

## Whole Berries versus Berry Anthocyanins: Interactions with Dietary Fat Levels in the C57BL/6J Mouse Model of Obesity

RONALD L. PRIOR,<sup>\*,†,§</sup> XIANLI WU,<sup>†</sup> LIWEI GU,<sup>†</sup> TIFFANY J. HAGER,<sup>†,§</sup>  
 AARON HAGER,<sup>†</sup> AND LUKE R. HOWARD<sup>†</sup>

USDA Arkansas Children's Nutrition Center, 1212 Marshall Street, Little Rock, Arkansas 72202, and  
 Department of Food Science, University of Arkansas, Fayetteville, Arkansas 72704

Male C57BL/6J mice received diets with either 10% of calories from fat (LF) or a high-fat diet [45% (HF45) or 60% (HF60) calories from fat] for 92 days (expt 1) or 70 days (expt 2). These were given with or without freeze-dried powders from whole blueberries (BB) or strawberries (SB) (expt 1) or purified anthocyanin extracts from BB or SB (expt 2). Body composition was determined utilizing Echo MRI. Berries added to the LF diet did not alter weight gain, final body weights, body fat, or protein (percent body weight) or diet (grams) or energy (kilocalories) intake. However, in both HF45- and HF60-fed mice, weight gain, final weights, body fat (percent), and epididymal fat weights increased and body protein decreased ( $p < 0.01$ ) compared to LF mice. In mice fed HF45 diet plus BB, body weight gains, body fat (percent of BW), and epididymal fat weights were significantly greater than those in the HF45-fed controls, whereas weights of mice fed SB HF were similar to those of HF controls. SB or BB feeding did not alter glucose tolerance, although glucose tolerance decreased with age and in HF45 versus LF mice. Baseline plasma glucose was lower in SB- versus HF45-fed mice. After 8 weeks, mice fed the HF60 diet plus purified anthocyanins from BB in the drinking water had lower body weight gains and body fat than the HF60-fed controls. Anthocyanins fed as the whole blueberry did not prevent and may have actually increased obesity. However, feeding purified anthocyanins from blueberries or strawberries reduced obesity.

**KEYWORDS:** Anthocyanin; blueberry; body composition; cyanidin; glucose tolerance; obesity; pelargonidin; strawberry

### INTRODUCTION

Anthocyanins are water-soluble plant secondary metabolites responsible for the blue, purple, and red colors of many plant tissues. They occur primarily as glycosides of their respective aglycone anthocyanidin-chromophores, with the sugar moiety mainly attached at the 3-position on the C-ring or possibly at the 5,7-position on the A-ring. Berries are particularly rich sources of anthocyanins. The specific health effects that anthocyanins might have in vivo are not known, although there are several possibilities related to obesity, cardiovascular disease, and cancer (1). We have studied the absorption/metabolism of anthocyanins using freeze-dried powders of elderberry, blueberry, Marion blackberry, chokeberry, and black currant (2–5). The total anthocyanin content in these freeze-dried berries varies considerably, with cyanidin-based anthocyanins being predomi-

nant in all of the berries except for strawberries (6). The strawberry is about the only berry that has pelargonidin as a primary anthocyanidin. Also, it appears that pelargonidin is much more stable in vivo, and even though the concentration of pelargonidin-based anthocyanins in strawberries is low relative to that of anthocyanins in other berries, its apparent absorption may be as much as 10 times higher than that of cyanidin-based anthocyanins (4).

Recent observations by Tsuda et al. (7) indicated that anthocyanins from purple corn (PC) (rich in cyanidin-3-glucoside) prevented obesity in C57BL/6J mice fed a high-fat (HF) diet compared to a HF diet with no anthocyanins. Mice fed a HF diet containing anthocyanins had decreased serum levels of glucose, insulin, and leptin concentrations compared to the HF-fed control mice. Anthocyanins from PC lowered liver total lipid and triglycerides in HF-fed mice relative to control mice fed a HF diet with no anthocyanins. In subsequent work (8), gene expression of adiponectin was shown to be up-regulated in white adipose tissue in mice fed an anthocyanin-supplemented diet, although the elevation of adiponectin gene expression by anthocyanins was not reflected in the serum

\* Address correspondence to this author at the USDA Arkansas Children's Nutrition Center, 1120 Marshall St., Little Rock, AR 72202 [e-mail priorronald@uams.edu; telephone (501) 354-2747; fax (501) 364-2818].

