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Chemoprevention of Esophageal Tumorigenesis by Dietary Administration of Lyophilized Black Raspberries[1]

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ABSTRACT

Fruit and vegetable consumption has consistently been associated with decreased risk of a number of aerodigestive tract cancers, including esophageal cancer. We have taken a "food-based" chemopreventive approach to evaluate the inhibitory potential of lyophilized black raspberries (LBRs) against *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in the F344 rat, during initiation and postinitiation phases of carcinogenesis. Anti-initiation studies included a 30-week tumorigenicity bioassay, quantification of DNA adducts, and NMBA metabolism study. Feeding 5 and 10% LBRs, for 2 weeks prior to NMBA treatment (0.25 mg/kg, weekly for 15 weeks) and throughout a 30-week bioassay, significantly reduced tumor multiplicity (39 and 49%, respectively). In a short-term bioassay, 5 and 10% LBRs inhibited formation of the promutagenic adduct *O*^{6}-methylguanine (O^6-meGua) by 73 and 80%, respectively, after a single dose of NMBA at 0.25 mg/kg. Feeding 5% LBRs also significantly inhibited adduct formation (64%) after NMBA administration at 0.50 mg/kg. The postinitiation inhibitory potential of berries was evaluated in a second bioassay with sacrifices at 15, 25, and 35 weeks. Administration of LBRs began after NMBA treatment (0.25 mg/kg, three times per week for 5 weeks). LBRs inhibited tumor progression as evidenced by significant reductions in the formation of preneoplastic esophageal lesions, decreased tumor incidence and multiplicity, and reduced cellular proliferation. At 25 weeks, both 5 and 10% LBRs significantly reduced tumor incidence (54 and 46%, respectively), tumor multiplicity (62 and 43%, respectively), proliferation rates, and preneoplastic lesion development. Yet, at 35 weeks, only 5% LBRs significantly reduced tumor incidence and multiplicity, proliferation indices and preneoplastic lesion formation. In conclusion, dietary administration of LBRs inhibited events associated with both the initiation and promotion/progression stages of carcinogenesis, which is promising considering the limited number of chemopreventives with this potential.

INTRODUCTION

Worldwide, esophageal cancer is the eighth most common incident cancer and the fifth most common cause of cancer death (1, 2). The prognosis for those diagnosed with esophageal cancer is poor with relative 5-year survival rates ranging from 8 to 12% (3). These statistics reflect the insidious nature of this malignancy and support the need to develop improved treatment and preventive strategies. Chemoprevention is one viable approach under investigation, especially for individuals at high risk for esophageal cancer development. Next to tobacco and alcohol, diet and nutrition are among the factors most strongly associated with aerodigestive tract cancers. Furthermore, in areas of high esophageal cancer incidence, the role of nutritional factors appears paramount to that of either smoking or alcohol consumption.

Regions of the world with the highest esophageal cancer rates include the "esophageal cancer belt," an area extending from Northern China westward into Iran (4–7). Residents of high-risk areas tend to have monotonous and restricted diets with seasonal variations in consumption of fresh fruit, vegetables, and specific nutrients (8, 9). Furthermore, research conducted in high esophageal cancer incident areas of China and South Africa suggest that *N*-nitroso compounds and their precursors are probable etiological factors in esophageal cancer development (10). Relatively high levels of nitrosamines and their precursors have been detected in the drinking water and food supplies of residents living in Linxian, China (10, 11). In addition to nitrosamine detection in saliva, gastric juices, and urine, elevated levels of O^6-meGua[3] adducts have been detected among China's residents (12–14). Although it is unlikely that poor diet alone would be sufficient to cause esophageal cancer, evidence supports the theory that dietary insufficiencies could render the esophageal mucosa more susceptible to insult by other agents, including carcinogens, mutagens, and other irritants (15).

Traditionally, the cancer-inhibitory effects of food-derived chemopreventive agents have been assessed either individually or as a few putative active constituents and often at pharmacological doses. Yet, epidemiological data supports the theory that consumption of fruits and vegetables, within a range that is behaviorally possible, decreases cancer risk at a number of sites, including the esophagus (1, 16). A number of human case-control and cohort studies specifically support a protective relationship between high levels of fruit consumption and decreased esophageal cancer risk (4, 17–25). In the present study, we used a food-based approach to evaluate the inhibitory potential of whole freeze-dried black raspberries against nitrosamine-induced initiation and postinitiation events in the F344 rat esophagus. This rat strain has been extensively studied and experiences progressive histopathological changes, similar to those in humans, ranging from normal esophageal epithelium to squamous cell carcinoma (26, 27). Black raspberries are rich in nutrients as well as nonnutritive phytochemicals, which may act individually or in combination to inhibit carcinogenic processes. Earlier work by Daniel *et al.* (28) found berries to be rich in EA, a plant polyphenol known to have antimutagenic and anticarcinogenic properties. EA inhibits carcinogenesis through multiple mechanisms, against various classes of chemical carcinogens, and in different target organs, including the rat esophagus (29–32). Raspberry ellagitannins have also been found to be inhibitory against TPA-induced ornithine decarboxylase activity, TPA-stimulated hydroperoxide production and TPA-stimulated DNA synthesis (30). The present study expands on our earlier work, which found that freeze-dried strawberries inhibit NMBA-induced esophageal tumorigenesis in the F344 rat model (33). In contrast to the

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[3] The abbreviations used are: O^6-meGua, *O*^{6}-methylguanine; NMBA, *N*-nitrosomethylbenzylamine; LBR, lyophilized black raspberry; PCNA, proliferating cell nuclear antigen; LI, labeling index; EA, ellagic acid; HPLC, high-performance liquid chromatography.

strawberry postinitiation study, the present study used a lower dose of NMBA, had multiple sacrifice time points, categorized and quantified preneoplastic lesions differently, and assessed PCNA and cyclin D1 expression via immunohistochemical techniques. Black raspberries, although similar to strawberries in composition, generally have higher levels of phenolics (ellagic and ferulic acids), sterols, calcium, iron, zinc, and antioxidant activity (Table 1), which may influence their chemopreventive potential. This knowledge coupled with epidemiological data associating increased fruit consumption with decreased esophageal cancer risk, led to the present investigation evaluating LBRs as inhibitors of carcinogen-induced tumor development in the rat esophagus.

