Suppression of colon carcinogenesis by bioactive compounds in grapefruit

Jairam Vanamala[1,2], Tety Leonardi[1],
Bhimanagouda S.Patil[2], Stella S.Taddeo[1],
Mary E.Murphy[3], Leonard M.Pike[2], Robert S.Chapkin[1],
Joanne R.Lupton[1] and Nancy D.Turner[1,*]

[1]Department of Nutrition and Food Science, [2]Vegetable and Fruit
Improvement Center, Department of Horticultural Sciences and
[3]Department of Statistics, Texas A&M University, College Station,
TX-77843, USA

[*]To whom correspondence should be addressed. Tel: +1 979-847-8714;
Fax: +1 979-862-1862;
Email: n-turner@tamu.edu

This study evaluated the hypothesis that untreated and
irradiated grapefruit as well as the isolated citrus com-
pounds naringin and limonin would protect against
azoxymethane (AOM)-induced aberrant crypt foci (ACF)
by suppressing proliferation and elevating apoptosis
through anti-inflammatory activities. Male Sprague–
Dawley rats ($n = 100$) were provided one of five diets:
control (without added grapefruit components), untreated
or irradiated (300 Gy, ^{137}Cs) grapefruit pulp powder
(13.7 g/kg), naringin (200 mg/kg) or limonin (200 mg/kg).
Rats were injected with saline or AOM (15 mg/kg) during
the third and fourth week and colons were resected
(6 weeks post second injection) for evaluation of ACF,
proliferation, apoptosis, and cyclooxygenase (COX)-2 and
inducible nitric oxide synthase (iNOS) protein levels.
Experimental diets had no effect on the variables measured
in saline-injected rats. However, in AOM-injected rats, the
experimental diets suppressed ($P \leq 0.02$) aberrant crypt
and high multiplicity ACF (HMACF; $P \leq 0.01$) formation
and the proliferative index ($P \leq 0.02$) compared with the
control diet. Only untreated grapefruit and limonin sup-
pressed ($P \leq 0.04$) HMACF/cm and expansion ($P \leq 0.008$)
of the proliferative zone that occurred in the AOM-injected
rats consuming the control diet. All diets elevated ($P \leq 0.05$)
the apoptotic index in AOM-injected rats, compared with
the control diet; however, the greatest enhancement was
seen with untreated grapefruit and limonin. Untreated
grapefruit and limonin diets suppressed elevation of both
iNOS ($P \leq 0.003$) and COX-2 ($P \leq 0.032$) levels observed in
AOM-injected rats consuming the control diet. Although
irradiated grapefruit and naringin suppressed iNOS levels
in AOM-injected rats, no effect was observed with respect
to COX-2 levels. Thus, lower levels of iNOS and COX-2
are associated with suppression of proliferation and
upregulation of apoptosis, which may have contributed to
a decrease in the number of HMACF in rats provided with
untreated grapefruit and limonin. These results suggest
that consumption of grapefruit or limonin may help to
suppress colon cancer development.

Abbreviations: AC, aberrant crypt; ACF, aberrant crypt foci; AOM, azoxymethane; COX-2, cyclooxygenase-2; HMACF, high multiplicity aberrant crypt foci; iNOS, inducible nitric oxide synthase.

Introduction

Evidence from epidemiological studies suggest that diets rich in fruits and vegetables are protective against a number of different cancers, including colon cancer (1). Mortality due to non-hereditary colon cancer appears to be reduced with appropriate changes in diet and modifiable non-dietary factors, such as smoking (2). Despite a few recent publications that suggest little protection against cancer with fruit and vegetable consumption (3,4), well-designed experiments with animal models demonstrate a protective effect of fruits and vegetables on colon cancer development.

Aberrant crypt foci (ACF), a surrogate biomarker for colon cancer induction by a colon specific carcinogen, azoxy-methane (AOM), was reduced in rats consuming freeze dried vegetables (peas, spinach, sprouts and broccoli) (5). Additional studies have reported the chemoprotective effects of tomato and onion on ACF formation in AOM-induced colon cancer (6,7).

Grapefruits are a rich source of bioactive compounds, which may serve as cancer chemopreventive agents (8). The red-fleshed varieties (e.g. Rio Red) contain flavonoids, limonoids and their glucosides, vitamin C, folic acid, caroten-oids (e.g. lycopene and beta-carotene), coumarin-related com-pounds (e.g. auraptene), soluble fiber and potassium. Limonin and obacunone isolated from grapefruit have been reported to reduce the incidence of colonic adenocarcinomas induced by AOM in male F344 rats (9). Part of the protection against colon cancer is likely derived from the ability of certain isol-ated compounds to influence cell cycle activity. Hesperidin, a citrus flavanone, reduced proliferation and induced apoptosis in colonocytes, and suppressed AOM-induced colon carcino-genesis (10). One mechanism whereby citrus flavonoids may influence proliferation and apoptosis is through their effects on the expression and activity of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (11,12).

