JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Blueberry (*Vaccinium ashei*) on DNA Damage, Lipid Peroxidation, and Phase II Enzyme Activities in Rats

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Blueberry extracts have high antioxidant potential and increase phase II enzyme activities in vitro. This study tested the hypothesis that blueberries would reduce DNA damage and lipid peroxidation and increase phase II enzyme activities in vivo. Young, healthy male Sprague−Dawley rats ($n = 8$ per group) were fed control AIN-93 diets or AIN-93 diets supplemented with blueberries or blueberry extracts for 3 weeks. Diets were supplemented with 10% freeze-dried whole blueberries, blueberry polyphenol extract and sugars to match the 10% blueberry diet, or 1 and 0.2% blueberry flavonoids, which were primarily anthocyanins. Liver and colon mucosa glutathione-*S*-transferase (GST), quinone reductase, and UDP-glucuronosyltransferase activities in colon mucosa and liver were not significantly increased by freeze-dried whole blueberries or blueberry fractions. Liver GST activity, however, was approximately 25% higher than controls for the freeze-dried whole blueberry, blueberry polyphenol, and 1% flavonoid groups. DNA damage was significantly lower than control only in the liver of animals fed the 1% flavonoid diet. The level of urinary F_2-isoprostanes, a measure of lipid peroxidation, was unaffected. In summary, in healthy rats, short-term supplementation with freeze-dried whole blueberries, blueberry polyphenols, or blueberry flavonoids did not significantly increase phase II enzyme activities. However, supplementation with 1% blueberry flavonoids did decrease oxidative DNA damage in the liver.

KEYWORDS: Blueberry (*Vaccinium ashei*); polyphenols; oxidative stress; phase II enzymes

INTRODUCTION

The association between diets rich in fruits and vegetables and reduced chronic disease risk is now well established. Dietary antioxidants found in fruits and vegetables, such as polyphenols, may contribute to their beneficial effects (*1*). The blueberry is one fruit that has received attention due to its high antioxidant potential (*2, 3*). Sellappan et al. (*4*) reported that blueberries contained on average 556.14 mg of total polyphenols/100 g of fresh weight and had Trolox equivalent antioxidant capacity (TEAC) values of 27.60 μM/g of fresh weight, which is considered to be high. Much of the blueberry's protective nature may be attributed to the flavonoids found in the fruit, particularly anthocyanins (*1, 2, 5, 6*). Polyphenols, such as flavonoids, can reduce oxidative stress by directly scavenging free radical species and chelating transition metals (*6, 7*). Blueberry may also affect chronic disease through antiinflammatory and antitumor mechanisms (*2, 5, 6*). The focus of this study was to examine the impact of blueberry on DNA damage, lipid peroxidation, and detoxification enzyme systems in rats.

It is well documented that blueberries have a high antioxidant capacity (*3, 4, 6*). In vitro studies have shown that blueberry anthocyanins have the capacity to reduce H_2O_2-induced reactive oxygen species in endothelial cells and red blood cells (*8, 9*). A number of studies have examined the impact of blueberries or blueberry fractions on measures of oxidative stress in vivo (*2*). Supplementation of rats with blueberry anthocyanins reduced isolated red blood cell susceptibility to free radical damage (*8*). Blueberry supplementation can also reduce age-associated losses of neuronal and behavioral function (*10*) and ischemia-induced brain damage (*11, 12*) in rats. Both antioxidant and anti-inflammatory mechanisms may be involved in the protection of neural tissue by blueberry (*10, 13, 14*). Improved cognitive function in blueberry-supplemented aged rats was correlated with normalized levels of oxidative stress-responsive

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10.1021/jf802405y CCC: $40.75
Published on Web 11/26/2008

Table 1. Composition (Grams per Kilogram) of Diets[a]

ingredient	diet 1, control	diet 2, 10% blueberry	diet 3, carbohydrate match	diet 4, total polyphenol	diet 5, 1% flavonoid	diet 6, 0.2% flavonoid
casein	200.0	200.0	200.0	200.0	198.0	199.6
cornstarch	529.0	529.0	529.0	529.0	524.0	528.4
soybean oil	70.0	70.0	70.0	70.0	69.3	69.7
fiber	50.0	33.0	50.0	50.0	49.5	49.9
mineral mix	35.0	35.0	35.0	35.0	34.7	34.9
vitamin mix	10.0	10.0	10.0	10.0	9.9	10.0
L-cysteine	3.0	3.0	3.0	3.0	3.0	3.0
choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
sucrose	100.0	18.0	26.2	24.3	99.0	99.8
fructose			37.0	28.5		
glucose			37.0	28.5		
blueberries		100.0				
polyphenols				20.0		
flavonoids					10.0	2.0