MATERIALS AND METHODS

Chemicals. Unlabeled NMBA was purchased from Ash Stevens Inc. (Detroit, MI) and solubilized in 20% DMSO. [^{3}H]NMBA (specific activity, 2.48 Ci/mmol) was prepared by Dr. Lisa Peterson (University of Minnesota, Minneapolis, MN) as described previously (34). EA and DMSO were purchased from Aldrich Chemical Company (Milwaukee, WI). Purity analysis by reverse-phase HPLC indicated that the EA and NMBA used were greater than 98% pure.

Animals. All of the experimental protocols were in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee of The Ohio State University. Five-to-6-week-old male F344 rats were purchased from Harlan Industries (Indianapolis, IN). Animals were group housed and maintained under standard conditions (20 ± 2°C; 50 ± 10% relative humidity; 12-h light/dark cycle). Diet and water were available *ad libitum* throughout the studies. Body weight and food consumption data were recorded weekly.

Diet Preparation. Fresh frozen black raspberries of the Driscol variety were purchased from Coloma Frozen Foods (Benton Harbor, MI) and used in all studies, except in the NMBA metabolism study, which used Bristol variety berries supplied by the Stokes Fruit Farm (Wilmington, OH). Berries were shipped frozen to Van Drunen Farms (Momence, IL) for freeze-drying. Black raspberries were processed as described previously (35) with the exception that berry seeds were repulped and added back to the berry slurry prior to freeze-drying. Berries were analyzed for a number of nutrients and potential chemopreventive components (Table 1). EA content was determined in our laboratory as described previously (28), and other component analysis was conducted by Covance Laboratories, Inc. (Madison, WI). Lyophilized berries were mixed into modified AIN-76A diet weekly at the expense of cornstarch and stored at 4°C until fed to the animals.

Table 1 *Levels of nutrients and potential chemopreventive components present in LBRs*

Components	Berry samples analyzed[a]	
	LBRs1[b]	LBRs2[c]
Minerals		
Calcium	245.00	215.00
Copper	0.52	0.55
Iron	13.20	10.10
Magnesium	169.00	153.00
Manganese	3.60	4.68
Phosphorus	222.00	170.00
Potassium	1200.00	1300.00
Zinc	2.69	2.17
Selenium	<5.00	<5.00
Vitamins		
Ascorbic Acid	<1.00	4.40
α-carotene	<0.02	<0.02
β-carotene	<0.02	<0.02
Folate	0.07	0.06
Sterols		
β-sitosterol	89.10	80.10
Campesterol	4.30	3.40
Stigmasterol	<3.00	<3.00
Cholesterol	<1.00	<1.00
Phenolics		
Ellagic Acid	185.00	166.30
Ferulic Acid	32.40	17.60
P-Coumeric Acid	7.94	9.23

[a] Components reported in mg/100 g sample, except selenium levels reported in μg/100 g.
[b] LBRs1 were administered in the diet during the complete carcinogenesis bioassay and DNA adduct study.
[c] LBRs2 were administered in the diet during the post-initiation bioassay.

Complete Carcinogenesis Intervention with LBRs. LBRs were administered in the diet at 5 and 10% before, during, and after NMBA treatment to evaluate black raspberries as inhibitors of initiation and postinitiation events. Male F344 rats (7 to 8 weeks of age) were randomized into five groups of 15 animals each and were fed modified AIN-76A diet, or modified AIN-76A diet containing 5 or 10% LBRs. Animals were maintained on their respective diets throughout the 30-week bioassay. Two weeks after initiation of experimental diets, rats in the positive control group (Group 3) and in the berry test groups (Groups 4 and 5) received NMBA, which was administered s.c. at a concentration of 0.25 mg/kg body weight once per week for 15 weeks. Control animals received either the vehicle (Group 1) or the highest concentration of berries (Group 2). Animals were killed at 30 weeks, complete necropsies were performed, and tissues were harvested. Esophagi were excised and fixed in 10% neutral buffered formalin, and tumors greater than 0.5 mm in diameter were counted, mapped, and sized.

O^6-meGua DNA Adduct Study. Groups of 24–26 male F344 rats (7 to 8 weeks of age) were randomized into eight experimental groups and placed on experimental diets. Animals were fed modified AIN-76A diet, modified AIN-76A diet containing either 5 or 10% LBRs, or modified AIN-76A diet containing either 0.004% (equivalent to 0.4 g/kg) or 0.04% (equivalent to 4.0 g/kg) EA for 15 days. On day 14, rats received a single s.c. injection of NMBA at a concentration of either 0.25 mg/kg or 0.50 mg/kg body weight. Twenty-four h after NMBA administration, rats were killed by CO_2 asphyxiation. Esophagi were harvested, split longitudinally, stripped of the underlying layers of muscle and submucosa, immediately frozen in liquid nitrogen, and stored at −80°C. The esophagi of four to six rats were pooled to yield a single sample totaling five samples per treatment group. DNA was isolated, purified, and quantified as described previously (36), except that O^6-meGua and guanine were detected using a Waters 470 fluorescence detector (excitation wavelength, 290 nm; emission wavelength, 360 nm).