Both iNOS and COX-2 are involved in chronic inflam-mation, which creates a microenvironment conducive for colon carcinogenesis (13). iNOS and COX-2 protein levels are enhanced in colorectal adenomas and adenocarcinomas in humans and chemically induced colonic tumors in rats (14–17). Excessive nitric oxide (NO) production can reduce DNA repair enzymes and suppress apoptosis by nitrosylation of caspases (18,19). NO has also been implicated in the upregulation of COX-2 activity (20,21). COX-2 contributes to pathological processes, such as inflammation, abnormal cell proliferation and reduced apoptosis found in colon cancer (22). Therefore, the suppression of iNOS and COX-2 levels or activity may protect against colon cancer development. Indeed, iNOS and COX-2 inhibitors reduce ACF in rats

Table I. Composition of the diets

Diet components	Control diet	Untreated grapefruit	Irradiated grapefruit	Naringin[a]	Limonin[b]
Dextrose[c] (g/100 g)	51.06	49.87	49.87	51.04	51.04
Casein[c] (g/100 g)	22.35	22.35	22.35	22.35	22.35
Pectin[d] (g/100 g)	6.00	5.82	5.82	6.00	6.00
Mineral mix[e] (g/100 g)	3.91	3.91	3.91	3.91	3.91
Vitamin mix[e] (g/100 g)	1.12	1.12	1.12	1.12	1.12
D,L-methionine[c] (g/100 g)	0.34	0.34	0.34	0.34	0.34
Choline bitartrate[e] (g/100 g)	0.22	0.22	0.22	0.22	0.22
Corn oil[f] (g/100 g)	15.00	15.00	15.00	15.00	15.00
Treatment components (g/100 g)	0.00	1.37	1.37	0.02	0.02

[a]Sigma-Aldrich, St Louis, MO.
[b]Limonin with 95% or greater purity (supplied by B.S.P., Texas A&M University, College Station, TX).
[c]Bio-Serv, Frenchtown, NJ.
[d]Danisco Cultor, New Century, KS.
[e]Harlan, Indianapolis, IN.
[f]Donated by Traco Labs, Champaign, IL.

(23–25). However, little information is available on the effect of citrus bioactive compounds on iNOS and COX-2 levels in relation to *in vivo* models of colon carcinogenesis.

This study evaluated the chemopreventive ability of pulp from untreated grapefruit, irradiated grapefruit (300 Gy, [137]Cs—a proposed quarantine dose against fruit flies), and two isolated compounds (naringin and limonin) against chemically induced colon carcinogenesis measured at the promotion stage. Specifically, the effect of grapefruit pulps and isolated grapefruit compounds on ACF, proliferation, apoptosis, and COX-2 and iNOS protein levels in Sprague–Dawley rats was determined.

Materials and methods

Animals, diets and study design
The animal use protocol was approved by the University Laboratory Animal Care Committee of Texas A&M University and conformed to NIH guidelines. This experiment utilized a 5×2 factorial design comprising five diets (Table I) and two injection protocols: AOM (Midwest Research Institute, Kansas City, MO) or saline injection. One hundred male Sprague–Dawley rats (Harlan Sprague–Dawley, Houston, TX) were individually housed and maintained in a temperature and humidity controlled animal facility with a 12 h light cycle. Twenty-one-day rats were acclimated for 4 days prior to receiving the defined diets for 10 week. Rats were stratified by body weight so that mean initial body weight was similar among the treatment groups ($n = 10$ rats/group).

Grapefruit pulp ($-80°C$) devoid of seeds, albedo and flavado was freeze dried at NASA, Shuttle and ISS Food Systems, Johnson Space Center, Houston, TX to improve the stability and uniformity of the pulp. Freeze dried pulp was stored under nitrogen at $-20°C$ prior to preparation of experimental diets. Freeze dried grapefruit pulp was powdered to ensure uniform diet mixing (Hobart Mixer). We calculated the energy composition of the grapefruit powders using the USDA Data Bank Values (Nutrient Composition Tables indicate that 100 g of peeled grapefruit contains 47 kcal). We determined that 100 g of fresh grapefruit results in 12.5 g of dry matter. Therefore, when we calculated the diet compositions, the removal of dextrose (54 kcal) was approximately equal to the energy contained in the grapefruit pulp (51.5 kcal). The levels of flavonoids, limonoids, carotenoids and terpenoids in freeze dried untreated and irradiated grapefruits have been reported previously (26).

The untreated freeze dried grapefruit pulp powder (13.5 g/kg) was incorporated into a modified AIN-76A diet to achieve a naringin concentration in the diet of 200 mg/kg. To evaluate the effect of proposed quarantine doses of irradiation (300 Gy, ^{137}Cs, Husman model 521A, Isomedix, Whippany, NJ) for grapefruit, another diet was prepared by adding the same amount (13.5 g/kg) of irradiated freeze dried grapefruit pulp powder. Purified naringin or limonin (200 mg/kg) was added to the remaining two diets. Grapefruit pulps or isolated compounds were added to the diet at the expense of dextrose. The amount of isolated pectin added to the diets was reduced for the grapefruit pulp diets so

that all diets contained the same amount of pectin. Diets were stored at $-20°C$ through the experimental period. Rats received fresh food every day and drinking water was provided *ad libitum*. Animals were injected with AOM (15 mg/kg body wt), a colon carcinogen, or saline, 3 weeks after starting the experimental diets. One week after the first injection, a second AOM or saline injection was given to the rats. For each animal, 48 h diet intakes and body weights were recorded before the injections and at termination.

