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Berry Ellagitannins May Not Be Sufficient for Prevention of Tumors in the Rodent Esophagus†

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Abstract

Biodirected fractionation is used to identify the active inhibitory constituents in berries for esophageal cancer in rats. The present study was undertaken to determine if ellagitannins contribute to the chemopreventive activity of an alcohol/water-insoluble (residue) fraction of berries. Rats consumed diets containing residue fractions of three berry types, that is, black raspberries (BRBs), strawberries (STRWs), and blueberries (BBs), that differ in their content of ellagitannins in the order BRB > STRW > BB. Animals were fed residue diets beginning 2 weeks before treatment with the esophageal carcinogen *N*-nitrosomethylbenzylamine (NMBA) and throughout the 30-week bioassay. Residue fractions from all three berry types were about equally effective in reducing NMBA tumorigenesis in the rat esophagus irrespective of their ellagitannin content (0.01–0.62 g/kg of diet). These results suggest that the ellagitannins may not be responsible for the chemopreventive effects of the alcohol/water-insoluble fraction of berries.

Keywords

Ellagitannins; biodirected fractionation; esophageal cancer; *N*-nitrosomethylbenzylamine

INTRODUCTION

The Fischer 344 (F344) rat has been used extensively as a model for squamous cell carcinoma (SCC) of the esophagus, the most prevalent type of esophageal cancer worldwide (1). In this model, esophageal tumors are induced routinely by treatment of rats with the nitrosamine carcinogen *N*-nitrosomethylbenzylamine (NMBA) (2). In a typical bioassay, subcutaneous (sc) injections of NMBA at 0.25–0.5 mg/kg of body weight (bw) three times a week for 5 weeks or once per week for 15 weeks result in 100% tumor incidence by 20–25 weeks (3). Our laboratory has used this model since the early 1980s to identify and determine mechanism(s) of action of putative chemopreventive agents for esophageal cancer (4). We reported that the addition of black raspberry (BRB) powder to the diet of NMBA-treated rats at concentrations of 5 or 10% results in a 39–64% reduction, respectively, in the number of esophageal tumors (5). More recently, diets containing either 5% whole black raspberry (BRB) powder, an alcohol/water-soluble extract of BRBs, or an anthocyanin-rich fraction of BRBs (all three diets contained ~3.8 μmol of anthocyanins/g) were found to be

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about equally effective in reducing NMBA tumorigenesis in the esophagus (6). These results suggested that the anthocyanins are responsible for some of the chemopreventive potential of BRBs. In this same study, however, a diet containing the alcohol/water-insoluble (residue) fraction of BRBs containing only 0.02 μmol of anthocyanins/g was nearly as effective as the anthocyanin diets in preventing esophageal tumorigenesis, suggesting that components other than the anthocyanins may be chemopreventive. The residue fraction of BRBs represents about 45% of whole BRB powder and likely contains cellulose, hemicelluloses, pectins, lignans, and protein (7). Chemical analysis of the residue indicated that it also contains ellagitannins (8).

The ellagitannins are complex polyphenols in which the compound hexahydroxydiphenic acid forms diesters with sugars (most often β-D-glucose) (9). Ellagitannins form polymers that can reach molecular weights of up to 4000 and, when hydrolyzed with acids or bases, yield ellagic acid. Because the ellagitannins and anthocyanins have antioxidant potential and are among the most prevalent compounds in berries, collectively, they are thought to be responsible for much of the antioxidant activity of berries (10–12). Ellagitannins have been shown to possess chemopreventive potential in multiple model systems in vitro and in vivo. For example, the ellagitannins in raspberry extract were responsible for reducing the proliferation rate of cultured human cervical cancer (HeLa) cells (8). Our laboratory reported that pure ellagic acid added to a rat diet inhibits the metabolic activation of NMBA as well as NMBA-induced tumorigenesis in the rat esophagus (13,14). In a study in which the ellagic acid content of different fruits was measured, BRBs were found to have the highest content (1500 μg/g of dry weight), strawberries (STRWs) were intermediate (630 μg/g of dry weight), and blueberries (BBs) had among the lowest contents (<100 μg/g of dry weight) (15). As indicated above, the residue fraction of BRBs was found to be chemopreventive and to contain ellagitannins (6). The present study was designed to determine if the ellagitannins in the residue fraction of berries might be responsible for chemopreventive effects or lack thereof. On the basis of their relative contents of ellagitannins, we expected that the chemopreventive activity of the residue fractions of BRBs, STRWs, and BBs would be in the order BRB > STRW > BB.