NMBA Metabolism Study in Esophageal Explant Cultures. Groups of six male F344 rats (7–8 weeks of age) were randomized into three groups and fed modified AIN-76A diet or modified AIN-76A diet with either 5% or 10% LBRs for 2 weeks. The animals were euthanized on day 14, and esophagi were aseptically removed and immersed in Leibovitz's L-15 medium (Life Technologies, Inc., Grand Island, NY) with added penicillin and streptomycin. Esophagi were opened longitudinally, cut into two equal halves, and placed mucosal side up in 60-mm tissue culture dishes. Esophagi were moistened with 3 ml of Pasadena Foundation Medical Research-4 culture medium (BRFF, Ijamsville, MD) with growth factors added as described previously (37). Each dish contained 0 or 10 μM [^{3}H]NMBA. Samples were incubated for 8 h at 37°C. Aliquots were removed at 2, 4, and 8 h and were analyzed by reverse-phase HPLC as described by Morse *et al.* (38). Authentic standards of NMBA, benzyl alcohol, benzaldehyde, and benzoic acid were coinjected with aliquots of each sample. Chromatographic run time was 50 min.

Postinitiation Intervention with LBRs. To evaluate berries as inhibitors of postinitiation tumorigenic events, LBRs were administered in the diet at 5 and 10% after completion of NMBA treatment. Groups of 28–38 male F344 rats (5–8 weeks of age) were randomized to vehicle control (Group 1), 10% LBRs (Group 2), NMBA control (Group 3), or NMBA and LBR groups (Groups 4 and 5). NMBA was administered s.c. three times per week for 5 weeks at a concentration of 0.25 mg/kg of body weight (Groups 3–5). Vehicle controls (Group 1) received 20% DMSO:H_2O, and rats in Group 2 were nondosed, high-concentration LBR controls. Animals were maintained on their respective experimental diets until killed at 15 (n = 40), 25 (n = 64), or 35 (n = 65) weeks of study. At each death, a gross necropsy was performed on each rat before harvesting esophageal and colon tissues.

Histological Grading of Preneoplastic Lesions. Each esophagus was cut into thirds and paraffin embedded on edge. Serial 4-μm sections were cut and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). A H&E stained slide of each esophagus was prepared and the entire esophagus of each animal scanned at 100-X magnification. Each viewing field was categorized into one of five histological categories: normal epithelium, epithelial hyperplasia, low-grade dysplasia, high-grade dysplasia, and squamous cell papilloma (Fig. 2). The classification scheme used was a modification of criteria developed by Pozharisski *et al.* (26), with consideration toward the gross and

Table 2 *Complete carcinogenesis bioassay evaluating the effects of dietary administration of LBRs on NMBA-induced rat esophageal tumorigenesis*

Group	NMBA 0.25 mg/kg	Diet administered	Rats (*n*)	Tumor incidence (%)	Tumor multiplicity (± SE)	Tumor size mm^2 (± SE)
1	−	AIN-76A	15	0	0	0
2	−	10% LBRs	14	0	0	0
3	+	AIN-76A	13	100	3.15 (0.31)	2.28 (0.59)
4	+	5% LBRs	14	78.6	1.93[a] (0.40)	2.91 (0.68)
5	+	10% LBRs	13	92.3	1.61[a] (0.29)	3.67 (0.71)

[a] Statistically significant relative to NMBA controls (Group 3) ($P < 0.05$).

microscopic descriptions of hyperplasia and dysplasia given in Cotran *et al.* (39).

Immunohistochemical Analysis. At each study time point, the entire esophagi from five rats per group were stained for PCNA. Tissues were antigen retrieved by microwaving at 80% power for 12 min in 10 mM citrate buffer (pH 6.0). Tissues were blocked with 3% H_2O_2 for 20 min, casein for 15 min, goat serum for 20 min, and avidin/biotin for 30 min. Slides were incubated with the primary antibody, monoclonal mouse anti-PCNA for 30 min (prediluted antibody from BioGenex, Inc.). Rat-adsorbed link (biotinylated anti-immunoglobulin) followed for 20 min, streptavidin-horseradish peroxidase label for 20 min, and a final incubation with 3,3′-diaminobenzidine for 3.5 min to permit biomarker visualization. Slides were counterstained with hematoxylin, dehydrated, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). Positive (colon) and negative controls (mouse antiserum) were included in each staining run.

Computer-assisted Image Analysis. PCNA stained slides were viewed at ×200 with a Nikon bright-field microscope mounted with a high-resolution spot camera. The camera was interfaced with a computer containing a matrox frame grabber board and image analysis software (Simple PCI Imaging Systems by Compix Inc., Cranberry Township, PA). The basal cell layer of each esophagus was scanned, and a minimum of 10 fields (1500–2000 cells) were quantified to determine the mean LI. The LI was calculated by dividing the positive nuclear-stained area by the total nuclear area; the result was expressed as a percentage. Papillomas were similarly quantified, but at a magnification of ×100 to allow for greater visualization in a single field.

Statistical Analysis. Body weights, food consumption, tumor multiplicity, tumor size, and microscopic preneoplastic lesion data were compared using ANOVA followed by Newman-Keuls' multiple comparison test ($P < 0.05$) when appropriate. DNA adduct levels were analyzed by linear regression and ANOVA to detect differences between means and to calculate SEs. The χ^2 test was used for statistical analysis of tumor incidence data.