Tissue sample collection
Rats were euthanized 10 weeks after starting the diets using CO_2 and the colons were removed and cleaned with RNase free phosphate-buffered saline (PBS). Sections (1 cm) from the most proximal and distal portions of the colon were fixed in 4% paraformaldehyde (PFA) or 70% ethanol. The remaining mid-section of the colon was cut open vertically; one half was used for the ACF assay and the other half was used for protein isolation.

ACF assay
ACF represent a cluster of morphologically altered crypts in AOM-injected rodent (27) and human colons (28). Half of the colon mid-section to be used for determination of ACF was placed in a folded piece of Whatman no. 1 filter paper and fixed in 70% ethanol for 24 h. Fixed colons were dipped in a 0.5% solution of methylene blue in distilled water for 45 s and placed on a microscope slide with the mucosal surface up.

Aberrant crypts (AC) were easily detected using a light microscope at $\times 40$ magnification, as they were visibly enlarged and had increased pericryptal area, and thicker epithelial lining compared with surrounding crypts (27,29). The number of AC and high multiplicity ACF (HMACF; ACF consisting of four or more AC) were recorded.

Immunohistopathological analyses for proliferating cell nuclear antigen
Cell proliferation was measured using the methods described by Zheng *et al.* (30) with a monoclonal antibody (anti-PC-10, Signet Laboratories, Dedham, MA). The bound primary antibodies were detected by applying a peroxidase-conjugated antibody to biotinylated anti-mouse immunoglobulin using a Vectastain ABC Elite Kit (Vector Lab, Burlingame, CA). The intact antibody–antigen complex was made visible by placing slides into a solution containing 0.5 mg diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical, St Louis, MO) per ml PBS plus 0.05% H_2O_2 (added immediately before staining). Negative slides were prepared by substituting anti-PC 10 with the same volume of PBS. Only cells with intense proliferating cell nuclear antigen (PCNA) staining were measured. Total number of proliferating cells, position of the highest proliferating cell and total cell number/crypt column (crypt column height) were determined in 25 crypt columns/rat. Proliferative index (number of proliferating cells/crypt column height) and proliferative zone (the highest proliferating cell/crypt column height) were calculated.

Apoptosis assay
The TUNEL (terminal transferase dUTP nick end labeling) procedure was performed to determine the effect of diet on apoptosis (31). Colon tissue sections were pretreated with proteinase K (Ambion, Austin, TX) for 3 min at 37°C. A working solution of TdT-reaction buffer (125 mM Tris–HCl, 1 M Sodium Cacodylate, 1.25 mg/ml bovine serum albumin, pH 6.6) (1:80) was applied to the sections and incubated for 1 h in a pre-warmed 37°C humidified chamber. After incubation, methyl green was used to counterstain the tissue
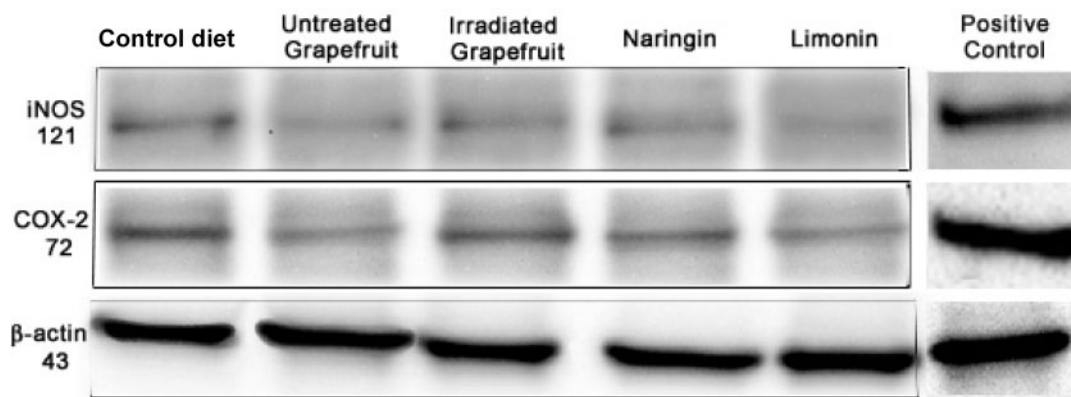
Fig. 1. A representative immunoblot of iNOS and COX-2 in the azoxymethane (AOM)-injected rat colonic mucosa ($n = 5$ rats/group). The elevation in iNOS in AOM-injected rats receiving the control diet was not seen with rats on experimental diets. COX-2 levels were not elevated in AOM-injected rats consuming untreated grapefruit and limonin diets compared with the rats provided with control diet.

sections. Positive control sections were prepared by nicking DNA with DNase 1 for 5 min. Rat colon sections obtained 12 h after injecting AOM served as a biological positive control, as this time point invariably contains numerous apoptotic cells (31). PBS was substituted for 'TdT' in the working solution for developing negative control tissue sections. Total number of apoptotic cells and total number of cells/crypt column were determined in 50 crypt columns/rat. Apoptotic index (apoptotic cells/crypt column height) was calculated.

