

Apigenin and naringenin suppress colon carcinogenesis through the aberrant crypt stage in azoxymethane-treated rats

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Abstract

Epidemiological evidence suggests that a diet abundant in fruits and vegetables may protect against colon cancer. Bioactive compounds, including flavonoids and limonoids, have been shown to possess antiproliferative and antitumorigenic effects in various cancer models. This experiment investigated the effects of four citrus flavonoids and one limonoid mixture at the promotion stage of chemically induced colon cancer in rats. Male Sprague–Dawley rats ($n = 10$ rats/group) were randomly allocated to one of six diets formulated to contain 0.1% apigenin, 0.02% naringenin, 0.1% hesperidin, 0.01% nobiletin, 0.035% limonin glucoside/obacunone glucoside mixture or a control diet (0% flavonoid/limonoid). Rats received experimental diets for 10 weeks and were injected with azoxymethane (15 mg/kg) at weeks 3 and 4. Excised colons were evaluated for aberrant crypt foci (ACF) formation, colonocyte proliferation (proliferating cell nuclear antigen assay), apoptosis (terminal deoxynucleotidyl transferase dUTP nick end labeling assay) and expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (immunoblotting). When compared with the control diet, apigenin lowered the number of high multiplicity ACF (HMACF >4 aberrant crypts/focus) by 57% ($P < 0.05$), while naringenin lowered both the number of HMACF by 51% ($P < 0.05$) and the proliferative index by 32% ($P < 0.05$). Both apigenin and naringenin increased apoptosis of luminal surface colonocytes (78% and 97%, respectively; $P < 0.05$) when compared with the control diet. Hesperidin, nobiletin and the limonin glucoside/obacunone glucoside mixture did not affect these variables. The colonic mucosal protein levels of iNOS or COX-2 were not different among the six diet groups. The ability of dietary apigenin and naringenin to reduce HMACF, lower proliferation (naringenin only) and increase apoptosis may contribute toward colon cancer prevention. However, these effects were not due to mitigation of iNOS and COX-2 protein levels at the ACF stage of colon cancer.

Keywords: citrus, limonoids, flavonoids, colon cancer

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Introduction

Aging of the ever-increasing US population is estimated to cause a 45% increase in overall cancer incidence rates, which includes a 52% increase in the incidence of colon cancer by 2030.¹ Despite the prominence of colon cancer mortality rates, colon cancer may be preventable when risk factors such as diet and lifestyle are modified,² as evidenced by studies of individuals migrating from low- to high-risk areas.^{3,4} The revised The World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR)⁵ report on cancer reiterated the relative chemoprotection derived from diets containing larger proportions of plant-derived foods. However, not all

the evidence supports these conclusions, as some studies have found little or no relationship between consumption of fruits and vegetables and colon cancer.^{6,7}

One of the studies indicating protection by diets with elevated fruit and vegetable servings is a prospective cohort study of Swedish women, in which the risk of colon cancer was greatest for those with the lowest intake of fruits and vegetables. Fruit consumption had a greater association with lowered risk.⁴ Similarly, a population study by Risch *et al.*⁸ suggested that fruits, especially citrus fruits, contain biologically active compounds besides

vitamin C that may play a role in preventing many chronic diseases including cancer. For example, orange juice⁹ or a 15% orange-pulp diet¹⁰ was able to suppress chemically induced colon tumors in rats. Among the constituents in citrus that may provide protection are the bioactive flavonoids and limonoids.^{11,12}

Au *et al.*¹³ found that ornithine decarboxylase activity and aberrant crypt foci (ACF) numbers were reduced by 0.1% apigenin, but it only modestly reduced tumor formation in azoxymethane (AOM)-injected rats and failed to reduce tumors in Min-mice. ACF in rats are also reduced by diets containing 20 mg/kg hesperetin (the aglycone of hesperidin).¹⁴ A mixture of 20 mg apigenin and 20 mg epigallocatechin-gallate suppressed colon neoplasia recurrence rates in human subjects with resected colon cancers.¹⁵ Similarly, we have shown that grapefruit pulp or isolated limonin and naringin suppress high multiplicity ACF (HMACF) because of lower levels of proliferation and enhanced apoptosis.¹⁶ These effects occurred in parallel with lower levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in response to limonin in the diet and a lower level of iNOS in response to naringin in the diet, suggesting changes in nitric oxide and/or prostaglandin synthesis may be mediating the benefits derived from these dietary interventions. Kohno *et al.*¹⁷ found that 0.01% nobiletin decreased prostaglandin E₂ (PGE₂) production in rats.