RESULTS

General Observations. No significant differences were detected in animal body weights between the NMBA control group and NMBA-treated animals consuming 5 or 10% LBRs. However, during the last 3 weeks of the postinitiation bioassay, NMBA-treated animals consuming 10% LBRs (Group 5) had significantly lower body weights compared with vehicle controls ($P < 0.05$; data not shown). Group 5 animals also consumed significantly less food compared with untreated controls during weeks 26, 29, and 31 of study ($P < 0.05$; data not shown). Overall, berries were well tolerated and did not produce any gross or histological abnormalities in the esophagus, liver, kidneys, spleen, stomach, or intestinal tract of the rats.

Inhibition by LBRs in a Complete Carcinogenesis Bioassay. The chemopreventive effects of LBRs on esophageal tumor incidence, multiplicity, and size are summarized in Table 2. Administration of 5 and 10% LBRs before, during, and after NMBA treatment significantly reduced tumor multiplicity to 1.9 and 1.6, respectively, compared with 3.2 in NMBA-treated controls ($P < 0.05$; Table 2). Tumor incidence was 100% among NMBA controls, whereas nonsignificant declines of 21.4 and 7.7%, respectively, occurred in groups that consumed 5 and 10% LBRs. As expected, non-NMBA-treated animals (Groups 1 and 2) had no esophageal tumors. There were no significant differences in tumor size among the carcinogen-treated groups (Groups 3–5).

Inhibition of O^6-meGua Adducts. The effects of dietary administration of LBRs and EA on NMBA-induced DNA adduct formation in the F344 rat esophagus is summarized in Table 3. Levels of O^6-meGua were not detectable in esophagi from rats treated with vehicle alone. O^6-meGua levels were 4.4 and 7.5 pmol/mg DNA in rats treated with NMBA at 0.25 and 0.50 mg/kg of body weight, respectively. Dietary administration of LBRs and EA significantly inhibited O^6-meGua formation compared with rats consuming control diet ($P < 0.05$). EA-treated groups were run in parallel with berry-treated groups in an effort to account for the chemopreventive contribution of EA among berry treatments. Administration of 5 and 10% LBRs prior to treatment with 0.25 mg/kg NMBA significantly inhibited esophageal O^6-meGua levels by 73 and 80%, respectively. Interestingly, feeding 5% LBRs but not 10%, significantly inhibited O^6-meGua adduct formation after treatment with 0.50 mg/kg NMBA. EA fed at 4.0 g/kg of diet significantly lowered esophageal adduct formation, but to a lesser extent than did LBRs. EA pretreatment at 0.04% in the diet followed by NMBA treatment at 0.25 and 0.50 mg/kg of body weight reduced adduct formation by 48 and 57%, respectively. Lower dietary levels of EA (0.004%) did not result in significant declines in esophageal O^6-meGua adduct levels.

Effects of LBR Pretreatment on NMBA Metabolism in Esophageal Explants. The principal NMBA metabolites produced by esophageal explants were two unidentified peaks (peaks 1 and 2), benzyl alcohol, and benzoic acid (Fig. 1). Two-week dietary pretreatment with 5 and 10% LBRs did not significantly alter the metabolism of NMBA or the formation of NMBA metabolites at 2, 4, or 8 h postincubation (data not shown). Incubation periods of 2, 4, and 8 h resulted in 46, 65, and 86% metabolism of NMBA by explants pretreated with modified AIN-76A diet. Levels of NMBA and NMBA metabolites among groups pretreated with 5 and 10% LBRs were essentially unchanged from those of AIN-76A-fed animals (data not shown).

Inhibition by LBRs in a Postinitiation Bioassay. The postinitiation inhibitory effects of LBRs were evaluated in a second bioassay,

Table 3 *Effects of dietary administration of LBRs and EA on NMBA-induced DNA adduct formation in the F344 rat esophagus*

Experimental group	Adduct level (pmol O^6-meGua/mg DNA) mean ± SE	% inhibition[a]
0.25 mg/kg NMBA	4.4 ± 0.9	
0.25 mg/kg NMBA + 5% LBR	1.2 ± 0.3[b]	73
0.25 mg/kg NMBA + 10% LBR	0.9 ± 0.2[b]	80
0.50 mg/kg NMBA	7.5 ± 1.7	
0.50 mg/kg NMBA + 5% LBR	2.7 ± 0.9[b]	64
0.50 mg/kg NMBA + 10% LBR	6.4 ± 2.1	15
0.25 mg/kg NMBA	4.4 ± 0.9	
0.25 mg/kg NMBA + 0.004% EA	2.9 ± 1.1	35
0.25 mg/kg NMBA + 0.04% EA	2.3 ± 0.9[b]	48
0.50 mg/kg NMBA	7.5 ± 1.7	
0.50 mg/kg NMBA + 0.004% EA	4.2 ± 1.9	44
0.50 mg/kg NMBA + 0.04% EA	3.2 ± 0.9[b]	57

[a] The percentage inhibition in berry-treated groups is relative to NMBA controls.
[b] Statistically significant relative to NMBA controls (Group 3) ($P < 0.05$).