MATERIALS AND METHODS

Sources of Berries

Black raspberries (*Rubus occidentalis*) of the Jewel variety were obtained from a single farm in Ohio, strawberries (*Fragaria* × *ananassa*) from the California Strawberry Commission, and blueberries (*Vaccinium corymbosum*) from Watershed Foods (Gridley, IL). All three berry types were freeze-dried and processed into powder, and the powders were analyzed for content of multiple vitamins, minerals, simple and complex phenols, carotenoids, and phytosterols as described before (5). Berry powders were shipped frozen to the laboratory of Dr. Stephen Hecht for preparation of the residue extracts as described below. The remaining berry powders were stored frozen for use in an esophageal carcinogenesis bioassay conducted at The Ohio State University.

Preparation of the Ethanol/H_2O-Insoluble (Residue) Fraction from Berries

Freeze-dried berries (500 g) were placed in a 2500 mL beaker, and 1500 mL of 200 proof USP ethanol/H_2O (80:20) was added. The mixture was sonicated for 10min, and the slurry was stirred for 10 min and then sonicated again for 10 min. It was then filtered using a Buchner funnel with vacuum. The extraction procedure was repeated three additional times (total ethanol/H_2O = 6000 mL). The filtrate (berry mass) was allowed to dry under vacuum for 3 days at room temperature and then was stored at 4 °C until use.

Measurement of Ellagitannins in Berries

The ellagitannins in the residue fractions were determined by methanolysis. Residue (50 mg) was added to 4 mL of freshly prepared 19% acetyl chloride in methanol. The reaction vial with a Teflon-lined cap was placed behind a safety shield and heated to 160 °C for 60 min (16). An aliquot of the hydrolysate was analyzed by HPLC with UV detection at 260 nm essentially as described (17). The ellagitannins were quantified using standard curves of ellagic acid and the relative absorptivities of ellagic acid and methyl sanguisorbate. The contents of anthocyanins in the residue fractions were not measured because preliminary studies revealed that ~99% of the anthocyanins in BRB powder are extracted after only a single treatment with ethanol/water (80:20) (unpublished data).

Chemicals

NMBA, obtained from Ash Stevens (Detroit, MI), was >98% pure as determined by HPLC. Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO).

Animals

Male F344 rats, 4–5 weeks old, were obtained from Harlan Sprague–Dawley (Indianapolis, IN). The animals were housed two per cage under standard conditions (20 ± 2 °C, 50 ± 10% relative humidity, 12 h light/dark cycle). Food and water were available ad libitum. Hygienic conditions were maintained by twice weekly cage changes. The animals were fed a modified American Institute of Nutrition-76A (AIN-76A) synthetic diet (Dyets, Inc., Bethlehem, PA). Body weights and food intake were recorded weekly after administration of the various diets. The animals were housed and maintained according to the recommendations of the American Association of Laboratory Animal Care (AALAC).

Animal Bioassay

Two weeks after arrival in the animal facility, rats were randomly assigned into 17 groups of 15 animals each and placed on control AIN-76A diet or AIN-76A diet containing berry powder or its residue fraction for the entire 30 week bioassay. The amount of each fraction added to the diet was based on the weight percent contribution of each residue fraction to berries (Tables 1 and 2). The groups were as follows: (1) no additions to the diet (diet control), (2) residue from 10% black raspberry (BRB) powder, (3) residue from 10% strawberry (STRW) powder, and (4) residue from 10% blueberry (BB) powder. Groups 1–4 were not treated with NMBA. Rats in groups 5–17 received sc injections of NMBA and were treated with different diets as follows: (5) no additions to the diet and treatment with NMBA (NMBA control), (6) residue from 10% BRB powder and NMBA, (7) residue from 10% STRW powder and NMBA, (8) residue from 10% BB powder and NMBA, (9) residue from 5% BRB powder and NMBA, (10) residue from 5% STRW powder and NMBA, (11) residue from 5% BB powder and NMBA, (12) 10% BRB powder and NMBA, (13) 10% STRW powder and NMBA, (14) 10% BB powder and NMBA, (15) 5% BRB powder and NMBA, (16) 5% STRW powder and NMBA, and (17) 5% BB powder and NMBA.

To maintain an isocaloric diet, the starch in the diet of rats fed 5 and 10% berry powders was reduced by 5 and 10%, respectively. All berry residue fractions were mixed with regular AIN-76A diet. After 2 weeks on their respective diets, rats in groups 1–4 were injected sc with 0.2 mL of a solution containing 20% DMSO in water (the vehicle for NMBA) once per week for 15 weeks. Rats in groups 5–17 were injected sc with NMBA (0.3 mg/kg of bw) in 0.2 mL of vehicle once per week for 15 weeks. At 30 weeks, the animals were killed by CO_2 asphyxiation, the esophagus of each animal was opened longitudinally, and the surface tumors were mapped, counted, and sized. Lesions >0.5 mm in a single dimension (length, width, or height) were considered to be tumors. Tumor volume was calculated using the

formula for a prolate spheroid, length × width × height × $p/6$, and expressed in cubic millimeters; this can also be considered as an estimation of tumor size.