Based on this evidence, we hypothesized that citrus flavonoids (hesperidin, nobiletin, apigenin, naringenin) and limonoids (a limonin glucoside/obacunone glucoside mixture) would act as chemopreventive agents at the promotion stage of colon carcinogenesis. The potential mechanisms were theorized to involve regulation of proliferation and apoptosis caused by lower levels of expression of the proinflammatory mediators iNOS and COX-2.

Materials and methods

Animals and study design

The animal use protocol for this work was approved by the University Laboratory Animal Care Committee of Texas A&M University and conformed to the National Institute of Health guidelines. Sixty male weanling (21-d) Sprague-Dawley rats (Harlan, Houston, TX, USA) were individually housed in a temperature- and humidity-controlled animal facility with a 12-h light-dark photoperiod. Food and water were freely available at all times. After acclimation to the facility, the rats were stratified by body weight to one of six diets (10 rats/diet), which they consumed for a total of 10 weeks. All rats were injected with the colon carcinogen AOM (Midwest Research Institute, Kansas City, MO, USA; 15 mg/kg) on days 21 and 28 of the experiment. Body weights and intake were recorded on days 7, 21, 56 and 77. Rats were terminated six weeks after the last AOM injection and colon tissues collected.

Diets

The six diets (control, 0.1% hesperidin, 0.01% nobiletin, 0.02% naringenin, 0.1% apigenin and 0.035% limonin

glucoside/obacunone glucoside mixture) contained levels of experimental compounds previously shown to suppress either colon or oral cancer.¹⁷⁻¹⁹ We have also demonstrated protection against colon cancer by 0.02% naringin,¹⁶ and in order to compare those results with the current work, we used the same concentration for naringenin (the aglycone of naringin).

The experimental compounds were added to the diets at the expense of dextrose. Hesperidin (97% pure), naringenin (96% pure) and apigenin (97% pure) were isolated from methanol extracts of powdered dried peel from *Citrus reticulata*, *Citrus junos* and *Citrus aurantium* L., respectively.²⁰ Nobiletin (97% pure) was crystallized from hexane extracts of dried citrus peel²¹ and the limonin glucoside/obacunone glucoside mixture (76% pure) was purified from ethanol (EtOH) extracts of citrus seeds.²² Diets were mixed and stored in a -20°C freezer until use. The diets were analyzed by high-performance liquid chromatography (HPLC) as previously described²³ to verify the final diet concentrations of the experimental compounds.

Collection of tissue samples

Six weeks after the second AOM injection, each rat was euthanized, the colon excised and two 1-cm sections of the distal colon were fixed in EtOH (70%) or paraformaldehyde (4% PFA). After flushing with ice-cold phosphate-buffered saline (PBS), the remaining colon was split open and cut in half longitudinally. One half was fixed in 70% EtOH for ACF analysis and the other half was scraped to prepare protein isolates. The mucosal scrapings were homogenized in a protein buffer (50 mmol/L Tris-HCl (pH 7.2), 250 mmol/L sucrose, 2 mmol/L EDTA, 1 mmol/L ethylene glycol tetraacetic acid, 50 µmol/L NaF, 1% Triton X-100, 100 µmol/L sodium orthovanadate, 4% Sigma Protease Inhibitor Cocktail and 10 mmol/L beta-mercaptoethanol), lysed and centrifuged at 15,000g for 20 min at 4°C. The supernatant was stored at -80°C until use.

Aberrant crypt foci

This assay was performed using our standard procedures,¹⁶ in which the fixed colon tissue was stained with 0.5% methylene blue and examined at 40× to quantify aberrant crypt (AC) numbers. ACF containing more than four ACs were categorized as HMACF, as they are more likely to correlate with subsequent formation of adenomas and/or adenocarcinomas.^{24,25}

Colonocyte proliferation

Proliferation was measured with the proliferating cell nuclear antigen (PCNA) assay using reported procedures.¹⁶ EtOH-fixed colon tissue sections were incubated with a murine anti-PC-10 monoclonal antibody (dilution 1:50; Signet Lab, Dedham, MA, USA) followed by incubation with biotinylated anti-mouse IgG from the Vectastain ABC Elite kit (Vector Lab, Burlingame, CA, USA). Negative control tissues were prepared by incubating with PBS instead of anti-PC-10 antibody. Tissue sections were then

stained with diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical, St Louis, MO, USA) and counterstained with hematoxylin (Sigma Chemical). PCNA-containing nuclei were counted in 25 crypt columns per rat, with the total number of proliferating cells/crypt column, the proportion of proliferating cells and the position of the highest proliferating cell (proliferative zone) recorded.