[a] Diet 1 was an AIN-93 M diet; diet 2 was 10% freeze-dried blueberries, which are 17% fiber and 82% sugar. Added fiber and sucrose were adjusted so total values would match control. Diet 2 had 0.24% polyphenols based on freeze-dried blueberry analysis. Diet 3 contained sugars to match the composition of blueberries, 10% sucrose and 45% each of glucose and fructose. Diet 4 contained crude polyphenol extract and sugars to match the 10% blueberry diet. Diet 4 had 0.13% polyphenols based on fraction analysis. Diet 5 consisted of 99% control diet and 1% flavonoids consisting mainly of anthocyanins and tannins. Diet 6 was 99.8% control diet and 0.2% purified flavonoids consisting mainly of anthocyanins and tannins.

NF-κB by Goyarzu et al. (*15*), whereas Barros et al. (*16*) was able to show both enhanced memory and decreased neural DNA damage in mice supplemented with blueberry extract. Rats fed whole blueberry and subjected to an inflammatory insult tended to have less liver lipid peroxidation than controls, although reductions were not significant (*17*). Human studies have also been conducted. Chronic smokers who consumed 250 g of blueberries daily for 3 weeks had reduced plasma lipid hydroperoxide levels, but not F_2-isoprostanes; plasma antioxidant potential was not increased (*18*). In contrast, healthy adults who consumed a blueberry/apple juice for 4 weeks had increased serum antioxidant capacity and reduced oxidative DNA damage in lymphocytes. Effects were dependent on genetic polymorphisms (*19*).

Polyphenols can also up-regulate detoxification enzymes, which process free radical species and facilitate excretion of foreign compounds. Thus, another potential protective mechanism for blueberry is to increase phase II enzymes involved in detoxification of substances that are harmful or foreign to the body, such as drugs, toxins, or carcinogens. Detoxification enzymes are highly expressed in the liver, which is the primary site for detoxification (*20*). Glutathione *S*-transferases (GST), UDP-glucuronosyltransferases (UGT), and NAD(P)H:quinone reductase (QR) are some of the most common detoxification enzymes. GSTs catalyze the reaction of electrophilic or radical compounds with glutathione, which inactivates the compounds and facilitates excretion. UGTs conjugate glucuronic acid to lipophilic compounds, tagging them for excretion. QR facilitates the transfer of electron pairs to quinones and some azo dyes and, thus, prevents the formation of superoxide radical intermediates (*21*). Phase II enzymes play an important role in cancer prevention, and have been shown to be induced by a wide range of polyphenols (*22*).

Supplementations with blueberry and other fruit extracts have produced mixed results on enzyme induction in both in vitro and in vivo models. Wild blueberry flavonoids significantly increased (*23*) or had no effect (*24*) on QR activity in Hepa 1c1c7 cells, a murine hepatoma cell line. A previous study in our laboratory (*25*) showed that rabbiteye blueberry anthocyanins (50−150 μg/mL) decreased or had no effect on QR activity of HT-29 colon cancer cells, with response dependent on cultivar. GST activity, however, was decreased in a dose-dependent manner by anthocyanins from all cultivars tested. Fischer 344 rats treated with azoxymethane to induce colon cancer had increased hepatic GST activity after supplementation with 5% freeze-dried blueberries for 13 weeks and also had fewer precancerous aberrant crypt foci (*26*). Similarly, rats fed a diet of 5 or 10% freeze-dried black raspberries, another fruit high in anthocyanins, for 3 weeks had an approximately 45% increase in liver GST activity above the control (*27*). Ronis et al. (*28*) reported that blueberry increased activities of liver GST α and QR.

Research on the effect of blueberry supplementation on oxidative stress and phase II enzymes in vivo is limited. In this study we tested the hypothesis that whole blueberries and blueberry fractions would decrease basal levels of DNA damage and lipid peroxidation but increase activities of the phase II enzymes GST, UGT, and QR in a rodent model.