Statistical Analysis

Body weight, food consumption, and tumor number and volume were compared using ANOVA and an unpaired *t* test Stat View (SAS Institute). A p value of < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

General Observations

No significant differences in animal body weights and food consumption were found among any of the groups during the entire 30 week bioassay. Each tumor was examined by light microscopy for histopathologic features of squamous cell carcinoma; for example, loss of cell polarity, nuclear atypia, keratin “pearl” formation, cellular invasion through the basement membrane into the underlying stroma, blood vessels, and lymphatics. None of the tumors had these features; all tumors resembled papillomas. Typically, in this model system, and at the dose of NMBA used, the animals succumb to the occlusive effects of papillomas in the esophagus before carcinomas develop.

Effects of Diets Containing Different Amounts of Ellagitannins on NMBA-Induced Rat Esophageal Tumorigenesis

The effects of the different diets on the number and volume of NMBA-induced esophageal papillomas at 30 weeks are shown in Tables 1 and 2, respectively. As expected, BRBs contained the highest amount of ellagitannins among the three berry types, followed by STRWs and BBs. Importantly, all groups of NMBA-treated rats fed either the residue diets or the whole berry powder diets (groups 6–17) had fewer and smaller papillomas than the NMBA control group (group 5). However, the berry residue and berry powder diets did not differ significantly in their ability to reduce the number of NMBA-induced tumors in the esophagus (Table 1). With respect to BRBs, this result confirmed our previous observation that the BRB residue is as effective in the chemoprevention of esophageal cancer as whole BRBs (6). The present study extends this observation to both STRWs and BBs; residues from STRWs and BBs were equally as effective as whole STRW or BB powders in reducing NMBA-induced esophageal tumors. In the NMBA control group (group 5), 25% of the animals had tumor volumes that exceeded 100 mm^3, whereas 6–17% of animals had smaller tumors resembling those in groups 6–17 (Table 2).

Overall, the results of this study suggest that the inhibitory effects of the alcohol/water-insoluble (residue) fractions of BRBs, STRWs, and BBs on NMBA tumorigenesis in the rat esophagus may not be due solely to their content of ellagitannins. The concentrations of ellagitannins in the different berry diets (groups 6–17) varied up to 62-fold, that is, ranging from a low of 0.01 g/kg to a high of 0.62 g/kg of diet, yet these diets all produced an average 42–56% overall reduction in tumor multiplicity. Moreover, none of the berry residue or whole berry powder groups was more effective in chemoprevention than the other. These results suggest that other constituents in the residue fractions from the three berry types are responsible for at least a substantial portion of their chemopreventive effects. These constituents could be small molecular weight nutrients and non-nutritive compounds that may be well absorbed and have substantial chemopreventive potential. It is also possible that the fibrous component of the residues, which likely contains lignans, cellulose, pectin, complex carbohydrates, etc., contributes to their chemopreventive effects because they represent a major portion of the residue fractions and the residues comprise 58, 27, and 33% of whole BRBs, STRWs, and BBs, respectively. In particular, lignans with antioxidant and

cytoprotective activities might possess chemopreventive potential (18). Dietary fibers reach the large bowel and are attacked by colonic microflora, yielding short-chain fatty acids, hydrogen, carbon dioxide, and methane as fermentation products. Short-chain fatty acids may contribute to the chemopreventive effects of berries (19). In addition, some indigestible carbohydrates (short-chain fructo-oligosaccharides) have been shown to reduce colon tumor incidence in Apc+/Min mice, and this effect was associated with stimulation of T-cell function (20).

Alternatively, it is possible that differences in the chemopreventive potential of the berry powders and the residue fractions used in the present study could have been demonstrated if lower concentrations of the test agents had been used. At the lowest concentrations used, absorption of the active constituents may have been saturated, leading to maximum effects. In view of this, we are conducting another study to determine if differences in chemopreventive potential of the berry powders and residue fractions can be demonstrated at half and one-fourth of the lowest concentrations used in the present study.

In summary, results from the present study suggest that the inhibitory effects of the residue fractions of the three berry types on tumor development in carcinogen-treated rat esophagus are not due solely to their content of ellagitannins. Currently, we are attempting to identify other constituents in the residue fractions of these berries that might be responsible for chemoprevention.