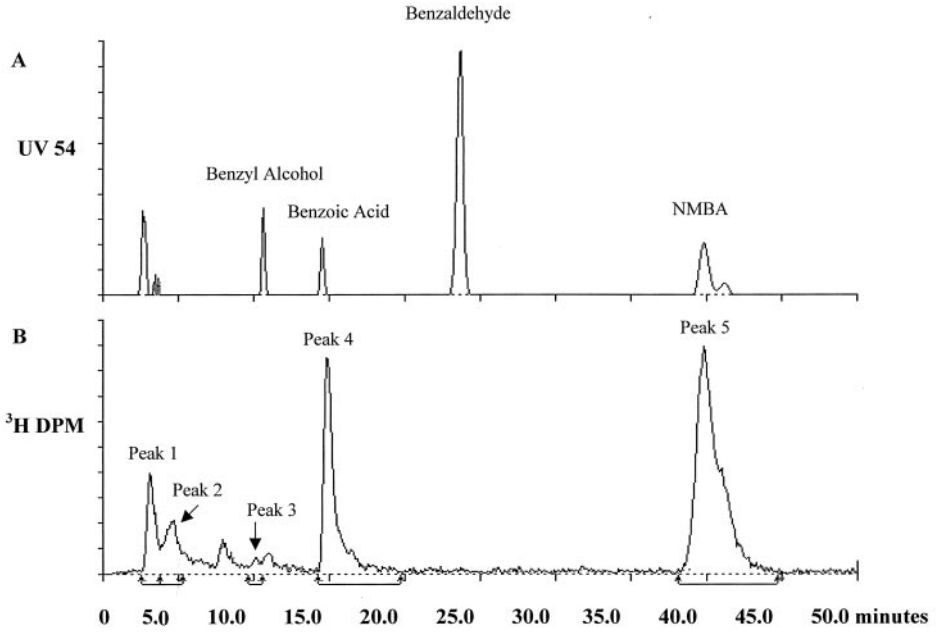

Fig. 1. HPLC analysis of NMBA metabolism by rat esophageal explant cultures. Chromatogram *A*, authentic standards; chromatogram *B*, analysis from a test sample.

in which the administration of LBRs began after the completion of NMBA treatment. The inhibitory effects of LBRs on the formation NMBA-induced preneoplastic lesions in the esophagus are summarized in Table 4. At 15 weeks of study, dietary administration of 5 and 10% LBRs significantly increased the proportion of fields appearing as normal epithelium and decreased high-grade esophageal dysplasia, compared with NMBA controls ($P < 0.05$). Additionally, at week 15, animals consuming 5% LBRs experienced a 21.2% decline in low-grade dysplasia, relative to NMBA controls. At 25 weeks, feeding 5 and 10% LBRs inhibited high-grade dysplasia by 52.3 and 71.3%, respectively, compared with NMBA controls. At 35 weeks of study, dietary administration of 5% LBRs produced significant declines in the formation of both low- and high-grade dysplastic esophageal lesions, relative to NMBA controls. At this time point, administration of 10% LBRs did not significantly reduce preneoplastic lesion development. Dietary administration of LBRs, especially at 5%, resulted in nonsignificant increases in normal esophagi.

Tumor incidence, multiplicity and size data from the 35-week postinitiation bioassay are summarized in Table 5. Non-NMBA-treated rats had no esophageal tumors at 15, 25, or 35 weeks of the bioassay. At 15 weeks, administration of 5 and 10% LBRs appeared to decrease tumor incidence, multiplicity, and size. Although these changes were not significant, the small sample size may have negated our ability to make statistically meaningful comparisons between experimental groups. At 25 weeks, dietary administration of 5 and 10% LBRs significantly reduced tumor incidence and tumor multiplicity; however, these declines were not dose dependent. LBRs fed at 5 and 10%, significantly reduced tumor incidence to 40 and 46.7%, respectively, compared with 86.6% among NMBA controls. At week 25, tumor multiplicity decreased to 0.53 and 0.80 in groups fed 5 and 10% LBRs, respectively, compared with 1.40 in NMBA controls. In addition, berry-treated animals experienced nonsignificant declines in tumor size compared with NMBA controls at 25 weeks. At 35 weeks, 5% LBRs produced significant declines in tumor incidence and tumor multiplicity compared with NMBA controls ($P < 0.05$); tumor incidence declined about 40.0% and tumor multiplicity was inhibited by 66.5%.

PCNA. PCNA LIs for the various experimental groups are reported in Table 6, and photomicrographs are depicted in Fig. 2. The mean PCNA LI in NMBA-treated controls was 31.5, 32.2, and 36.0%

Table 4 *Effects of dietary administration of LBR on NMBA-induced preneoplastic esophageal lesion formation in the F344 rat*

Group	NMBA 0.25 mg/kg	Diet administered	% normal or preneoplastic esophageal lesions (± SE)			
			Normal	Epithelial hyperplasia	Low-grade dysplasia	High-grade dysplasia
Week 15 of study						
1	−	AIN-76A	76.8 (1.5)	21.6 (1.3)	1.7 (0.7)	0
2	−	10% LBRs	71.6 (1.8)	25.6 (1.5)	2.9 (0.8)	0
3	+	AIN-76A	42.6 (2.8)	39.9 (2.3)	11.7 (1.3)	5.7 (0.8)
4	+	5% LBRs	52.7[a] (3.1)	37.5 (2.9)	7.1[a] (1.7)	2.7[a] (0.7)
5	+	10% LBRs	51.3[a] (1.4)	37.9 (1.4)	9.2 (0.9)	1.6[a] (0.5)
Week 25 of study						
1	−	AIN-76A	79.1 (3.0)	18.8 (2.4)	2.0 (0.8)	0
2	−	10% LBRs	76.5 (3.5)	20.8 (2.9)	2.7 (1.0)	0
3	+	AIN-76A	38.6 (2.5)	44.7 (1.7)	9.5 (0.8)	7.2 (0.9)
4	+	5% LBRs	47.0 (2.0)	40.6 (2.0)	8.3 (0.9)	4.1[a] (0.6)
5	+	10% LBRs	44.6 (3.0)	41.7 (2.8)	8.7 (1.0)	4.9[a] (0.8)
Week 35 of study						
1	−	AIN-76A	87.4 (2.2)	11.1 (1.7)	1.5 (0.6)	0
2	−	10% LBRs	87.2 (1.7)	11.4 (1.5)	1.4 (0.7)	0
3	+	AIN-76A	37.6 (2.8)	43.2 (1.8)	10.1 (1.1)	9.0 (1.1)
4	+	5% LBRs	46.3 (3.0)	41.7 (1.9)	6.1[a] (1.0)	5.9[a] (0.8)
5	+	10% LBRs	42.5 (3.3)	41.1 (1.9)	8.9 (1.3)	7.5 (1.1)