Quantification of iNOS and COX-2 by immunoblot

Protein was extracted from rat colon mucosa as described previously (32) and protein concentrations were determined by a BCA Protein Assay kit (Pierce, Rockford, IL). Protein extracts (30 μg) were separated by Novex® 4–12% Tris–Glycine gels (Invitrogen, Carlsbad, CA) and electrophoretically transferred to Invitrolon PVDF membranes (Invitrogen). To identify the relative positions of the desired protein bands, a prestained protein molecular weight color marker mix (TriChromRanger, Pierce) was loaded into one of the wells of the gel. After transfer, the membranes were rinsed with Millipore™ water, dipped in methanol (100%) and air dried for 15 min. Membranes were cut into three pieces based on molecular weight using the color bands from TriChromRanger. Each membrane strip contained one protein band of interest. The membrane strips were saturated in methanol (100%) and blocked with 2% bovine serum albumin for 1 h at room temperature (Fisher, Pittsburg, PA). The individual strips were incubated with either goat polyclonal anti-β-actin antibody (1:10 000; Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal anti-COX-2 antibody (1:1000; Santa Cruz Biotechnology) or rabbit polyclonal anti-iNOS antibody (1:375; Cayman Chemicals, Ann Arbor, MI) overnight at 4°C. Membranes were subsequently probed with bovine anti-goat (β-actin 1:300 000; COX-2 1:60 000; Santa Cruz Biotechnology) or goat anti-rabbit IgG–HRP (horseradish peroxidase) conjugate (iNOS 1:60 000; Cayman Chemicals). Target proteins were detected with West Femto Maximum Sensitivity Substrate (Pierce). The membrane was scanned and quantified with a Bio-Rad Fluor-Imager (Bio-Rad Lab, Hercules, CA) using Quantity One software (Bio-Rad Lab). β-actin served as a loading control. A representative immunoblot of iNOS and COX-2 in the AOM-injected rat colonic mucosa is presented in Figure 1.

Statistical analysis

Effect of GFPP, IGFPP, naringin and limonin supplemented diets on ACF development were evaluated using the GLM and MIXED procedures in SAS (SAS Institute, Cary, NC). Data on apoptosis, COX-2, iNOS, proliferative index and extent of proliferative zone were analyzed using the same statistical procedures.

Results

General observations

Naringin concentration in untreated grapefruit, irradiated grapefruit and naringin diets was 196, 192 and 196 mg/kg, respectively. Limonin content in the limonin diet was 194 mg/kg. Limonin content in the untreated and irradiated grapefruit diets were 14.7 and 12.5 mg/kg, respectively. Food intake (17 ± 0.43 g/d) and body weight gain (311 ± 7.5 g; over

Table II. Incidence of AOM-induced AC, HMACF and HMACF/cm of colon in male Sprague–Dawley rats

Treatment	Total AC	HMACF*	HMACF/cm
Control Diet	184.9[a]	13.9[c]	1.15[e]
Untreated Grapefruit	110.7[b]	5.7[d]	0.53[f]
Irradiated Grapefruit	111.5[b]	6.7[d]	0.64[ef]
Naringin	114.9[b]	6.7[d]	0.63[ef]
Limonin	108.1[b]	4.8[d]	0.46[f]
SEM	19.7	1.8	0.17

*HMACF, Foci with ≥ 4 AC/focus; SEM, Standard Error of the Mean. Values are least square means ($n = 10$ rats/group). Values in a column without the same superscript differ [ab]$P \leq 0.02$; [cd]$P \leq 0.01$; [ef]$P \leq 0.04$.

a 10-week period) did not differ among the groups suggesting the experimental diets were well tolerated and supported normal growth in rats.

ACF

Saline-injected rats showed no microscopically observable changes in colonic morphology. Experimental diets reduced the total number of AC ($P \leq 0.02$) and HMACF ($P \leq 0.01$) compared with the control diet (Table II). However, only the untreated grapefruit and limonin diets lowered ($P \leq 0.04$) HMACF/cm of colon compared with the control diet.

Proliferation

Proliferative index was significantly ($P \leq 0.005$) higher in AOM-injected rats receiving the control diet, compared with the saline-injected rats (Figure 2). All the experimental diets reduced ($P \leq 0.02$) the proliferative index in AOM-injected animals to levels similar to those in the saline-injected rats. The proliferative zone was ($P \leq 0.008$) larger in AOM-injected rats consuming the control diet, compared with those in the saline-injected rats (Figure 3). Only diets containing untreated grapefruit and limonin prevented ($P \leq 0.03$) the AOM-induced expansion ($P \leq 0.008$) of the proliferative zone that occurred with the control diet. Proliferative index and proliferative zone were not different among diet groups in saline-injected rats.