Colonocyte apoptosis

The incidence of apoptosis in the colon epithelia was measured by terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling assay according to the method of Hong *et al.*²⁶ Samples (4% PFA fixed) from the distal colon were pretreated with proteinase K (Ambion, Austin, TX, USA) to remove cross-linking caused by PFA. A working solution containing terminal TdT-reaction buffer was incubated with tissue sections in a prewarmed humidified chamber, with the subsequent addition of anti-digoxigenin peroxidase. Visualization of the antibody-antigen complex was accomplished by staining with DAB. Positive control tissues were prepared by nicking DNA with deoxyribonuclease I (Ambion) and negative control tissues were prepared by substituting PBS for TdT in the working solution. Apoptotic cells were quantified both within the crypt and on the luminal surface for 50 crypt columns per rat. For each rat, the proportion of apoptotic cells within each crypt column and corresponding mucosal surface cells were recorded.

Immunoblot analysis of iNOS and COX-2

Protein concentrations of the supernatants extracted from the colonic mucosal homogenates were determined by a BCA Protein Assay kit using the manufacturer's instructions (Pierce, Rockford, IL, USA). Samples (30 μ g protein) were separated by Novex[®] 4–12% Tris-glycine gels for 3.5 h at 15 mA and 150 V and electrophoretically transferred to Invitrolon polyvinylidene fluoride membranes (Invitrogen, Carlsbad, CA, USA) for two hours at 300 mA and 25 V, both at 4°C.¹⁶ TriChromRanger prestained molecular weight marker mix (Pierce) was loaded into one of the wells of each gel for the identification of the relative positions of the desired protein bands. After the transfer procedure, membranes were thoroughly rinsed in Millipore[™] water, dipped into 100% methanol and air dried for 15 min. The membranes were then cut horizontally into three pieces, using the bands of the color marker as a guide, so that each piece of the membrane contained one protein band of interest. The three membrane pieces were individually rehydrated with methanol and processed separately. After blocking with 2% bovine serum albumin for one hour at room temperature (Fisher, Pittsburgh, PA, USA), the membranes were incubated overnight at 4°C with goat polyclonal anti-COX-2 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-iNOS antibody (1:375; Cayman Chemicals, Ann Arbor, MI, USA) or goat polyclonal anti- β -actin (1:10,000; Santa Cruz Biotechnology). The membranes were then incubated for one hour at room temperature with a bovine

anti-goat (COX-2 1:60,000; β -actin 1:300,000; Santa Cruz Biotechnology) or a goat anti-rabbit IgG-horseradish peroxidase conjugate (iNOS 1:60,000; Cayman Chemicals). The antibody-antigen complexes were developed with West Femo Maximum Sensitivity Substrate (Pierce), and the band intensities were scanned and quantified with a Bio-Rad Fluor-Imager (Bio-Rad Lab, Hercules, CA, USA) and Quantity One software (Bio-Rad Lab). The loading control was β -actin.

Statistical analysis

Animal weight and diet intake data were analyzed by one-way analysis of variance (ANOVA) or a repeated-measures model using the general linear model or Mixed Procedures in SAS (SAS Institute Inc, Cary, NC, USA). Evaluation of ACF, proliferation and apoptotic indices, and iNOS and COX-2 protein expression were analyzed by one-way ANOVA using the Proc Mixed method in SAS. Least squares means were separated using a Fisher's protected least significant difference for multiple comparisons. Pearson's correlation analyses in SAS were used for the evaluation of the relationships between ACF formation and proliferation or apoptosis, and between proliferation or apoptosis and iNOS or COX-2.