MATERIALS AND METHODS

Chemicals and Supplies. Commercial kits were used for the comet assay (Trevigen, Gaithersburg, MD) and urinary 15-F_{2t}-isoprostanes (Oxford Biomedical, Oxford, MI). Diet ingredients were purchased from Harlan Teklad (Madison, WI). Pure standards of gallic acid, (+)-catechin, (−)-epicatechin, quercetin, and kaempferol were purchased from Sigma (St. Louis, MO). Anthocyanin standards were purchased from Polyphenols Laboratories (AS) (Sandnes, Norway). These standards were delphinidin 3-*O*-β-glucopyranoside (Dp-Glc), cyanidin 3-*O*-β-galactopyranoside (Cy-Gal), cyanidin 3-*O*-β-glucopyranoside (Cy-Glc), petunidin 3-*O*-β-glucopyranoside (Pt-Glc), peonidin 3-*O*-β-galactopyranoside (Pn-Gal), peonidin 3-*O*-β-glucopyranoside (Pn-Glc), malvidin 3-*O*-β-glucopyranoside (Mv-Glc), and peonidin 3-*O*-α-arabinopyranoside (Pn-Ara). All other chemicals were obtained from Sigma.

Blueberry Extract and Diet Preparation. Rats were fed an AIN-93 diet supplemented with 10% freeze-dried whole blueberries, blueberry polyphenol extract added at a concentration to be equivalent to the 10% freeze-dried whole blueberry, or 1 or 0.2% blueberry flavonoid extract for 3 weeks (**Table 1**). Flash-frozen fresh Tifblue rabbiteye blueberries were donated by the Georgia Blueberry Growers Association and were stored at −40 °C until use. A 10% freeze-dried whole blueberry concentration was chosen on the basis of previous studies suggesting that 5−10% blueberry was associated with changes in QR and GST activities in rat liver (*26, 28*). The blueberry flavonoid extract was high in anthocyanins, and the 0.2% concentration was chosen on the basis of research showing that anthocyanin concentrations of 0.1−0.2% of the diet can produce some antioxidant effects in vivo (*6*). A higher 1% concentration was also added to examine dose−response effects for this extract. These dietary concentrations are much higher than typically consumed by humans, even for those who consume a

high amount of fruit (*6*). We did not assess bioavailability in this study, but it has been shown that anthocyanin bioavailability is low in both humans (*6*) and rats (*29*). Prior (*6*) reviewed human studies showing maximum plasma anthocyanin concentrations of 1−120 nmol/L resulting following anthocyanin intake of 0.7−10.9 mg/kg of body weight. Talavéra et al. (*29*) demonstrated that supplementing rats with blackberry anthocyanin extract at 1.5% of the diet resulted in plasma anthocyanin concentrations of 360 ± 20 nmol/L.

Whole blueberries were freeze-dried and incorporated into the diet at a 10% level for one diet. The total polyphenol and flavonoid extracts were obtained using a procedure described by Yi et al. (*5*). The polyphenol extract was prepared by homogenizing blueberries with a solvent consisting of a 40:40:20:0.1 proportion by volume of acetone/methanol/water/formic acid solution. The extract was first centrifuged, then concentrated using a rotary evaporator to remove the organic solvents, and finally freeze-dried to remove water before it was added to the diet. The flavonoid extract was obtained by further purifying the polyphenol extract using an Oasis HLB cartridge and then eluting with acidified methanol.

Freeze-dried whole blueberry and flavonoid extracts were analyzed for polyphenol content, flavonols, and anthocyanins using procedures previously reported by our laboratory (*4, 5*). HPLC analysis was performed with a Hewlett-Packard (Avondale, PA), model 1100 liquid chromatograph with quaternary pumps and a diode array UV−visible detector. The column was a Beckman Ultrasphere C18 ODS 4.6 × 250 mm, and the column temperature was 40 °C. Phenolic compounds were detected at wavelengths of 280, 320, 360, and 520 nm. For the anthocyanin analysis, the mobile phases were, solvent A, *O*-phosphoric acid/methanol/water (5:10:85, v/v/v), and, solvent B, acetonitrile. The flow rate was 0.5 mL/min. The gradient for the separation was as follows: 100% solvent A at 0 min, 90% solvent A and 10% solvent B at 5 min, 50% solvent A and 50% solvent B at 25 min, with 5 min postrun with HPLC-grade water. For other phenolic compounds including epicatechin, quercetin, and kaempferol, extracts were hydrolyzed in methanol containing 1.2 N HCl under 80 °C to convert phenolic glycosides to aglycones. The mobile phases were (A) water/methanol (70:30 v/v) with 1% formic acid, (B) methanol, and (C) 1% formic acid in water. The flow rates were 1.3 mL/min from 0 to 5 min and 1.0 mL/min from 5.01 to 75 min. The gradient was as follows: at 0 min, 100% solvent C; at 5 min, 100% solvent C; at 5.01 min, 50% solvent C; at 10 min, 15% solvent B; at 10.01 min, 5% solvent B and 45% solvent C; at 20 min, 15% solvent B; at 25 min, 15% solvent B; at 60 min, 50% solvent B; at 60.01 min, 100% solvent B; at 65 min, 100% solvent B; at 65.01 min, 50% solvent C; at 75 min, 50% solvent C.