[a] Statistically significant relative to NMBA controls (Group 3) as determined by analysis of variance ($P < 0.05$).

Table 5 *Postinitiation effects of dietary administration of LBR on NMBA-induced esophageal tumorigenesis in the F344 rat*

Group	NMBA 0.25 mg/kg	Diet administered	Rats (*n*)	Tumor incidence %	Tumor multiplicity mean (± SE)	Tumor size mm^3 mean (± SE)
Week 15 of study						
1	−	AIN-76A	8	0	0	0
2	−	10% LBRs	8	0	0	0
3	+	AIN-76A	8	50.0	0.75 (0.31)	0.34 (0.16)
4	+	5% LBRs	8	25.0	0.38 (0.26)	0.12 (0.08)
5	+	10% LBRs	8	12.5	0.13 0.13)	0.04
Week 25 of study						
1	−	AIN-76A	10	0	0	0
2	−	10% LBRs	10	0	0	0
3	+	AIN-76A	15	86.6	1.40 (0.25)	1.2 (0.71)
4	+	5% LBRs	15	40.0[a]	0.53[b] (0.19)	0.29 (0.15)
5	+	10% LBRs	15	46.7[a]	0.80[b] (0.30)	0.54 (0.21)
Week 35 of study						
1	−	AIN-76A	10	0	0	0
2	−	10% LBRs	10	0	0	0
3	+	AIN-76A	14	92.9	2.00 (0.31)	3.91 (1.57)
4	+	5% LBRs	14	53.3[a]	0.67[b] (0.19)	3.60 (2.74)
5	+	10% LBRs	15	80.0	1.53 (0.32)	1.01 (0.66)

[a] Significantly lower than Group 3 as determined by χ^2 test ($P < 0.05$).
[b] Significantly lower than Group 3 as determined by analysis of variance ($P < 0.05$).

at 15, 25, and 35 weeks of study, respectively. In contrast, PCNA LIs for non-NMBA-dosed rats ranged from 18.3 to 21.3%, and there were no detectable differences between groups consuming modified AIN-76A control diet and control diet with 10% LBRs. Dietary administration of both 5 and 10% LBRs significantly lowered the PCNA LI in NMBA-treated animals at 15 and 25 weeks of study compared with rats that were treated with NMBA and consumed the control diet ($P < 0.05$). The PCNA LI was reduced by ~29% among berry-treated groups at 15 weeks of study. At 25 weeks, 5 and 10% LBRs inhibited PCNA LIs by 29.6 and 20.0%, respectively, compared with levels in NMBA controls. At 35 weeks, the PCNA LI declined 38% in rats consuming 5% LBRs relative to NMBA controls. PCNA LIs in papillomas at 25 and 35 weeks of study averaged 30.7 and 39.1%, respectively.

DISCUSSION

In this series of studies we used a food-based chemopreventive approach to evaluate the potential inhibitory effects of LBRs against nitrosamine-induced esophageal tumorigenesis. To our knowledge, the present investigation is the first to report suppression of esophageal carcinogenesis by dietary administration of LBRs. Especially promising is the finding that LBRs inhibit events associated with the initiation and postinitiation phases of esophageal tumorigenesis.

A 30-week complete carcinogenesis bioassay was conducted followed by adduct and metabolism studies aimed at evaluating potential mechanisms through which LBRs may exert anti-initiation effects. Dietary administration of 5 and 10% LBRs before, during, and subsequent to NMBA treatment resulted in significant reductions in tumor multiplicity as well as nonsignificant and non-dose-dependent declines in tumor incidence. Noteworthy, the tendency of 5% LBRs to impart greater inhibition than 10% LBRs emerged in subsequent studies.

NMBA, like many nitrosamines, requires metabolic activation to exert its carcinogenic effect. NMBA activation is mediated by cytochrome P-450 mixed-function oxidases forming benzaldehyde and a methylating species, which preferentially methylates the O^6- and *N*7-positions of guanine (40, 41). O^6-meGua is considered a critical promutagenic adduct resulting from NMBA activation, and studies indicate that its formation and persistence is closely linked to esophageal tumor induction (42). In the present study, dietary administration of 5% LBRs significantly inhibited O^6-meGua adduct formation after NMBA treatment at both 0.25 and 0.50 mg/kg of body weight. In contrast, feeding 10% LBRs resulted in adduct inhibition after treatment with 0.25 mg/kg NMBA only. This lack of dose-responsive adduct inhibition awaits further investigation, but we hypothesize that these differential results may be linked to the composition of black raspberries. LBRs are rich in antioxidants and contain appreciable levels of phenolics, sterols and other micronutrients, including iron. Antioxidants reportedly exert pro-oxidant effects under conditions of high oxygen pressure, during increased oxidative stress, or in the presence of metal ions (43–45). It is conceivable that feeding LBRs at 10% followed by exposure to NMBA at the higher concentration alters the cellular environment and shifts the balance from one of detoxification and DNA repair to one favoring increased DNA damage. One hypothesis concerning the increases in lung cancer rates among heavy smokers taking high supplemental doses of β-carotene is that β-carotene undergoes oxidative attack and acts as a promoting agent. Thus, the combination of high-dose supplementation and high exposure has proven problematic and potentially promotional in two major trials aimed at preventing lung cancer though β-carotene supplementation (44, 46). Other investigations of natural products have found lower concentrations of inhibitors to be more effective than high concentrations. A recent report by Narisawa *et al.* (47) found capsanthin-rich paprika juice administered at 2 ppm imparted greater