Results

Diet analysis

In order to verify the concentrations of experimental compounds in the diets, aliquots collected throughout the experiment were analyzed by HPLC using the concentrations and retention times of known standards. Quantification of the limonin glucoside/obacunone glucoside mixture in the final diet was not possible, as no standards existed. However, using available limonoid glucoside standards and HPLC methods we identified that the mixture primarily contained limonin glucoside and obacunone glucoside.²² The measured concentrations of experimental compounds were 0.096% for hesperidin, 0.007% for nobiletin, 0.037% for naringenin and 0.115% for apigenin. Except for naringenin, the concentrations were similar to the intended concentrations (0.1% for hesperidin, 0.01% for nobiletin, 0.02% for naringenin and 0.1% for apigenin).

Food intake and weight gain

Intake and weight gain were recorded throughout the experiment in order to determine if the experimental compounds influenced these variables. Rats tolerated the diets well as there were no differences in food intake among any of the diet groups overall or at any of the time points measured. However, the body weight of rats in the apigenin group was less than the body weight of rats from the hesperidin, nobiletin, naringenin and limonin glucoside/obacunone glucoside mixture groups on day 56, and from all diet groups on day 77 (Table 1). When weight gain over the experimental period was considered, apigenin

rats gained less than rats consuming the limonin glucoside/obacunone glucoside mixture diet (Table 1).

Incidence of ACF

Development of preneoplastic lesions (ACs) is a hallmark of colon carcinogenesis in both humans and animal models of the disease,²⁷ with HMAF being predictive of eventual tumor formation.²⁸ In rats provided with the experimental compounds, the total number of AC and the number of ACs per centimeter of colon were not different from those in rats provided with the control diet. In rats provided with the control diet, AOM induced an average of 3.94 HMAF per rat (Figure 1), with naringenin and apigenin yielding fewer (51% and 57%, respectively, $P < 0.05$) HMAF than in the control rats. The number of HMAF in rats provided with hesperidin, nobiletin and limonin glucoside/obacunone glucoside mixture were not different from the rats provided with the control diet.

Colonocyte proliferation

Loss of control over proliferation is a well-recognized transformation occurring during carcinogenesis. Therefore, we documented changes in the number, position and proportion of proliferating cells within the colon crypts. Naringenin lowered ($P < 0.05$) both the number of proliferating colonocytes and the proliferative index compared with the control diet (Table 2). In addition, rats provided with naringenin had a smaller ($P < 0.05$) proliferative zone (18% lower) when compared with rats in the control group (Table 2). There were no differences in the proliferative index or proliferative zone in rats consuming diets containing hesperidin, nobiletin or apigenin compared with rats provided the control diet. However, rats provided with the limonin glucoside/obacunone glucoside mixture did have a lower position of the highest labeled cell compared with the control diet (Table 2). Colonic crypt heights were not different among the diets.

Apoptosis

Induction of apoptosis among transformed cells is one mechanism whereby dietary compounds may protect

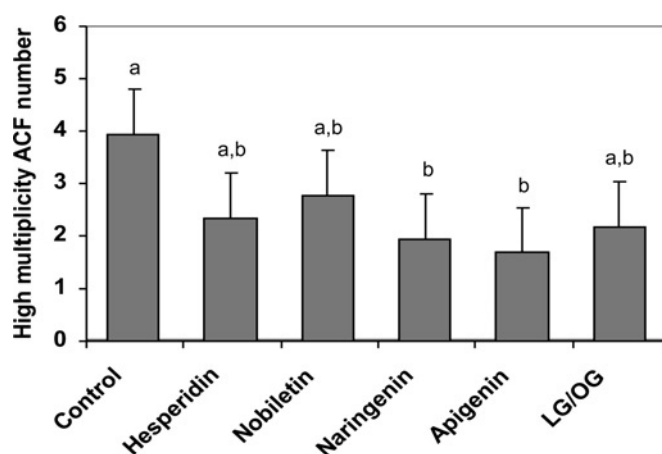


Figure 1 Effects of experimental diets on incidence of high multiplicity aberrant crypt foci (HMAF >4 aberrant crypts/focus) per colon. Values are least significant means $\pm$ standard error of mean, $n = 10$ rats/diet. ^{a,b}Bars with different letters are significantly different, $P < 0.05$ LG/OG, limonin glucoside/obacunone glucoside

against colon carcinogenesis. In order to determine if the experimental compounds were affecting colonocyte apoptosis, we documented the number and proportion of apoptotic cells within the crypt columns and along the luminal surface adjacent to the selected crypt columns. The number of apoptotic cells in the colonic crypts of all rats was only different between the naringenin and limonin glucoside/obacunone glucoside mixture diets (Table 3). Because extensive staining of apoptotic cells was observed on the luminal surface, the luminal surface colon cells were evaluated for the extent of apoptosis as well. The luminal surface apoptotic index for rats provided with dietary naringenin was 97% and apigenin was 78% higher ($P < 0.05$) than rats provided with the control diet (Table 3). The extent of apoptosis in luminal surface cells in rats consuming hesperidin, nobiletin or the limonin glucoside/obacunone glucoside mixture were not different from control rats.