The total polyphenol content (gallic acid equivalents) of freeze-dried whole blueberries was 2.4%, following the method reported by Singleton and Rossi (*30*). Total polyphenols were expressed as gallic acid equivalents (GAE), and the total polyphenol content was calculated as GAE/(extract weight) × 100%. Flavonol and anthocyanin contents of freeze-dried whole blueberry are shown in **Table 2**. The remaining phenolic compounds in freeze-dried whole blueberry include phenolic acids and tannins. The total polyphenol content in the blueberry polyphenols extract was 6.4% (gallic acid equivalents). In addition to flavonols and anthocyanins (**Table 2**), the remaining phenolic compounds include phenolic acids and tannins, whereas the major nonphenolic content in the polyphenols extract was sugars. The blueberry flavonoids extract was predominantly anthocyanins but also contained 20.1% tannins (catechin equivalents), measured using the vanillin assay (*31*) and flavonols. The total polyphenol content in the flavonoids extract was 92.7%. The identified individual compounds, including epicatechin, quercetin, kaempferol, and seven anthocyanins, account for 54.8% of this extract.

The freeze-dried whole blueberry and polyphenol and flavonoid extracts were mixed into the respective diets after all other ingredients had been thoroughly blended. The diets were mixed in 15-day batches and stored at −20 °C to prevent degradation.

Animal Study. All animal procedures were approved by the University of Georgia Institutional Animal Care and Use Committee. Forty-eight male Sprague−Dawley rats (Harlan, Indianapolis, IN), 75−99 g, were divided into six weight-matched treatment groups of eight. The rats were housed individually in wire-bottom cages and kept at 21.1 °C in a humidity- and light-controlled environment (12 h light/dark cycle). The rats had unlimited access to their assigned diet and distilled water for 3 weeks. Diet was provided fresh daily. Body weight was recorded weekly, and food consumption was recorded daily.

Table 2. Major Individual Flavonoid Compounds in Freeze-Dried Blueberries and Blueberry Extracts (Weight Percent)[a]

compound	freeze-dried blueberries	blueberry polyphenols	blueberry flavonoids
epicatechin	0.03	0.07	0.48
quercetin	0.09	0.22	1.51
kaempferol	0.07	0.17	1.17
Dp-Glc	0.17	0.41	4.56
Cy-Gal	0.27	0.63	8.94
Cy-Glc	0.11	0.25	3.54
Pt-Glc	0.37	0.87	12.24
Pn-Gal	0.16	0.37	5.16
Pn-Glc	0.38	0.87	12.30
Mv-Glc	0.15	0.35	4.86

[a] Values are weight percentage of dry mass. Dp-Gl, delphinidin 3-*O*-*β*-glucopyranoside; Cy-Gal, cyanidin 3-*O*-*β*-galactopyranoside; Cy-Glc, cyanidin 3-*O*-*β*-glucopyranoside; Pt-Glc, petunidin 3-*O*-*β*-glucopyranoside; Pn-Gal, peonidin 3-*O*-*β*-galactopyranoside; Pn-Glc, peonidin 3-*O*-*β*-glucopyranoside; Mv-Glc, malvidin 3-*O*-*β*-glucopyranoside.

On the day preceding sacrifice, urine was collected overnight and frozen at −80 °C until analysis. The rats were sacrificed using CO_2. Blood was collected via cardiac puncture. Plasma lymphocytes and serum were immediately isolated with Histopaque 1083 and frozen at −80 °C until analysis. Livers were removed, sectioned, and frozen at −80 °C until analysis. Colons were removed, opened, and rinsed with saline, and the mucosa was obtained by gentle scraping with a microscope slide. The mucosal samples were then frozen at −80 °C until analysis.