Table 6 *Effects of dietary administration of LBRs on PCNA LIs in the F344 rat esophagus*

Group	NMBA 0.25 mg/kg	Diet	% positive PCNA LI (± SE)	% inhibition[a]
Week 15 of study				
1	−	AIN-76A	18.5 (0.8)	
2	−	10% LBRs	18.3 (1.7)	
3	+	AIN-76A	31.5 (1.3)	
4	+	5% LBRs	22.4 (1.4)[b]	29
5	+	10% LBRs	22.6 (0.7)[b]	28
Week 25 of study				
1	−	AIN-76A	21.3 (1.2)	
2	−	10% LBRs	20.0 (1.2)	
3	+	AIN-76A	32.2 (1.8)	
4	+	5% LBRs	22.7 (0.9)[b]	30
5	+	10% LBRs	25.7 (0.9)[b]	20
Week 35 of study				
1	−	AIN-76A	20.7 (0.9)	
2	−	10% LBRs	20.8 (1.6)	
3	+	AIN-76A	36.0 (1.4)	
4	+	5% LBRs	22.3 (1.1)[b]	38
5	+	10% LBRs	34.3 (1.5)	5

[a] The percentage inhibition in berry-treated groups is relative to NMBA controls (Group 3).
[b] Statistically significant relative to NMBA controls (Group 3) ($P < 0.05$).

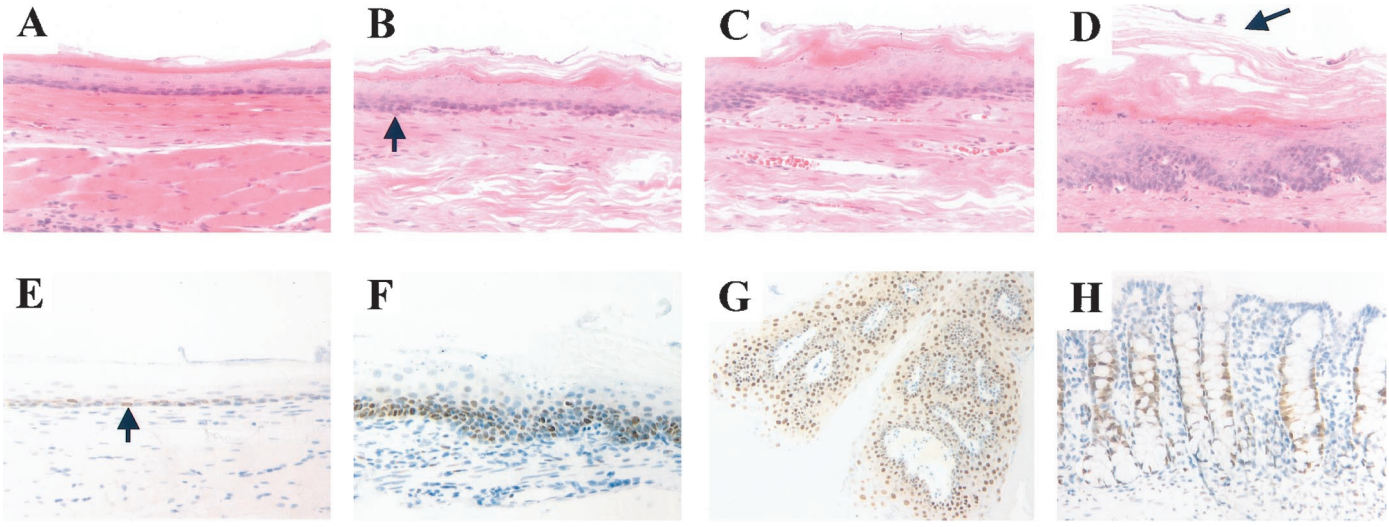

Fig. 2. Esophageal tissue sections stained with H&E and PCNA. *A*, normal esophageal epithelium composed of an orderly mucosa and basal layer one to two cells in thickness (H&E; ×200). *B*, early focal hyperplasia as evidenced by slight thickening of the basal cell and keratin layers (H&E; ×200). *C*, low-grade dysplasia typified by greater hyperplasia (H&E; ×200). *D*, high-grade dysplasia characterized by increased cellular atypia, a disorderly epidermal hyperplasia, and increased thickening of the keratin layer (*arrow*; H&E; ×200). *E*, low-level PCNA staining in normal esophageal epithelium. *F*, increased PCNA nuclear reactivity in dysplastic esophageal epithelium. *G*, intense PCNA staining in a NMBA-induced papilloma at week 35 (H&E; ×100). *H*, PCNA positive staining in rat colon control tissue (H&E; ×200).

protection against chemically induced colon carcinogenesis than paprika juice containing 10 ppm capsanthin. Additionally, capsanthin extracted from paprika and administered alone at 2 ppm. and 10 ppm was not effective as a chemopreventive. We tested two levels of EA, a component of LBRs, as an inhibitor of O^6-meGua adducts. Black raspberries are rich in EA, contributing ~0.180 mg/g of diet or about 0.002% (Table 1) when fed at 10% in the diet. Notably, this level is far below the low-dose concentration of EA (0.004% or 0.4 mg/g of diet) used in the adduct study, a dose that did not reduce adduct levels. EA fed at 0.04% significantly reduced adduct levels after NMBA treatment, but to a lesser extent than LBRs. These results support the theory that EA alone cannot account for the inhibitory potential of LBRs, and that one or more additional berry components must be contributory. Similar findings have been published comparing lycopene alone *versus* administration of tomato juice, which is lycopene rich (48).