<sup>†</sup> USDA Arkansas Children's Nutrition Center.

<sup>§</sup> University of Arkansas.

adiponectin protein level *in vivo*. These authors suggested that anthocyanins may have important implications in preventing obesity and diabetes. What is not clear from these studies is whether this effect is due to only cyanidin-3-glucoside, which is reported to be the predominant anthocyanin in purple corn, or whether other anthocyanins have similar effects.

More recently, anthocyanins (primarily cyanidin-3-glucoside) from Cornelian cherry (*Cornus mas*) when added to the diet (1 g/kg) appeared to ameliorate obesity and insulin resistance in C57BL/6 mice fed a high-fat diet (9). Anthocyanin-treated mice fed a HF diet had a >100-fold increase in blood insulin levels compared to non-anthocyanin-treated HF-fed mice; however, pancreatic islets from anthocyanin-treated mice stained intensely for insulin similar to islets in mice fed the LF diet, which was in contrast to the non-anthocyanin-treated mice fed a HF diet, which had enlarged islets and diffused staining (9).

In our first study with C57BL/6J mice, we chose to use the whole berry (blueberry and strawberry) rather than an extract of the anthocyanins. Blueberries contain a mixture of more than 20 anthocyanins including 5 of the 6 most common aglycones, whereas strawberries contain pelargonidin as the primary aglycone (10). The objectives of these experiments were to determine whether anthocyanins and/or other components in the whole berries have effects on obesity in a mouse model similar to that previously observed with anthocyanins from PC and cherry. On the basis of results from our first study, we conducted a second study in which we compared whole berry versus purified anthocyanin as well as the effect of 45 versus 60% of kilocalories from fat in the diet.

## MATERIALS AND METHODS

**Standards and Solvents.** Standards of the 3-*O*- $\beta$ -glucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (six mixed anthocyanin standard, HPLC grade) were obtained from Polyphenols Laboratories (Sandnes, Norway). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO). All other solvents were purchased from Fisher (Fair Lawn, NJ).

**Preparation of Purified Anthocyanins.** Freeze-dried blueberry (BB) and strawberry (SB) powders were provided by FutureCeuticals Inc. (Mokenca, IL) and the Oregon Raspberry and Blackberry Commission, respectively. Berry powders were weighed (500 g for BB or 2000 g for SB) and extracted two times with methanol/water/formic acid (85:15:0.5, v/v). The filtrates were combined and subjected to vacuum evaporation (Büchi) to remove methanol. The concentrated extracts were loaded onto an Amberlite XAD-7 resin (Sigma-Aldrich). The resin was washed with 0.5% formic acid, and subsequently the absorbed anthocyanins were recovered with 0.5% formic acid in methanol. The methanol eluent was subjected to vacuum evaporation again to remove most of the methanol. To remove other phenolic acids, the concentrated eluents were extracted three times with ethyl acetate (EtOAc). After EtOAc extraction, the aqueous layer was subjected to vacuum evaporation to remove residual organic solvents. The final concentrated extracts were analyzed with an Agilent 1100 series HPLC (Palo Alto, CA) equipped with an autosampler/injector and a diode array detector to determine the concentration of anthocyanins as well as other phenolic acids. The total volume of each final berry extract was measured, distributed appropriately, and lyophilized to yield dry purified anthocyanin powders.

**Animals and Diets.** Male C57BL/6J mice (21 days of age) were purchased from Jackson Laboratories (Bar Harbor, ME) and brought into the animal facility at the Arkansas Children's Nutrition Center. Control low-fat and high-fat diet (HF45 and HF60) and diets mixed with freeze-dried berry powders were produced by Research Diets (New Brunswick, NJ). All diets were pelleted. Purified anthocyanin powders of BB or SB were dissolved in drinking water. Stability tests of anthocyanins in water were determined before the experiment was

begun, and it was determined that no significant degradation occurred during a 48 h period at room temperature. Thus, drinking water containing anthocyanins was replaced every other day.

**Experiment Design.** Animal protocols were approved by the Animal Care and Use Committee of the University of Arkansas for Medical Sciences.