Preparation of the Cytosol and Microsome Fractions. Colon mucosal scrapings and 0.5 g liver samples were homogenized with 1:2 parts ice-cold Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 9500*g* for 20 min at 4 °C. The supernatant was transferred to a thick-walled polycarbonate tube and centrifuged at 100000*g* for 30 min. The cytosol supernatant was aliquoted and frozen at −80 °C until analysis. The microsomal pellet was washed with Tris-HCl buffer, then resuspended in stabilizing buffer (phosphate buffer with 1 mM EDTA, pH 7.4), and stored at −80 °C until analysis.

DNA Damage. DNA damage in lymphocytes and liver cells was assessed using a comet assay kit. Briefly, cells were prepared, embedded in agarose on a slide, and lysed in a basic solution. The slides were then electrophoresed and stained with the silver staining kit. Finally, the cells were visually scored according to degree of DNA damage. Visual scoring is consistent with computer image analysis (*32*). Cells were scored on a scale of 1−5, with 1 representing an undamaged cell with intact DNA and no comet tail and 5 representing a highly damaged cell with a large comet tail. Representative comet images by Tuo et al. (*33*) were used as a guide, and a DNA damage formula by Zhao et al. (*32*) was used. Two samples per rat were scored. For the lymphocyte scoring, five rats had unscorable samples. Slides were blinded prior to scoring to prevent bias.

GST Activity. Cytosolic GST activity of liver and colon mucosa samples was measured in duplicate at 25 °C using the methods of Habig et al. (*34*) with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. Briefly, 0.1 mL of diluted sample was used for the assay. The final 1 mL assay mixture included potassium phosphate buffer (0.1 mol/L, pH 6.5), 5 mmol/L glutathione, and 1 mmol/L CDNB. The conjugation of glutathione to CDNB at 25 °C was measured for 3 min at 340 nm in a spectrophotometer. The molar extinction coefficient for CDNB at 340 nm is 9.6 mM/cm.

QR Activity. QR activity in liver and colon mucosa was measured according to an established protocol described by Kore et al. (*35*) using 2,6-dichlorophenolindophenol (DPIP) as the substrate. The ability of the isolated cytosol to reduce DPIP was determined at 600 nm, and

the dicoumarol-sensitive portion of activity was used to estimate QR activity. Samples were run in triplicate. The molar extinction coefficient for DPIP at 600 nm is 21 mM/cm.

UGT Activity. UGT activity of the liver was determined using the methods of Bock et al. (*36*) with modifications by Letelier et al. (*37*). Microsomal samples were assayed in duplicate to assess the glucuronidation of *p*-nitrophenol. Approximately 1 mg of microsomal protein was incubated for 5 min at 37 °C with 0.01% w/v Triton X-100, 100 mM Tris-HCl, 5 mM $MgCl_2$, and 0.5 mM *p*-nitrophenol. The reaction was initiated by adding 3 mM UDP-glucuronic acid. Aliquots of 100 μL were taken at 0 and 15 min and combined with 2 mL of cold 5% TCA to stop the reaction. After centrifugation, 1 mL of supernatant was combined with 250 μL of 2 M NaOH. Absorbance was measured at 405 nm, with color being proportional to the remaining, unglucuronidated *p*-nitrophenol. A molar extinction coefficient of 18.1 mM/cm for *p*-nitrophenol at pH >10 was used to calculate activity. UGT activity was not measured in the colon mucosa because preliminary experiments showed that the activity levels were too low to detect.

15-F_{2t}-Isoprostane Assay. Urinary F_2-isoprostane levels were measured using glucuronidase sample treatment and urinary 15-F_{2t}-isoprostane kits (Oxford Biomedical, Oxford, MI). Urine samples were pretreated for 2 h at 37 °C with β-glucuronidase from *Helix pomatia* to release isoprostanes bound to glucuronic acid. An enhanced dilution buffer was then added to the sample to remove nonspecific binding interference. The diluted samples were incubated for 2 h with 15-F_{2t}-isoprostane conjugated to horseradish peroxidase in microplate wells with polyclonal antibody to 15-F_{2t}-isoprostane coated in the wells. The plates were washed three times to remove unbound contents. Substrate was then added to the wells for 30 min, to activate the horseradish peroxidase, which causes a blue color development. Sulfuric acid was added to the wells, changing the color to yellow. The plate absorbance was read at 450 nm, with color intensity being indirectly proportional to the amount of 15-F_{2t}-isoprostane in the samples. All samples were analyzed in duplicate. Concentrations were determined from a standard curve prepared at concentrations ranging from 0.05 to100 ng/mL.