Expression of iNOS and COX-2

Based on the literature describing the potential anti-inflammatory effects of compounds related to those used in this study, we sought to document the expression of iNOS and COX-2. There were no differences detected in the expression of iNOS and COX-2 enzymes in the colonic mucosa of rats among the diet groups because of the extensive variability in these measurements (Supplementary Figure). Interestingly, when the data for rats in all diet groups were combined, there was a positive correlation between the levels of iNOS and COX-2 enzyme expression ($r = 0.504$, $P < 0.05$).

Correlation analysis

The proliferative zone was negatively correlated with surface apoptotic index ($r = -0.279$, $P < 0.05$) when data for all diet groups were combined. In addition, there was a tendency for a negative relationship between proliferative index and surface cell apoptosis for the pooled data ($r = -0.237$, $P = 0.068$). In rats provided the naringenin

Table 1 Mean body weights and body weight gain^a

Diet group	Experiment day					Weight gain (g)
	0	7	21	56	77	
Control	66.7 ^a	124.6 ^a	194.0 ^a	332.4 ^{a,b}	381.0 ^a	313.8 ^{a,b}
Hesperidin	66.9 ^a	126.5 ^a	199.4 ^a	336.0 ^a	381.3 ^a	313.9 ^{a,b}
Nobiletin	67.1 ^a	126.9 ^a	198.3 ^a	336.5 ^a	383.5 ^a	315.8 ^{a,b}
Naringenin	67.1 ^a	126.6 ^a	197.5 ^a	338.5 ^a	384.4 ^a	316.8 ^{a,b}
Apigenin	66.7 ^a	125.0 ^a	194.5 ^a	320.9 ^b	365.3 ^b	298.0 ^a
LG/OG mixture	67.4 ^a	123.8 ^a	196.5 ^a	335.7 ^a	386.5 ^a	318.6 ^b
SEM	4.3	7.8	7.8	7.8	7.8	9.5

^{a,b}Values given are least significant means $\pm$ standard error of mean, $n = 10$ rats/group. Means in a column without a common letter differ, $P < 0.05$ LG/OG mixture, limonin glucoside/obacunone glucoside mixture

Table 2 Effect of experimental diets on number of proliferating cells, location of highest proliferating cell and crypt height in colon of Sprague–Dawley rats^a

Diet group	Proliferating cell number	Number of cells/crypt column	Proliferative index (%)	Location of highest proliferating cell (proliferative zone)
Control	6.64 ^a	32.05 ^a	20.74 ^a	14.98 ^a
Hesperidin	5.77 ^{a,b}	32.16 ^a	18.01 ^{a,b}	13.76 ^{a,b}
Nobiletin	4.94 ^{a,b}	31.21 ^a	15.85 ^{a,b}	12.58 ^{a,b}
Naringenin	4.47 ^b	31.98 ^a	14.03 ^b	12.12 ^b
Apigenin	4.77 ^{a,b}	32.11 ^a	14.98 ^{a,b}	12.78 ^{a,b}
LG/OG mixture	4.76 ^{a,b}	31.96 ^a	15.16 ^{a,b}	12.37 ^b
SEM	0.85	0.42	2.69	1.10

^{a,b}Values are least significant means $\pm$ standard error of mean, $n = 10$ rats/group. Means in a column without a common letter differ, $P < 0.05$

LG/OG mixture, limonin glucoside/obacunone glucoside mixture

diets, COX-2 level was positively correlated with proliferative index ($r = 0.896$, $P < 0.05$).