**Experiment 1.** Mice were randomized by weight and assigned to one of six dietary treatments: (1) control low fat (10% of calories from fat, LF); (2) LF + 10% freeze-dried blueberry powder; (3) LF + 10% freeze-dried strawberry powder; (4) control high fat (45% of calories from fat; HF45); (5) HF45 + 10% freeze-dried blueberry powder (BB-HF45); and (6) HF45 + 10% freeze-dried strawberry powder (SB-HF45) (Table 1). The relative proportions of each of the anthocyanins in the whole freeze-dried blueberry and strawberry are presented in Table 2. There were 12 animals per treatment with 3 mice housed per cage. Weekly body weights and estimates of feed intake were obtained. Body composition was determined on days 57 and 80 by Echo MRI techniques. On days 91–94 of the experiment, the animals were killed, and serum, liver, kidney, and adipose tissue (subcutaneous and epididymal) were collected and weighed.

The total anthocyanin contents in the freeze-dried blueberry and strawberry powders were 2.9 and 27.8 mg/g (Table 2). The compositions of the diets used in experiments 1 and 2 are presented in Table 1. The compositions of the anthocyanins in the diet were analyzed (10) before and after pelleting to determine if there was any significant degradation of anthocyanins due to pelleting. Some losses were observed (<10%) with pelleting, but we felt that the pelleted diet could be used as long as the content of anthocyanins was determined.

**Experiment 2.** Male C57BL/6J mice (25 days of age) were assigned at random to treatment such that there were 9 animals per treatment. Mice were housed three per cage. The treatments included (1) control low fat (10% of calories from fat) (C-LF) (same as expt 1), (2) control high-fat diet (45% of calories from fat) (HF45) (same as expt 1), (3) high-fat diet (45% of calories from fat) (BB-HF45) with 10% freeze-dried blueberry powder (same as expt 1), (4) high-fat diet (60% of calories from fat) (HF60) (Table 1), (5) diet 4 (HF60) plus purified anthocyanins from blueberry provided in the drinking water, and (6) diet 4 (HF60) plus purified anthocyanins from strawberry provided in the drinking water. All diets were pelleted. The relative proportions of each anthocyanin in the extracts of blueberry or strawberry added to the drinking water are presented in Table 2. Mice were weighed weekly. Fresh water containing anthocyanins was provided every other day. The volume of water consumed was determined.

**Body Composition.** Whole body composition (fat and lean tissues) was determined using nuclear magnetic resonance technology with an Echo MRI Analyzer system by Echo Medical Systems (Houston, TX). Because of the small size of mice, two mice from the same treatment were placed in the analytical chamber at the same time to get reliable body composition measurements.

**Glucose Tolerance Test.** Glucose tolerance tests were performed by injecting intraperitoneally 2 g of glucose/kg of body weight. Blood was collected into heparinized capillary tubes from the tail vein at baseline and at 15, 60, 90, and 120 min after glucose injection. The capillary tubes were centrifuged, and plasma was collected for glucose analysis using a Beckman Glucose Analyzer.

**Statistical Analysis.** Data were analyzed using analysis of variance (ANOVA) with a post hoc comparison using LSD.

## RESULTS

**Experiment 1.** Cumulative body weight gains for mice in expt 1 are shown in Figure 1A. Gains were significantly lower for the mice fed the low-fat diet containing 10% of calories from fat compared to a diet containing 45% of calories from fat (HF45). The addition of freeze-dried whole blueberry or strawberry powder did not alter weight gains within the low-fat dietary treatments. By day 60 of the experiment, the mice fed BB-HF45 gained significantly more weight than did mice given the other two HF45 treatments (Figure 1A). Three of the same dietary treatments (C-LF10, C-HF45, BB-HF45) were

**Table 1.** Composition of Low- (10% of Kilocalories from Fat) and High-Fat Diets [45% of Kilocalories from Fat (HF45) and 60% of Kilocalories from Fat (HF-60)] Used in Experiments 1 and 2