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Table 1

Effects of Diets Containing Different Amounts of Ellagitannins (ET) Derived from Black Raspberries (BRBs), Strawberries (STRWs), and Blueberries (BBs) on Tumor Number in NMBA-Treated Rat Esophagus

	group																
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
NMBA[a]	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
residue or whole berry[b]	−	residue	residue	residue	−	residue	residue	residue	residue	residue	residue	whole berry	whole berry	whole berry	whole berry	whole berry	whole berry
type of berry	−	BRB	STRW	BB	−	BRB	STRW	BB	BRB	STRW	BB	BRB	STRW	BB	BRB	STRW	BB
whole berry equivalent (%)	−	10	10	10	−	10	10	10	5	5	5	10	10	10	5	5	5
whole berry as residue (%)	−	58.3	27.3	33.2	−	58.3	27.3	33.2	58.3	27.3	33.2	58.3	27.3	33.2	58.3	27.3	33.2
g of ET/kg of diet	−	0.62	0.17	0.02	−	0.62	0.17	0.02	0.31	0.08	0.01	0.62	0.17	0.02	0.31	0.08	0.01
av tumor no. per rat[c]																	
mean	−	−	−	−	10.2	5.5*	6.2*	4.9*	4.4*	5.2*	5.8*	5.7*	4.5*	5.4*	4.8*	5.2*	5.4*
SE	−	−	−	−	1.1	0.7	0.6	0.7	0.5	0.6	1.0	0.7	0.8	1.0	1.0	0.6	0.8

[a] Animals in groups 1–4 received sc injections of 0.2 mL of 20% DMSO, the vehicle for NMBA, once per week for 15 weeks. Animals in groups 5–17 received sc injections of NMBA (0.3 mg/kg of bw) in0.2 mL of 20% DMSO, once per week for 15 weeks. The injections of vehicle or NMBA were started 2 weeks after initial administration of berry powders or residues to the diet.

[b] Animals in groups 1 and 5 were fed AIN-76A diet only. All other groups were fed AIN-76A containing either residue or whole berry diets throughout the entire 30 week bioassay.

[c] $n = 15$.

* significantly lower ($P < 0.05$) than rats treated with NMBA only (group 1).

Table 2

Effects of Diets Containing Different Amounts of Ellagitannins (ET) Derived from Black Raspberries (BRBs), Strawberries (STRWs), and Blueberries (BBs) on Tumor Volume in NMBA-Treated Rat Esophagus

	group																
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
NMBA[a]	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
residue or whole berry[b]	−	residue	residue	residue	−	residue	residue	residue	residue	residue	residue	whole berry	whole berry	whole berry	whole berry	whole berry	whole berry
type of berry	−	BRB	STRW	BB	−	BRB	STRW	BB	BRB	STRW	BB	BRB	STRW	BB	BRB	STRW	BB
whole berry equivalent (%)	−	10	10	10	−	10	10	10	5	5	5	10	10	10	5	5	5
whole berry as residue (%)	−	58.3	27.3	33.2	−	58.3	27.3	33.2	58.3	27.3	33.2	58.3	27.3	33.2	58.3	27.3	33.2
g of ET/kg of diet	−	0.62	0.17	0.02		0.62	0.17	0.02	0.31	0.08	0.01	0.62	0.17	0.02	0.31	0.08	0.01
av tumor vol per rat[c]																	
mean (mm^3)	−	−	−	−	85.7	47.3*	58.6*	48.9*	56.6*	44.5*	59.5*	33.4*	32.1*	26.1*	41.7*	54.0*	55.1*
SE	−	−	−	−	10.0	15.7	15.2	18.1	26.1	15.1	19.6	14.5	15.2	7.6	13.8	22.4	24.5
% of animals with tumors >100 mm^3	−	−	−	−	25	11	11	14	11	16	17	13	13	6	13	7	13
% of animals with tumors 51–100 mm^3	−	−	−	−	20	22	39	21	16	5	20	2	3	1	6	20	19
% of animals with tumors e50 mm^3	−	−	−	−	55	67	50	64	74	79	63	86	85	93	81	73	69

[a] Animals in groups 1–4 received sc injections of 0.2 mL of 20% DMSO, the vehicle for NMBA, once per week for 15 weeks. Animals in groups 5–17 received sc injections of NMBA (0.3 mg/kg of bw) in 0.2 mL of 20% DMSO, once per week for 15 weeks. The injections of vehicle or NMBA were started 2 weeks after initial administration of berry powders or residues to the diet.

[b] Animals in groups 1 and 5 were fed AIN-76A diet only. All other groups were fed AIN-76A containing either residue or whole berry diets throughout the entire 30-week bioassay.

[c] $n = 15$.

* significantly lower ($P < 0.05$) than rats treated with NMBA only (group 1). Percent of animals in each group having a tumor volume of either >100 mm^3, 51–100 mm^3, or ≤50 mm^3.