Apoptosis

The experimental diets did not influence apoptotic index of saline-injected rats (Figure 4). However, all the experimental diets increased ($P \leq 0.02$) apoptotic index, compared with
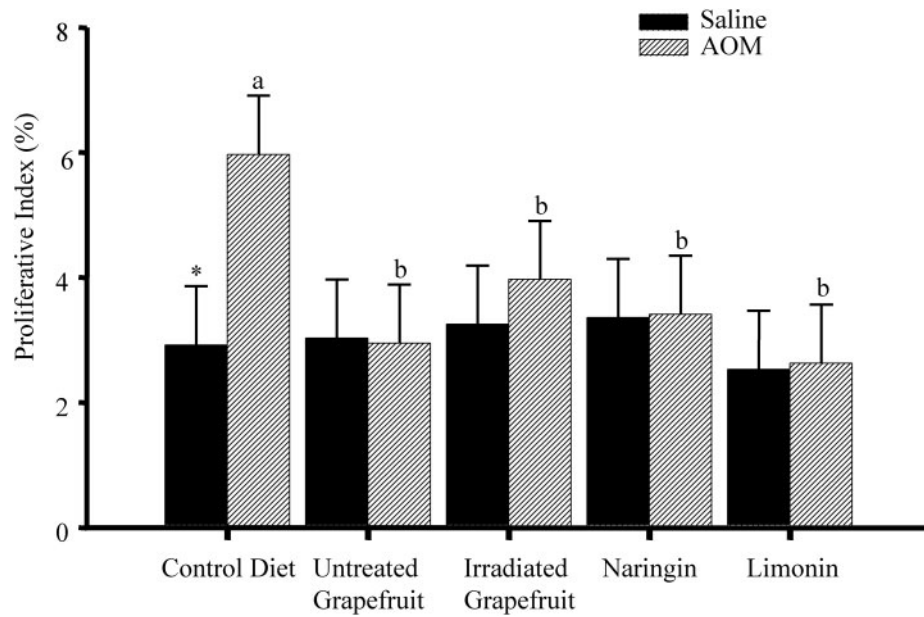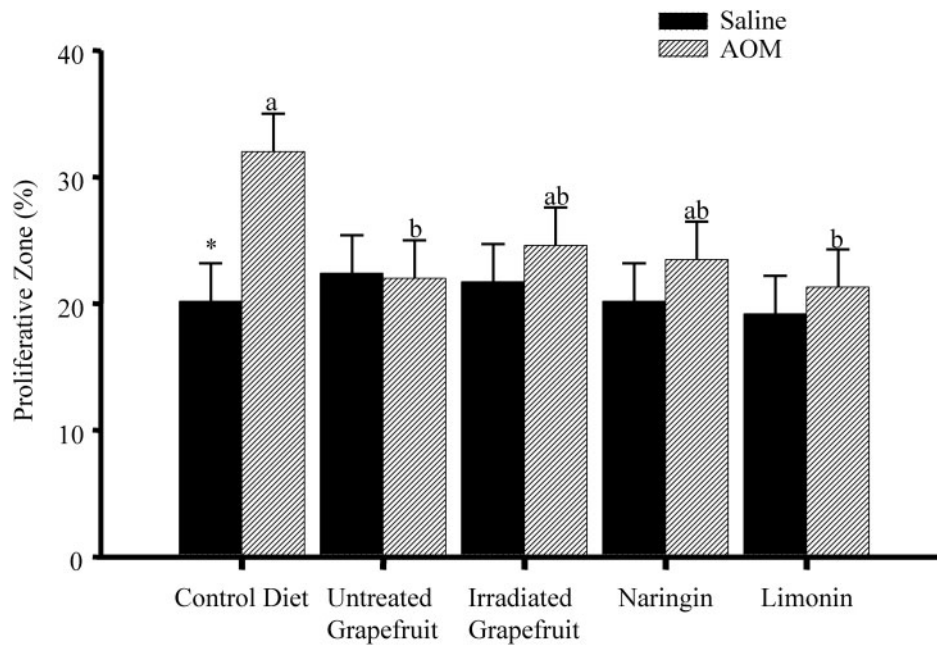
Fig. 2. Effect of experimental diets on proliferative index in saline- and AOM-injected rats. Proliferation index was calculated as the number of proliferating cells divided by the total number of cells in the crypt column. Different letters on the bars for AOM-injected animals represent significant differences ($P \leq 0.02$) among the diets. Asterisk (*) indicates a significant ($P \leq 0.005$) difference in the mean values of saline- and AOM-injected animals consuming the same diet. Each bar indicates the least square mean of 10 rats ± SEM.

Fig. 3. Effect of experimental diets on proliferative zone in saline- and AOM-injected rats. Proliferation zone was calculated as the position of the highest proliferating cell divided by the crypt column height in number of cells. Different letters on the bars for AOM-injected animals represent significant differences ($P \leq 0.03$) among the diets. Asterisk (*) indicates a significant ($P \leq 0.008$) difference in the mean values of saline- and AOM-injected animals for the same diet. Each bar indicates the least square mean of 10 rats ± SEM.

the control diet, in AOM-injected rats. Apoptotic index in AOM-injected rats consuming untreated grapefruit, naringin and limonin diets were ($P \leq 0.01$) greater than that of saline-injected rats consuming those diets.

COX-2 and iNOS

COX-2 and iNOS were not influenced by experimental diets in saline-injected rats (Figures 5 and 6). COX-2 was elevated ($P \leq 0.003$) in AOM versus saline-injected rats consuming