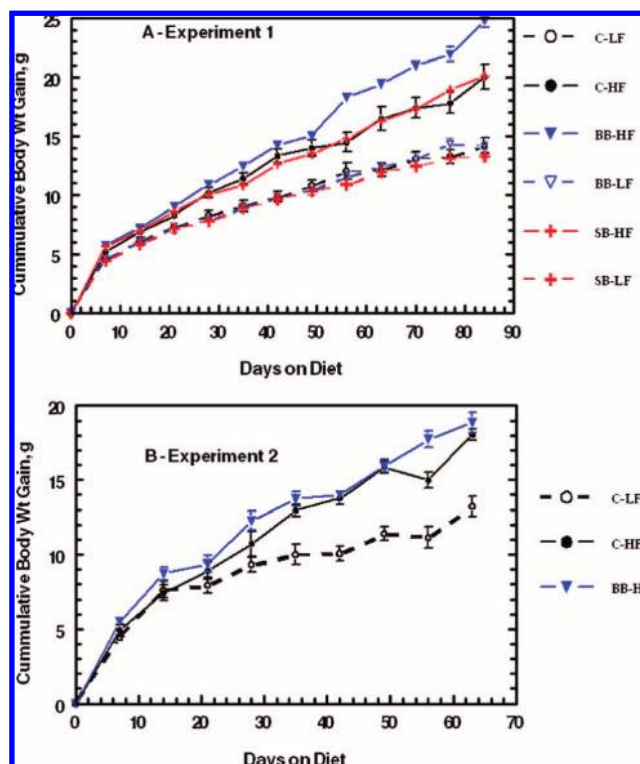
component	control (C-LF)	blueberry (BB-LF)	strawberry (SB-LF)	control (C-HF45)	blueberry (BB-HF45)	strawberry (SB-HF45)	control (C-HF60)
protein, g	19.2	18.9	18.9	23.7	23.1	23.1	26.2
carbohydrate, g	67.3	67.9	67.9	41.4	42.8	42.8	26.3
fat, g	4.3	4.2	4.2	23.6	23.1	23.1	34.9
total, g	90.8	91.0	91.0	88.7	89.0	89.0	87.5
anthocyanins, mg/kg	0.0	2528.5	267.0	0.0	3095.3	326.8	0.0
anthocyanins, mg/kcal	0.0	0.67	0.07	0.0	0.67	0.07	0.0
procyanidins, mg/kg	0.0	3012.1	786.0	0.0	3687.6	962.3	0.0
procyanidins, mg/kcal	0.0	0.798	0.208	0.0	0.798	0.208	0.0
kcal/g	3.85	3.77	3.77	4.73	4.62	4.62	5.24
casein, 80 mesh, g	200	200	200	200	200	200	200
L-cystine, g	3	3	3	3	3	3	3
corn starch, g	315	275	275	72.8	32.8	32.8	0
maltodextrin 10, g	35	35	35	100	100	100	125
sucrose, g	350	310	310	172.8	132.8	132.8	68.8
cellulose, BW200, g	50	50	50	50	50	50	50
soybean oil <sup>a</sup> , g	25	25	25	25	25	25	25
lard <sup>b</sup> , g	20	20	20	177.5	177.5	177.5	245
Mineral Mix S10026, g	10	10	10	10	10	10	10
dicalcium phosphate, g	13	13	13	13	13	13	13
calcium carbonate, g	5.5	5.5	5.5	5.5	5.5	5.5	5.5
potassium citrate, 1 H <sub>2</sub> O, g	16.5	16.5	16.5	16.5	16.5	16.5	16.5
Vitamin Mix V10001, g	10	10	10	10	10	10	10
choline bitartrate, g	2	2	2	2	2	2	2
blueberry powder, g	0	100	0	0	100	0	0
strawberry powder, g	0	0	100	0	0	100	0
total	1055.1	1075	1075	858.2	878.1	878.1	773.9

<sup>a</sup> Fatty acid composition of soybean oil: C16, 10.4%; C18, 3.8%; C18:1, 24.3%; C18:2, 53.5%; C18:3, 7.8%. <sup>b</sup> Fatty acid composition of lard: C10, 0.1%; C12, 0.2%; C14, 1.3%; C16, 23.8%; C16:1, 2.7%; C18, 13.5%; C18:1, 41.2%; C18:2, 10.2%; C18:3, 1.0%; C20:1, 1.0% (USDA Nutrient Database, NDB no. 04002).