Statistics. The Statistical Analysis System (SAS, 1996) was used to analyze the data. The data were tested for normality using the Shapiro−Wilk and Anderson−Darling tests. For normal data sets, differences among means were determined with analysis of variance and Fisher's least significant difference. For non-normal data sets, differences among treatment groups were determined using nonparametric methods. Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

The blueberry has been identified as a fruit with high antioxidant capacity that raises in vivo antioxidant status. However, few studies have examined whether or not the high antioxidant capacity provides added protection against oxidative stress in vivo. Furthermore, previous studies have suggested that fractions from blueberries may alter phase II detoxification enzymes in cell culture (*23*−*25*), but studies evaluating the effects of blueberry supplementation in vivo are limited (*26, 28*).

Initial and final body weights and food intake of the rats were measured. There were no differences in final body weight ($p = 0.88$) and food intake ($p = 0.82$) between the treatment groups. Increased liver weight is one symptom of toxicity. There were no statistically significant differences in mean liver weight per 100 g of body weight between treatment groups ($p = 0.69$). Therefore, it appears that blueberry extract supplementation did not enlarge the livers.

Overall, no significant increase in phase II enzyme activity was seen with blueberry supplementation at the concentrations used in this study. Others have reported significant increases in GST activity after supplementation with freeze-dried berries (*26, 27*). In the current study, supplementation with freeze-dried whole blueberry and blueberry extracts did not significantly elevate GST activity in liver ($p = 0.51$) or colon mucosa ($p = 0.74$), even though the whole blueberry, polyphenol, and 1% flavonoid groups had 28, 27, and 24% increases in liver GST activity, respectively, over the control group (**Figure 1**). This is in contrast to the results of Boetang et al. (*26*), who reported a significant increase in liver GST activity in rats supplemented with 5% freeze-dried blueberry for 13 weeks. It is possible that our results conflicted due to different processing methods or the shorter treatment time. In a similar study of another anthocyanin-rich fruit, Reen et al. (*27*) reported increased liver GST activity in rats after 3 weeks of supplementation with 5 and 10% freeze-dried black raspberry diets. The control GST activity was comparable to that in our study, and they noted a significant increase of 47% in GST activity for the 10% black raspberry treatment group compared to an approximate 27% increase in activity with the 10% freeze-dried whole blueberry and polyphenol groups in our study. Ronis et al. (*28*) found that when lyophilized powdered concord grapes and blueberries were fed to rats, the grapes were more potent than blueberries in induction of liver GSTα, one of the GST isoforms. Black raspberries and concord grapes may have a greater effect on GST activity than blueberries due to different polyphenolic profiles, or processing methods may have differed. However, further study with direct comparisons of extracts would be needed to test these conjectures. The freeze-dried whole blueberry and blueberry polyphenol diets may have contained a concentration of polyphenols that was too low to affect GST activity. However, we also did not see an increase in GST activity in rats supplemented with 1% blueberry flavonoids, which would have provided a higher concentration of polyphenols than either the freeze-dried whole blueberry or blueberry polyphenol diets.

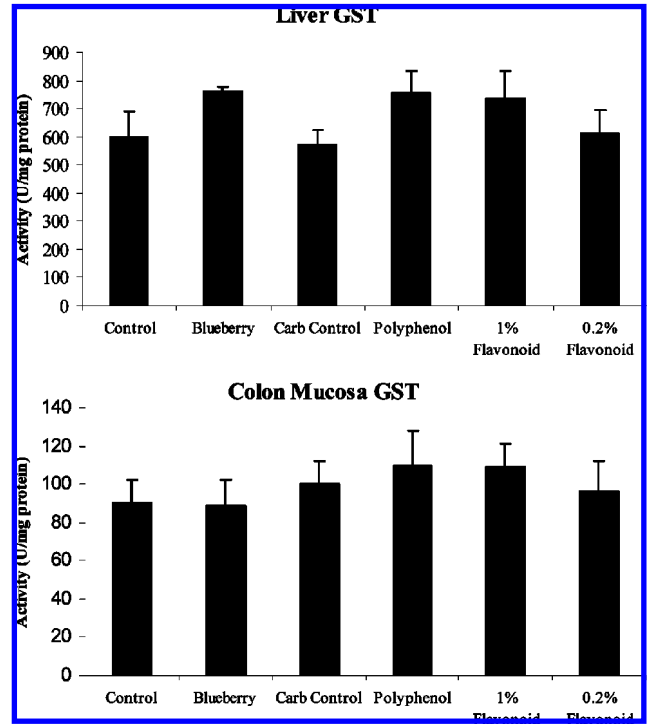

Figure 1. GST activity in liver and colon mucosa of rats fed control or blueberry-supplemented diets. Values are mean ± SE, $n = 8$ for all groups except $n = 7$ for colon mucosa for the polyphenol group. GST activity expressed as units per milligram of protein; 1 unit of activity is nanomoles of conjugate formed per minute. No groups were significantly different. Flavonoids for the 1 and 0.2% flavonoid diets consist mainly of anthocyanins and tannins.