the control diet. Similar elevations in COX-2 levels were not observed in AOM-injected rats consuming natural grapefruit and limonin diets. The elevation in iNOS levels observed in AOM-injected rats receiving the control diet was not seen with rats on experimental diets ($P \leq 0.003$). Levels of iNOS in AOM-injected rats consuming the control, untreated and irradiated grapefruit diets were greater ($P \leq 0.045$) than that of saline-injected rats consuming those diets.
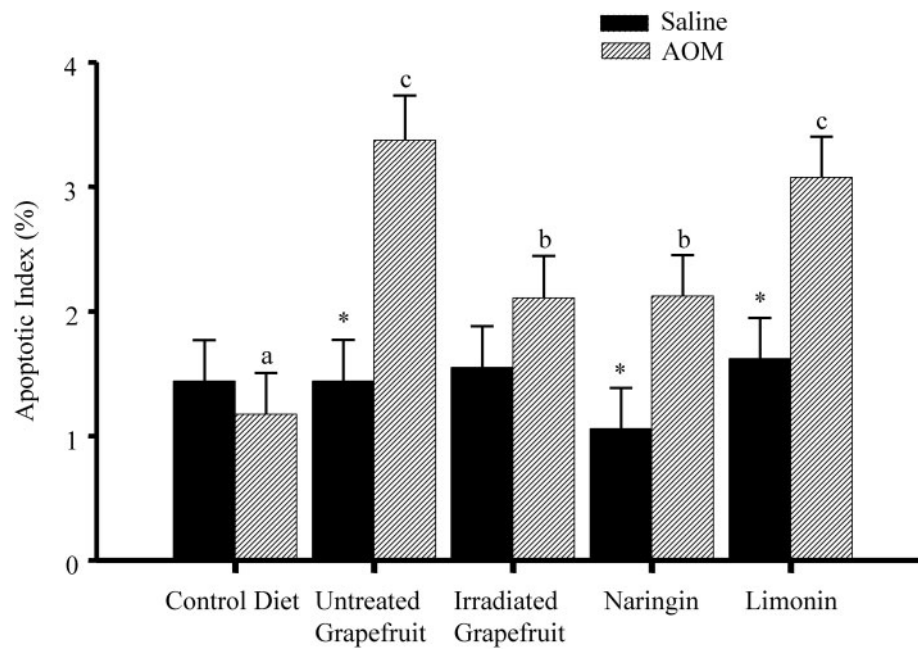
Fig. 4. Effect of experimental diets on apoptotic index in saline- and AOM-injected rats. The apoptotic index was calculated as the number of apoptotic cells divided by the number of cells per crypt column. Different letters on the bars for AOM-injected animals represent significant differences among the diets ($P \leq 0.02$). Asterisk (*) indicates a significant ($P \leq 0.01$) difference in the mean values of saline- and AOM-injected animals for the same diet. Each bar indicates the least square mean of 10 rats $\pm$ SEM.
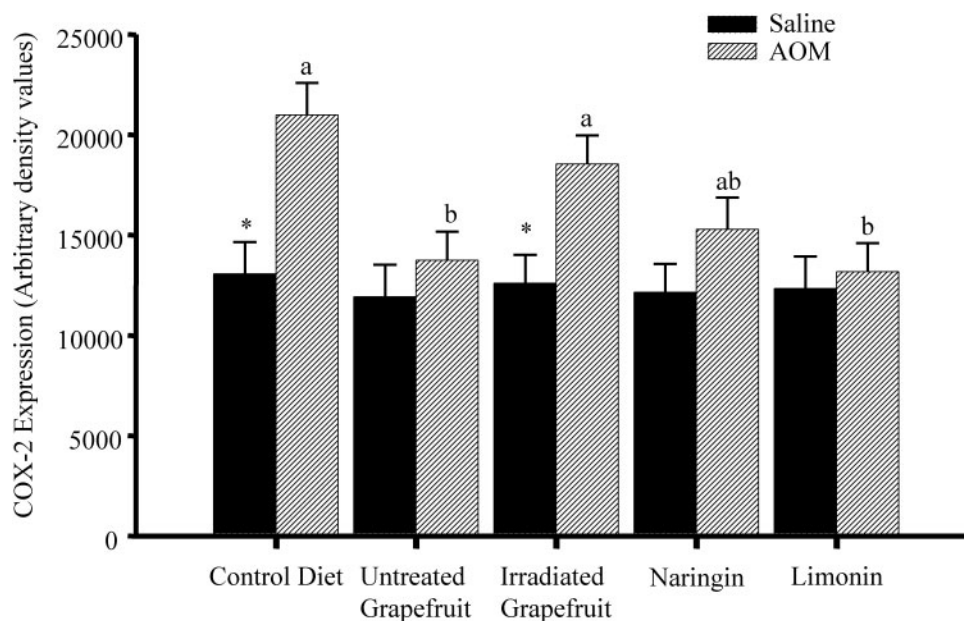

Fig. 5. Effect of experimental diets on cyclooxygenase-2 (COX-2) in saline- and AOM-injected rats. Different letters on the bars for AOM-injected animals represent significant differences among the diets ($P \leq 0.032$). There were no differences in COX-2 protein levels among saline-injected rats consuming different diets. Asterisk (*) indicates a significant ($P \leq 0.003$) difference in the mean values of saline- and AOM-injected animals for the same diet. Each bar indicates the least square mean of five rats $\pm$ SEM.

Discussion

This study was designed to determine whether grapefruit and isolated grapefruit compounds (naringin, a flavonoid and limonin, a limonoid) would suppress AOM-induced colon carcinogenesis in Sprague–Dawley rats measured at the promotion stage. The study also addressed the question of whether radiation treatment of grapefruit in order to control fruit flies would influence the responses.

