The antibodies, steptavidin, AEC, and Faramount were purchased from DA-KO (Carpinteria, CA). The sections were rinsed twice with 10 mM Tris buffered saline (pH 7.4) in between incubations with antibodies, steptavidin, and AEC. All incubations, except with PC-10, were done at room tem-

perature.

Ten crypts/rat were evaluated by two observers to determine cell proliferation parameters. Only full-length crypts extending from the muscularis mucosa to the lumen were counted. All nuclei lining one side of a crypt were numbered with the cell in the middle of the crypt base designated as cell number one and the cell at the top of the crypt mouth was assigned the highest number. In addition, each nucleus was recorded as PCNA positive or negative. Crypt height is the highest cell number and labeling index is the number of PCNA labeled nuclei divided by crypt height. Proliferation zone is the PCNA positive nucleus with the highest number divided by crypt height. Labeling index within each one-third of a crypt is the number of PCNA positive nuclei divided by the number of nuclei within that compartment.

4. Statistical analysis

Tumor incidence and tumor mulitplicity were analyzed using contingency table analysis and the χ^2 test. Tumor burden was analyzed by the Wilcoxon signed rank test. Data for initial and final weights and for cell proliferation parameters were compared by Student's t-test. The results were considered statistically significant when $p \le 0.05$.

RESULTS AND DISCUSSION

A previous study at our lab showed that the consumption of orange juice inhibited the early stages of AOM-induced colon cancer in rats suggesting that there are phytochemicals in orange with chemopreventive effects.[18] We hypothesized that feeding a combined diet of wheat bran, soy, flaxseed and orange juice containing a variety of phy-

Table 2. Weight gain and tumor data for rats fed control and orange juice diets[1]

Group	Weight gain (g)	Tumor		
		Incidence[2]	Multiplicity[3]	Burden[4]
Control	286.12 ± 2.4[a]	73[a]	1.2 ± 0.2[a]	210 ± 108[a]
Combined diet	276.79 ± 2.0[a]	53[b]	0.9 ± 0.2[a]	65 ± 22[a]

[1]Values are means ± SE. N = 30 rats/group. Means within a column with different superscripts are significantly different at p ≤ 0.05.
[2]Percentage of rats with one or more tumors.
[3]Number of tumors per rat.
[4]mg of tumor per rat

Table 3. Colon mucosa proliferation characteristics in rats fed the control and orange juice diets

Group	Labeling index	Proliferative zone	Labeling index in crypt compartments		
			Bottom 1/3	Middle 1/3	Top 1/3
Control	0.48[a]	0.63[a]	0.79[a]	0.58[a]	0.05[a]
Combined diet	0.46[b]	0.60[b]	0.79[a]	0.55[b]	0.04[b]
Pooled SE	0.011	0.013	0.021	0.026	0.010

[1]Values are means. N = 30 rats/group. Means within a column with different superscripts are significantly different at p ≤ 0.05.

tochemicals and dietary fibers would strongly inhibit colon carcinogensis. During the 28 wk feeding experimental period, growth was comparable and there was no significant difference in body weight gain between dietary treatments ($p > 0.05$, Table 2). Tumor incidence decreased 27% by feeding the combined diet ($p < 0.05$) compared to controls (Table 2). The reduction in tumor incidence and size indicates that the variety of phytochemicals and dietary fiber in the combined diet inhibited clonal expansion of initiated cells. In addition, feeding the combined diet produced a marginally significant reduction in tumor burden (p ≈ 0.08). That is, the phytochemicals inhibited accumulation of initiated cells, resulting in fewer rats with visible, smaller tumors.

Also the data suggest that the variety of phytochemicals in the combined diet did not have strong chemopreventive activity compared to feeding just orange juice.[18] Tumor incidence and tumor burden was only slightly lower in the combined diet group (53, 65 ± 22 mg of tumor per rat) compared to what was observed by feeding only orange juice (53, 118 ± 40 mg of tumor per rat).[18] The amount of phytochemicals in combined diet may not be sufficient to exert a strong preventive activity. Colon Cancer prevention by wheat bran,[19] flaxseed,[14] soy[7] or hesperidin[15] has been reported, but the combined effects from feeding a mixture of these phytochemicals on colon cancer had not previously been explored. Tumor multiplicity (average number of colon tumors per rat) was similar (0.90 vs 1.2) for both groups, but there was a strong trend ($p \approx 0.08$) towards a smaller average tumor burden (mg of tumor per rat) for the combined diet group (65 vs 210).

Colon mucosa cell proliferation indices are shown in Table 3. The percentage of PCNA positive nuclei (i.e., the labeling index) in colon crypts was reduced by feeding the combined diet ($p < 0.05$). In addition, the proliferative zone was lower ($p < 0.05$) in rats fed the combined diet. Labeling indices in middle 1/3 and top 1/3 of crypt compartments were lower in rats fed the combined diet ($p < 0.05$).

Abnormalities in epithelial cell proliferation, differentiation and maturation is one of the earliest indications of preneoplasia.[20,21] Studies in humans and animal models reveal that subjects with high risk of colon cancer have a larger proliferation zone and higher labeling indices than subjects at low risk for colon cancer.[20,21] Likewise, animals treated with a colon carcinogen have larger proliferation zones and labeling indices than animals treated with vehicle.[21] In this study, rats eating the combined diet had

smaller proliferation zones and lower labeling indices ($p \leq$ 0.05). The presence of proliferating cell nuclear antigen (PCNA) in the nucleous indicates that the cell is capable of further cell division while lack of nuclear PCNA indicates that the cell is no longer in the growth cycle and has differentiated. Therefore, feeding the combined diet caused more differentiation in the colon mucosa. Cell division in the bottom one third of the crypts was similar between groups ($p \geq 0.05$). However, feeding the combined diet reduced cell proliferation and enhanced cell differentiation in the upper two thirds of the crypt ($p \leq 0.05$). This suggests that the decreased tumor incidence was associated with altered cell proliferation patterns in the colon mucosa (decreased labeling index, increased cell differentiation, and reduced proliferation zone).

Whether feeding the combined diet could inhibit the initiation stage of colon carcinogenesis remains to be determined. However, phytochemicals in orange juice and flax have been shown to decrease the 'initiation stage' of AOM induced colon carcinogenesis.[14,15]

In conclusion, consumption of the combined diet containing a variety of phytochemicals from wheat bran, soy, flaxseed and orange juice was associated with a decrease in AOM induced colon tumor incidence in male rats. The reduction in tumor incidence was attributed to a decrease in cell proliferation, a smaller proliferation zone, and increased cell differentiation in colon crypts.

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