**Table 2.** Relative Anthocyanin Composition in Diets and in Freeze-Dried Blueberry (BB) and Strawberry (SB) Powders

	% of total	
	diet	extract
Blueberry Anthocyanins		
delphinidin-3-galactoside	8.01	7.88
delphinidin-3-glucoside	8.54	13.44
cyanidin-3-galactoside	3.96	2.20
delphinidin-3-arabinoside	5.32	3.97
cyanidin-3-glucoside	4.37	3.76
petunidin-3-galactoside	5.46	4.64
cyanidin-3-arabinoside	2.99	1.33
petunidin-3-glucoside	7.67	10.88
peonidin-3-galactoside	1.80	2.53
petunidin-3-arabinoside	3.23	1.94
peonidin-3-glucoside	13.14	10.53
malvidin-3-galactoside	0.00	7.94
malvidin-3-glucoside	13.89	20.67
malvidin-3-arabinoside	5.64	3.95
delphinidin-3-(6-acetyl)glucoside	2.73	1.22
peonidin-3-(6-acetyl)glucoside	0.30	
cyanidin-3-(6-acetyl)glucoside	1.78	0.39
malvidin-3-(6-acetyl)galactoside	2.19	0.50
petunidin-3-(6-acetyl)glucoside	2.24	0.52
peonidin-3-(6-acetyl)glucoside	1.16	
malvidin-3-(6-acetyl)glucoside	5.47	1.73
total, mg/g BB of powder, 27.8		
Strawberry Anthocyanins		
cyanidin-3-sophoroside	17.44	20.64
cyanidin-3-glucoside	9.76	6.01
cyanidin-3-rutinoside	2.43	
pelargonidin-3-glucoside	60.21	62.94
pelargonidin-3-rutinoside	1.86	3.41
pelargonidin-3-(malonoyl)glucoside	1.58	5.74
pelargonidin-3-(acetyl)glucoside	6.72	1.27
total, mg/g of SB powder, 2.9		

repeated in expt 2 (**Figure 1B**). The mice fed the high-fat diets gained significantly more than the C-LF treatment mice. The trend was for the BB-HF45 to mice to gain more weight than



**Figure 1.** Cumulative body weight gain (grams) of C57BL6 mice in experiments 1 and 2 fed a low-fat diet (10% of kilocalories from fat) (C-LF), a low-fat diet (10% of kilocalories from fat) containing blueberries (BB-LF), a high-fat diet (45% of kilocalories from fat) control (C-HF), or a high-fat diet (45% of kilocalories from fat) containing blueberries (BB-HF). Data are presented as means  $\pm$  SEM,  $N = 12$  and 9 mice per treatment for experiments 1 and 2, respectively.

**Table 3.** Average Food, Energy, Water, and Anthocyanin Intake in Mice Fed a Control Diet or High-Fat Diet (45 or 60% Calories from Fat)

treatment	food intake (g/day/mouse)	kcal intake (kcal/day/mouse)	water intake (mL/day/mouse)	anthocyanin intake (mg/day/mouse)
Experiment 1 <sup>a,c</sup>				
1. C-LF10%	2.6 ± 0.03	9.7 ± 0.12	ND <sup>d</sup>	0.0
2. C-HF45%	2.4 ± 0.11	10.9 ± 0.52	ND	0.0
3. BB-LF10%	2.6 ± 0.05	9.4 ± 0.19	ND	3.25
4. BB-HF45%	2.7 ± 0.15	12.2 ± 0.69	ND	3.75
5. SB-LF10%	2.5 ± 0.03	9.0 ± 0.12	ND	0.52
6. SB-HF45%	2.4 ± 0.06	10.7 ± 0.26	ND	0.58
Experiment 2 <sup>b,c</sup>				
1. C-LF10%	2.8 ± 0.03	10.9 ± 0.1	ND	0.0
2. C-HF45%	2.6 ± 0.06	12.1 ± 0.3	ND	0.0
4. BB-HF45%	2.8 ± 0.10	12.8 ± 0.5	ND	3.86
7. C-HF60%	2.3 ± 0.02	12.0 ± 0.1	2.29 ± 0.10	0.0
8. BB-HF60%	2.4 ± 0.20	12.7 ± 1.0	2.18 ± 0.20	2.83 ± 0.26
9. SB-HF60%	2.2 ± 0.05	11.5 ± 0.2	1.59 ± 0.05	1.59 ± 0.05