It is essential that potential chemoprotective agents are safe because long-term oral administration of these compounds is needed for future human clinical trials. Results from this study demonstrate that saline rats on experimental diets were essentially 'normal' because no alternations were observed in proliferation, apoptosis, iNOS and COX-2 levels. Therefore, the experimental diets had no apparent influence on the normal regulation of the pathways involved in controlling the above variables. Overall, untreated and irradiated grapefruit, and
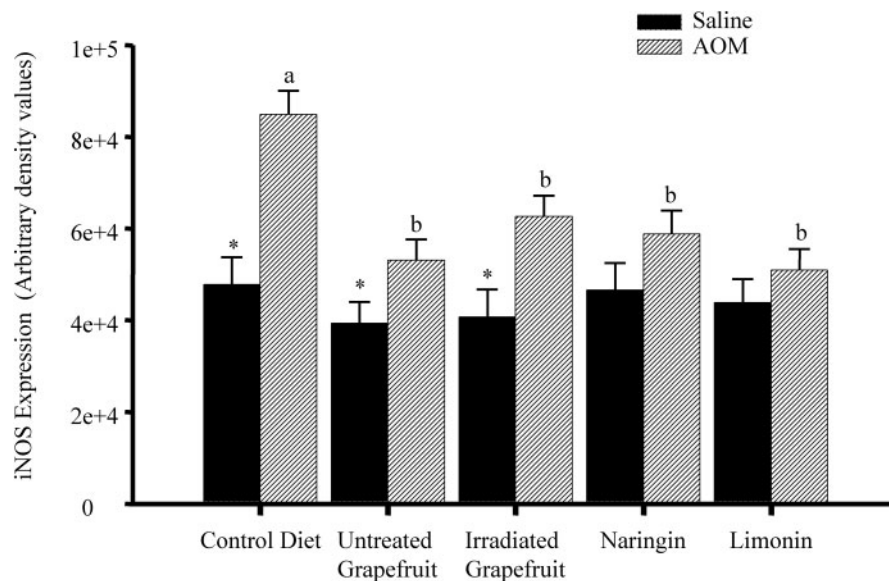
Fig. 6. Effect of experimental diets on inducible nitric oxide synthase (iNOS) in saline- and AOM-injected rats. Different letters on the bars for AOM-injected animals represent significant differences among the diets ($P \leq 0.003$). There were no differences in COX-2 protein levels among saline-injected rats consuming different diets. Asterisk (*) indicates a significant ($P \leq 0.045$) difference in the mean values of saline- and AOM-injected animals for the same diet. Each bar indicates the least square mean of five rats $\pm$ SEM.

relatively high levels of naringin and limonin had no effect on normal colon epithelia physiology. Moreover, experimental diets did not adversely affect body weight gain and food intake. Previous studies also indicated that limonin at 200 or 500 mg/kg was well tolerated and supported normal growth in Fisher rats and had no negative effect on weight gain and food intake (9). In another study, dietary administration of naringin up to 1250 mg/kg was well tolerated in male Sprague–Dawley rats (33). These data suggest that ingestion of these levels of grapefruit-derived compounds should not have deleterious effects on normal tissues.

Carcinogen injection led to the formation of AC, HMACF, increased proliferation, decreased apoptosis (not significant) and elevated iNOS and COX-2 levels in rats provided with the control diet. These results are in agreement with previous reports (15,27,34). All experimental diets suppressed AC and HMACF formation to levels that were 59–63% and 35–48% of the control diet, respectively. Untreated grapefruit and limonin suppressed ($P \leq 0.04$) HMACF/cm to 46–40% of the levels in rats receiving the control diet, whereas irradiated grapefruit and naringin caused only a non-significant reduction. Other studies have shown that feeding orange juice, rich in flavonoids and limonoids, significantly inhibited AOM-induced colon cancer in male Fisher 344 rats (35). In a recent study, obacunone and limonin (200 or 500 mg/kg for 4 weeks fed during initiation or post-initiation) inhibited AOM-induced ACF formation (55–65% reduction by 'initiation' feeding; 28–42% reduction by 'post-initiation' feeding) in Fisher rats (9). Because HMACF correlates with AOM-induced colon adenocarcinoma incidence (36,37), these results suggest that untreated and irradiated grapefruits, as well as isolated naringin and limonin would confer some protection against colon carcinogenesis.

Enhanced colonocyte proliferation and lower apoptosis are hallmarks of ACF (38–41). Both human and animal models suggests that one of the indicators of preneoplasia is abnormal epithelial cell proliferation (42,43). Animals injected with colon carcinogen had a greater proliferative index than

saline-injected rats (41). In this experiment, all the diets eliminated the increase in proliferative index caused by AOM in rats consuming the control diet. Previous reports also suggest that citrus juice (41) or isolated bioactive compounds (44) from citrus are potent suppressors of PCNA-positive cell numbers. Even though the mechanism of action through which citrus components suppress the proliferation is not clear, citrus flavonoids have been shown to suppress cyclin D1 levels (41). However, only untreated grapefruit and limonin suppressed the AOM-induced expansion ($P \leq 0.008$) of the proliferative zone that occurred with the control diet. Other studies indicated that both human subjects with increased risk of colon cancer and carcinogen-injected animals have a larger proliferative zone compared with subjects at low risk for colon cancer and animals injected with vehicle, respectively (24). The mild depression of apoptosis observed in control rats injected with AOM was eliminated with experimental diets. Even though all experimental diets elevated ($P \leq 0.02$) apoptosis compared with the control diet, untreated grapefruit and limonin were more effective compared with irradiated grapefruit and naringin. Apoptosis levels in AOM-injected rats provided with untreated grapefruit, naringin and limonin were greater than in saline-injected rats consuming the same diets. Previous reports indicate that limonin inhibited growth of MCF-7 human breast cancer cells through upregulation of apoptosis (45) and that naringenin, an aglycone of naringin, also induced apoptosis in both Caco-2 and HL-60 cells (46). However, the mechanism of action through which citrus components induce apoptosis is not clear. These results suggest that the experimental diets, particularly untreated grapefruit pulp and limonin, suppress ACF formation by preventing excessive proliferation that leads to an expansion of proliferative zone and by increasing apoptosis to remove initiated/mutated colonic epithelial cells.