A large amount of unmetabolized anthocyanins is excreted via the intestinal tract (*38, 39*); thus, the effects of blueberry fractions on colon mucosa were examined. There was a slight but nonsignificant increase in GST activity in colon mucosa for the blueberry polyphenol (23%) and 1% flavonoid (22%) groups, compared to controls, but the freeze-dried whole blueberry did not alter GST activity at all in the colon mucosa as it did in the liver (**Figure 1**). It is not uncommon to see phase II enzymes in liver and colon mucosa respond differently to a treatment (*35*). Phenolic concentrations and metabolites found in liver tissue may be different from what is present in the colon due to low bioavailability and tissue-specific metabolism. This has been demonstrated for anthocyanins (*29, 39*). These metabolites could have diverse effects on enzyme activity.

Our in vivo results also conflict with previous in vitro work in our laboratory. Srivastava et al. (*25*) found that blueberry anthocyanins (50−150 μg/mL) inhibited GST activity in HT-29 human colon cancer cells. The conflicting results may be due to differences in anthocyanin concentration and structure in the two models. Whereas native anthocyanins were used in cell culture, it is likely that tissues such as liver and colon would have been exposed to anthocyanin metabolites. Talevera et al. (*29*) found native and methylated anthocyanins and monoglucuronide derivatives in the plasma and liver of rats fed blackberry anthocyanins (1.5% of diet). Also, anthocyanin levels we tested in HT-29 cells were much higher than mean plasma and liver anthocyanin concentrations reported in rats by Talevera et al. (*29*).

There was no statistically significant difference in QR activity between diet groups for either liver ($p = 0.44$) or colon mucosa ($p = 0.67$). Likewise, the effects of berry supplementation on QR activity are mixed in the existing literature. Wild blueberry flavonoids increased (*23*) or had no effect (*24*) on QR activity at high doses in Hepa 1c1c7 cells. In our laboratory, as with GST activity, high concentrations of blueberry anthocyanins from some cultivars decreased QR activity (*25*). In vivo, and in contrast with our results, Ronis et al. (*28*) reported increases in QR activity with blueberry feeding. It is possible that the conflicting results are due to different berry extraction procedures, differences in blueberry concentrations, or in vitro versus in vivo effects. UGT activity in the liver was not significantly affected by blueberry supplementation ($p = 0.48$). The blueberry treatment group had UGT activity that was 26% higher than the control. However, this level was only slightly higher than the activity in the carbohydrate control group. Similarly, hepatic UGT activity was unchanged in rats after receiving a 20% fruit and vegetable diet for 6 weeks (*40*).

Although ex vivo studies clearly show antioxidant effects of isolated polyphenols and some plant extracts, it is critical to examine the in vivo effects of plant foods and fractions on oxidative stress. Because ROS can damage DNA, lipids, and proteins, it is necessary to measure damage of more than just one of these cellular components. DNA damage is the most severe form of oxidative damage because it can cause permanent mutations that are passed on to progeny cells. The comet assay was performed to determine DNA damage in liver tissue and lymphoctyes. The 1% flavonoid treatment group had the lowest DNA damage levels in both liver tissue and lymphocytes (**Table 3**), but there was a significant reduction in DNA damage from supplementation only in liver with the 1% flavonoid diet (22.5% reduction; $p < 0.05$). This supplementation level represents a higher polyphenol intake than any of the other treatments, including the 10% whole blueberry diet, which may be the reason why it was the only group that showed improvement.