Discussion

The aim of this study was to investigate the chemopreventive ability of four citrus flavonoids (hesperidin, nobiletin, naringenin and apigenin) and one citrus limonoid mixture (containing limonin glucoside and obacunone glucoside) at the promotion stage of AOM-induced colon carcinogenesis in Sprague–Dawley rats. Dietary naringenin (0.037%) and apigenin (0.115%) reduced ($P < 0.05$) the incidence of HMAF in the rat colon by 51% and 57%, respectively, relative to the control diet. Although apigenin has been shown to inhibit AOM-induced ACF in CF-1 mice,²⁹ to our knowledge, this is the first study to demonstrate the ability of naringenin, a major flavonoid found in grapefruit, to reduce AOM-induced HMAF in Sprague–Dawley rats. Since HMAF are likely to correlate with future tumor formation,²⁴ these results suggest that dietary naringenin and apigenin may protect against colon carcinogenesis, similar to their effects in other tumor models.^{30,31}

The concentrations of hesperidin, nobiletin and limonin glucoside/obacunone glucoside mixture used in this study did not significantly suppress HMAF formation compared with the control diet. These observations contrast two studies in which dietary hesperidin (0.1%) and nobiletin (0.01%) inhibited ACF in F344 rats.^{17,18} Contrasting results may be due to strain differences between Sprague–Dawley and F344 rats or the time points at which the

observations were made. Taylor *et al.*³² showed febrile differences between the two rat strains after lipopolysaccharide injections and Tennekkes *et al.*³³ found Sprague–Dawley rats were more predisposed to certain tumors than F344 rats. A pioneer of the ACF model reported that ACF appear within two weeks after carcinogen injection, with numbers peaking at eight weeks.^{34,35} Rats in the studies by Tanaka *et al.*¹⁸ and Kohno *et al.*¹⁷ were terminated two weeks after the last carcinogen injection, thus there may not have been sufficient time for the ACF to reach their peak development. Since our study sacrificed the rats six weeks after the last AOM injection, the higher numbers of ACF observed in the hesperidin and nobiletin diet groups may be more reflective of the peak stage of AC formation.

Some studies have found that some citrus limonoids have chemopreventive properties, which was not observed with the 0.035% limonin glucoside/obacunone glucoside mixture used in this study. Tanaka *et al.*¹² found the aglycone form of limonin or obacunone (each at 0.02% of the diet) inhibited ACF formation in F344 rats. Because we were unable to confirm the final concentration of these compounds in our mixed diet, we do not know if the disparity in responses were due to differences in the dose delivered. However, a more probable reason for the difference in responses between our study and those of Tanaka *et al.*¹² are due to the glycosidic form of limonin and obacunone not generating the same protection against AOM-induced ACF as the aglycones.

Increased proliferation and suppressed apoptosis are typical in tumorigenesis.³⁶ In the current study, rats provided with naringenin had a reduced proportion of proliferating colon cells and smaller expansion of the proliferative zone than the control diet. The antiproliferative effects of naringenin have also been demonstrated in HT29 colon cancer cells.¹¹ Even though hesperidin, nobiletin, apigenin and the limonin glucoside/obacunone glucoside mixture yielded reductions in proliferation ranging between 13.2% and 27.8%, these differences were not significant. Cell culture experiments have reported antiproliferative effects for hesperetin, the aglycone form of hesperidin,³⁰ nobiletin,³⁷ apigenin,³⁸ and a limonoid glucoside mixture.³⁹ The apparent discordance between *in vivo* and *in vitro* studies raises the question of whether the metabolism of these compounds alters their effectiveness and/or functionality in an *in vivo* system. For example, Hanske *et al.*⁴⁰ recently demonstrated that apigenin-7-glucoside is metabolized to not only

Table 3 Effect of experimental diets on apoptosis within colonic crypt columns^a

Diet group	Number of apoptotic cells/crypt column	Luminal surface apoptotic index (%)
Control	0.20 ^{a,b}	20.71 ^a
Hesperidin	0.19 ^{a,b}	27.88 ^{a,b}
Nobiletin	0.22 ^{a,b}	26.67 ^{a,b}
Naringenin	0.32 ^a	40.81 ^c
Apigenin	0.18 ^{a,b}	36.93 ^{b,c}
LG/OG mixture	0.14 ^b	24.50 ^{a,b}
SEM	0.06	3.81

^{a,b,c}Values given are least significant means $\pm$ standard error of mean, $n = 10$ rats/group. Means in a column without a common letter differ, $P < 0.05$

LG/OG mixture, limonin glucoside/obacunone glucoside mixture

the aglycone form of apigenin, but also to low levels of naringenin (and other compounds) *in vivo*. Thus, although apigenin may be effective in reducing proliferation *in vitro*, its capacity to effect this change *in vivo* may have been reduced by its metabolism within the intestinal tract.