To better understand the potential mechanisms through which berries inhibit events associated with cancer initiation, an NMBA metabolism study was conducted using esophageal explants. Two-week dietary pretreatment of rats with 5 and 10% LBRs did not alter esophageal NMBA metabolism, which suggests that LBRs may inhibit O^6-meGua adduct formation without inhibiting NMBA activation. An earlier study by Barch and Fox (49), evaluating the mechanism by which EA inhibited O^6-meGua adducts, found similar results. The authors suggested that EA binds to DNA, selectively inhibiting the attachment of methyl adducts to the O^6-position of guanine without a reduction in the microsomal metabolism of NMBA and without a reduction in activation of NMBA to a methylating agent. Similarly, black raspberries, or a component thereof, may selectively bind to DNA, blocking methylation at the O^6-position of guanine. Alternatively, berries may enhance the repair of promutagenic adducts, may stimulate detoxification of NMBA, or may simply need to be present in culture to exert inhibitory effects. Additional studies are warranted to more clearly understand the mechanism by which berries inhibit adducts without altering NMBA activation.

Chemopreventive agents are commonly classified based on the time period that inhibitory effects are exhibited, whether it be initiation, promotion, or progression stages (50). Thus, a second study was conducted specifically aimed at evaluating the postinitiation chemopreventive potential of LBRs. Promotion, in contrast to initiation and progression, is considered a reversible and generally slow process influencing the proliferation of initiated cells. The majority of adults are presumed to possess initiated cells, and residents of high-esophageal-cancer-incident areas may experience regular exposure to both initiators and promoters. Thus, agents that inhibit the promotion stage of carcinogenesis, or both initiation and promotion events, could offer one of the most effective methods of cancer prevention. The results of this study demonstrate that LBRs inhibit postinitiation tumorigenic events as evidenced by reductions in tumor incidence and multiplicity. Operative mechanisms that contribute to the postinitiation inhibitory effects of LBRs include reduced preneoplastic lesion formation coupled with increases in the percentage of esophagus with a normal histological appearance and decreases in cell proliferation rates. Interestingly, over time, there appears to be a change in the inhibitory potential imparted by the two doses of LBRs administered in the diet. At 25 weeks of study, LBRs administered at both 5 and 10% in the diet significantly reduced tumor incidence, tumor multiplicity, proliferation rates, and preneoplastic lesion development. However, at the final study time point administration only of 5% LBRs was found to significantly impact tumorigenicity, preneoplastic lesion formation, and proliferation indices. The precise reason for the change in chemopreventive potency of a given dose over time is unknown. One possibility is that the optimal chemopreventive dose varies as the animal ages. Secondly, over time, there may be an accumulation of nonprotective berry constituents limiting the chemopreventive utility of LBRs fed at 10% in the diet. A case-control study evaluating phytoestrogen intake and prostate cancer found coumestrol and daidzein to be inversely associated with prostate cancer risk, whereas the phytoestrogens, campesterol and stigmasterol, were positively associated with prostate cancer (51). Similarly, certain phytosterols present in berries could act differentially, exhibiting protective activity when administered at 5% in the diet, but not at 10%.

The present bioassay is the first to evaluate PCNA and cyclin D1 protein after an abbreviated dosing regimen delivering NMBA at 0.25 mg/kg three times a week for 5 weeks. Cell proliferation is known to play an important role in esophageal tumorigenesis (52–54) and appears to be inhibited by the dietary administration of LBRs. Our data support that the down-regulation of cell proliferation is not

related to overexpression of cyclin D1 (data not shown), but instead may be linked to berries inhibition of preneoplastic lesions. Cyclin D1 stained mainly late papillomas, with only minimal expression found in epithelial areas with focal dysplasia at 35 weeks of study. Thus, cyclin D1 does not appear to be a useful biomarker for evaluating chemopreventive agents after NMBA dosing at a low concentration.

To summarize, our data support the hypothesis that LBRs inhibit both initiation and postinitiation tumorigenic events as evidenced by decreases in tumor incidence and multiplicity, adduct inhibition, reduced proliferative indices, and inhibition of preneoplastic lesion formation. Overall, dietary patterns have been closely linked to decreasing cancer risk, with this correlation often lessening as we investigate particular foods and decreasing further at the level of individual nutrients (55, 56). Utilization of a food-based approach may provide a midpoint on the chemopreventive continuum with the traditional approach of testing high concentrations of single agents representing one end and nutritional interventions the other. On the basis of an average daily consumption of 1900 kilocalories among females and 2700 kilocalories among males, ~1.4 and 2.0 cups of fresh berries, respectively, would be required to consume a diet comprised of 5% fresh berries. Because black raspberries are ~86% water, 1.4 and 2.0 cups of fresh berries are approximately equivalent to 28 and 40 grams on a freeze-dried basis. Although, the level found to be inhibitory is larger than a standard serving size of fruit, it approaches behaviorally achievable levels. Thus, using fruits and vegetables in a freeze-dried form may serve as an alternative and natural chemopreventive option.

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