Table 3. Percent DNA Damage in Lymphocytes and Liver of Rats Fed Control or Blueberry-Supplemented Diets[a,b]

treatment	lymphocytes	liver[c]
control	6.53 ± 0.8	8.35 ± 0.54 a
crude blueberry	7.04 ± 0.9	7.43 ± 0.45 ab
carbohydrate control	6.11 ± 0.48	6.79 ± 0.48 ab
crude polyphenol	6.97 ± 0.38	7.80 ± 0.59 ab
1% flavonoid[d]	5.68 ± 0.67	6.47 ± 0.82 b
0.2% flavonoid[d]	6.87 ± 0.79	7.18 ± 0.32 ab

[a] Values are mean ± SE, $n = 8$ for all groups except $n = 7$ for lymphocyte control and carbohydrate control groups and $n = 5$ for lymphocyte blueberry group. [b] Cells were scored into five categories based on tail size. One hundred cells were scored per sample. Percent DNA in the tail was calculated as $(2.5 \times \text{cells}_1 + 12.5 \times \text{cells}_2 + 30 \times \text{cells}_3 + 60 \times \text{cells}_4 + 90 \times \text{cells}_5)/100$ according to the method of Zhao et al. (*32*). Percent DNA in the tail corresponds to percent DNA damage. [c] For liver, treatment groups with different letters are significantly different from each other ($p < 0.05$). [d] Flavonoids for the 1 and 0.2% flavonoid diets consist mainly of anthocyanins and tannins.

Similarly, rats fed diets supplemented with 3.85 g/kg diet monomeric anthocyanins from bilberry, chokeberry, and grapes, which would be comparable to our 0.2% flavonoid diet, had no change in DNA damage levels assessed by measuring urinary 8-OH deoxyguanosine after 14 weeks, despite lowered expression of COX-2 mRNA (*41*). In contrast, DNA damage levels were significantly reduced in the hippocampal regions of mice that had been supplemented with a much lower level of 2.6−3.2 mg/kg of body weight of blueberry anthocyanins in drinking water for 30 days (*16*).

One reason for the limited effect of blueberry supplementation on DNA damage in this study may have been the low baseline level of damage. Because the rats were young and healthy, it is likely they had very low DNA damage levels. Some studies have suggested that supplementation with polyphenols may be more successful in conditions of oxidative stress. Harris et al. (*42*) reported that 2.5−10% black raspberry supplementation reduced DNA damage in rats treated with a carcinogen, but not in control rats. Anthocyanin extracts from *Abies koreana* supplemented at a low level of 1 g/kg of diet reduced liver DNA damage and lipid hydroperoxides in vitamin E deficient rats (*43*). In addition, supplementation with 12 mg of mixed carotenoids for 56 days in postmenopausal women reduced endogenous DNA damage by 35.8% (*32*). It was noted that the older participants had higher baseline levels of damage and consequently showed greater improvement than younger subjects. Further studies in models of moderate to high DNA damage may be necessary to fully examine the effect of blueberry on DNA damage.

Oxidative lipid damage can compromise the cell membrane. Lipid peroxidation was determined by measuring urinary F_2-isoprostanes, which are produced by lipid peroxidation of arachidonic acid. F_2-Isoprostane levels are known to increase considerably in organisms subjected to high levels of oxidative stress and are regarded as one of the best methods to measure oxidative stress due to their stability and measurement reliability (*44*). Reduction of isoprostanes has been demonstrated in vivo in healthy humans with high fruit and vegetable consumption (*45*). Our study showed no effect of blueberry supplementation on basal urinary F_2-isoprostane levels. This may have been due to the use of healthy, young rats with low levels of oxidative stress. However, a study of chronic smokers, individuals who generally have higher levels of oxidative stress, who consumed 250 g of blueberries daily for 3 weeks, had reduced lipid hydroperoxide levels but not F_2-isoprostanes or plasma antioxidant potential (*18*). Future studies using rodents under

oxidative stress and with additional measures of lipid peroxidation are needed to more fully determine whether blueberry supplementation can reduce oxidative damage.

In summary, liver and/or colon GST, QR, and UGT activities were not significantly increased by short-term supplementation with blueberries or blueberry fractions in healthy rats. Likewise, lipid peroxidation assessed with F_2-isoprostanes was also not significantly decreased. There was a slight decrease in liver DNA damage for the 1% flavonoid group. This study shows that in vivo results are not always in agreement with in vitro findings and that very high intakes of blueberry may be required to elicit significant effects in some tissues. Furthermore, future studies should be conducted in animals under oxidative stress to determine if similar blueberry preparations are protective.

ACKNOWLEDGMENT

We thank the Georgia Blueberry Growers Association for their assistance.

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Received for review August 1, 2008. Revised manuscript received October 13, 2008. Accepted November 4, 2008. This research was funded by the State of Georgia's Traditional Industries Program for Food Processing Research Grant.

JF802405Y