The extent of apoptosis within the colonic crypt columns was limited, and there were no differences in the apoptotic index among the diet groups, except between the naringenin and limonin glucoside/obacunone glucoside mixture diets. Tumors often develop when epithelial cells do not undergo apoptosis and instead accumulate on the luminal surface of the colon;⁴¹ thus apoptosis was measured in surface cells in this study. When compared with the control diet, surface cell apoptosis was enhanced by 97% and 78% in rats provided with naringenin and apigenin, respectively. Because changes in apoptosis may be more important in predicting colon tumorigenesis than cell proliferation alone,⁴² the upregulation of apoptosis observed with naringenin and apigenin may provide a possible explanation for their ability to inhibit HMAF. The results from this study extend existing *in vitro* observations and demonstrate that naringenin and apigenin are able to induce apoptosis *in vivo*. Proapoptotic effects were not seen in rats from hesperidin, nobiletin and limonin glucoside/obacunone glucoside mixture diet groups.

While there were no significant correlations between HMAF and either cell proliferation or apoptosis in this study, there was a slight negative correlation between the extent of the proliferative zone and surface cell apoptosis when data for all six diets were pooled. There was also a tendency for a negative relationship between proliferative index within crypt column and surface cell apoptosis for the pooled data. These results suggest that cell kinetics such as cell proliferation and apoptosis may have been modulated, but the individual effects of each diet were not strong enough to achieve significance. In any case, the negative relationship between cell proliferation and apoptosis is in agreement with evidence from the literature that increased cell proliferation together with decreased apoptosis may serve as a permissive environment for cancer development.³⁶

Another contributor to cancer development may be the induction of proinflammatory processes.^{43,44} Increased expression of two proinflammatory enzymes, COX-2 and iNOS, has been reported in human colorectal adenomas and adenocarcinomas.⁴⁵⁻⁴⁶ Apigenin and naringenin suppressed COX-2 or iNOS expression as well as NO and PGE₂ levels in lipopolysaccharide-stimulated macrophages.⁴⁷ Yet, in the current study, no differences in the levels of COX-2 or iNOS proteins among the six diet groups were observed. The data suggest that the protective effects of these bioactive compounds do not result from changes in the level of COX-2 or iNOS proteins in this *in vivo* colon cancer model. One possible explanation for the discrepancies between the *in vitro* and *in vivo* studies may be that most cell culture assays use transformed cancer cells so the products of tumor-promoting genes (e.g. iNOS or COX-2) are highly expressed. Samples from animal models, as occurs in humans, include cells that are not all transformed or cancerous. As a result, any protective

effects in such scenarios would more likely be harder to detect. Because the current study only measured the protein levels but not activities of iNOS and COX-2, it is not possible to know if the levels of NO and PGE₂ were affected by the compounds. Interestingly, there was a positive correlation between COX-2 level and proliferative zone in rats provided with naringenin, which was expected based on the literature linking PGE₂ and cell proliferation.⁴⁸ There was also a positive correlation between the enzyme expression levels of iNOS and COX-2 in the current study, when the data for rats in all diet groups were combined. Several cell culture studies and animal inflammatory models have shown the co-induction or co-regulation of iNOS and COX-2 enzymes.⁴⁹

Together, the data from this study indicate that naringenin and apigenin provide promise as naturally occurring chemopreventive agents against colon carcinogenesis. Although hesperidin, nobiletin and the limonin glucoside/obacunone glucoside mixture did not exhibit protective effects, they should not be discounted from future consideration since they have demonstrated effectiveness in other disease models. Furthermore, the differences in effect or lack of effect between different rat strains provided with the same compounds highlights the fact that higher biological systems are heterogeneous, suggesting that individual compounds at a certain dosage may be beneficial for some individuals but not for others. Therefore, more research in this area is needed to determine the optimum nutritional profile if we wish to pursue the goal of individualized medicine and nutritional recommendations.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; TL, JV, SST, LAD and NDT conducted the experiments; MEM, NW and RJC performed statistical analyses; BSP provided the experimental compounds; and TL, JV, RSC, JRL and NDT materially participated in data interpretation and manuscript preparation.

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