In vitro studies have indicated that elevated levels of iNOS and COX-2 proteins may be partially responsible for suppression of apoptosis in colon cancer cells (47) and transcriptional upregulation of genes involved in cell proliferation (48–50).

Our current *in vivo* data indicate that the early stages of colon carcinogenesis involve an increase in iNOS and COX-2 expression, as seen in rats consuming the control diet. Other researchers have also reported elevated levels of COX-2 and iNOS in colon carcinogen treated rats at the promotion stage of carcinogenesis (25,51). Untreated grapefruit and limonin diets suppressed the elevation in both iNOS and COX-2 observed in rats after AOM injection. Even though our study did not explore how grapefruit pulp or limonin suppresses the elevated levels of iNOS and COX-2 observed in carcinogen-injected rats, there are a few possible explanations based on existing literature. By providing the experimental diets 3 weeks before carcinogen administration, it is possible that carcinogen metabolism may have been altered through changes in glutathione-*S*-transferase (GST) and quinone reductase (QR) in the intestine (52,53) of rats consuming the untreated grapefruit or limonin diets. Accumulating evidence suggests that the level of expression of GST is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals (54). One recent study reveals that variations in habitual consumption of fruits, particularly citrus fruits, may contribute to variations in human rectal GST enzyme activity (55). Grapefruit components and limonin may also have suppressed the transcriptional and/or translational activity of NFkB, which regulates both iNOS and COX-2 expression (56,57). Several natural compounds such as curcumin, resveratrol and flavonoids [naringenin, quercetin, (−) Epigallocatechin-3-gallate and apigenin] are shown to suppress both iNOS and COX-2 levels by regulating NFkB levels (56,57). Moreover, strawberry, lingonberries, pomegranate fruit, *Kochia scoparia (L.)* fruit, grape seed and black raspberry extracts were shown to suppress the transcriptional activation of NFkB in a variety of human cancer cell lines (58–63). Although irradiated grapefruit and naringin prevented elevation of iNOS in AOM-injected rats, they were not able to significantly suppress the elevation of COX-2.

Lower levels of COX-2 and iNOS in rats provided with untreated grapefruit and limonin were associated with suppression of proliferation and upregulation of apoptosis, which may have contributed to a decrease in the number of HMACF, a putative biomarker for colon cancer. However, the chemoprotective ability of irradiated grapefruit and naringin were not as pronounced as untreated grapefruit and limonin. Even though the concentration of naringin was similar (200 mg/kg) in both the naringin and untreated grapefruit diets, untreated grapefruit was more effective in suppressing HMACF/cm, proliferative zone and enhancing apoptosis, which were associated with lower levels of COX-2.

The relative effectiveness of the isolated compounds and the grapefruit pulp can be attributed to a variety of factors. Limonin and untreated grapefruit were similar, yet the concentration of limonin used was ~10–15 times greater than that found in the grapefruit diets. This indicates that isolated limonin could be used as a supplement to attain some of the benefits of grapefruit for individuals not capable of consuming this quantity of intact fruit. Although naringin provides much of the benefits derived from grapefruit pulp, it is unlikely the sole factor in grapefruit that protects against colon cancer. Irradiation of grapefruit may have induced oxidative stress in the fruits which led to a negative influence on the 'protective compounds', especially those involved in COX-2 suppression. A recent report suggests that proposed quarantine doses of radiation (300 Gy) reduce potent GST-inducing limonoids,

such as nomilin and limonin (26). Moreover, pectin, a highly fermentable fiber which yields chemoprotective butyrate in the colon is very sensitive to radiation and quickly depolymerizes even at low doses of irradiation (64).

Bioavailability of the phytochemicals in their active form is a function of their release from plant cells and subsequent metabolism or fermentation by colonic bacteria. Several studies have demonstrated that the concentration of bioactive plant compounds is higher in the colon compared with plasma after ingestion of food and beverages rich in these compounds (65) because their release from the plant cell can be limited until they reach the colon. Therefore, it is also possible that the bioactive compounds in grapefruit pulp, or their products from bacterial fermentation, may exert a positive effect upon release in the colon.

In conclusion, untreated and irradiated grapefruit or isolated naringin and limonin had protective effects against chemically induced colon carcinogenesis measured at the promotion stage. However, untreated grapefruit and limonin may serve as better chemopreventive agents compared with irradiated grapefruit and naringin. Results from these studies emphasize that it is important to document the effects of post-harvest treatments, such as irradiation, on bioactive compounds of fruits and vegetables. In order to further elucidate whether the biological activities measured in this experiment are due to the parent compounds or the metabolites, it will be important to conduct thorough metabolic studies, as there is little information available in this area.

Acknowledgements

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