<sup>a</sup> Freeze-dried powder of either whole blueberries or strawberries was included in the pelleted diets. Composition of the diets is presented in **Table 1**. Food intake was determined weekly from day 1 through day 77 of feeding the experimental diets. <sup>b</sup> Treatments 1, 2, and 4 were the same as corresponding treatments in expt 1. Treatments 3, 5, and 6 were not included in expt 2. Treatments 7–9 were fed the C-HF 60% diet, and treatments 8 and 9 were provided as an extract of the anthocyanins in the drinking water. Food intake was determined from day 1 to day 65 of feeding the experimental diets. <sup>c</sup> Data presented as means ± SEM. <sup>d</sup> Not determined.

**Table 4.** Total Body Weight and Percent Fat and Lean at Days 57 and 80 of Experiment in C57BL6 Mice Fed a High- or Low-Fat Diet (Experiment 1)<sup>a</sup>

dietary treatment	day 57			day 80		
	wt, g	% fat	% lean	wt, g	% fat	% lean
low fat, control (C-LF10)	25.0 ± 0.7	15.1 ± 1.3	69.9 ± 1.4	27.0 ± 0.8	18.0 ± 1.5	64.7 ± 0.9
low fat, blueberry (BB-LF10)	25.0 ± 0.5	15.5 ± 1.1	70.0 ± 1.3	26.9 ± 0.5	17.7 ± 1.3	64.8 ± 1.1
low fat, strawberry (SB-LF10)	24.6 ± 0.3	16.1 ± 0.8	70.0 ± 2.6	24.3 ± 0.4	18.0 ± 0.7	65.1 ± 0.6
high fat, control (C-HF45)	28.8 ± 1.2	23.0 ± 1.4	64.9 ± 2.5	32.6 ± 1.4	27.5 ± 1.4	58.3 ± 1.7
high fat, blueberry (BB-HF-45)	31.8 ± 0.8 <sup>c</sup>	29.0 ± 1.2 <sup>b</sup>	59.0 ± 1.7 <sup>c</sup>	37.2 ± 0.9	35.1 ± 1.0 <sup>b</sup>	52.4 ± 0.6 <sup>b</sup>
high fat, strawberry (SB-HF45)	28.4 ± 1.4	25.5 ± 1.6	61.9 ± 2.1	32.8 ± 1.8	30.4 ± 1.8	54.9 ± 1.4

<sup>a</sup> Data presented as means ± SEM of six observations per group. <sup>b</sup> Treatment mean differs ( $p < 0.05$ ) from control within level of fat in the diet using *t* test. <sup>c</sup> Treatment mean differs ( $p < 0.10$ ) from control within level of fat in the diet using *t* test.

**Table 5.** Effect of Dietary Fat and Blueberry Treatment on Body and Tissue Weights as a Percentage of Body Weight at the End of Experiment 1 on Days 91–94<sup>a</sup>

treatment	final wt, g	liver wt, % BW <sup>b</sup>	kidney wt, % BW	heart wt, % BW	epididymal fat wt, % BW	subcutaneous fat wt, % BW
control, LF10	28.4 ± 0.70 <sup>c</sup>	4.45 ± 0.08 <sup>d</sup>	1.20 ± 0.02	0.68 ± 0.02	2.75 ± 0.22 <sup>c</sup>	0.88 ± 0.07 <sup>c</sup>
blueberry (BB-LF10)	27.8 ± 0.80 <sup>c</sup>	4.73 ± 0.05 <sup>e</sup>	1.26 ± 0.03	0.69 ± 0.03	2.29 ± 0.20 <sup>c</sup>	0.81 ± 0.07 <sup>c</sup>
strawberry (SB-LF10)	27.5 ± 0.48 <sup>c</sup>	4.75 ± 0.11 <sup>de</sup>	1.27 ± 0.01	0.72 ± 0.02	2.61 ± 0.11 <sup>c</sup>	0.84 ± 0.05 <sup>c</sup>
control, HF45	33.4 ± 1.10 <sup>d</sup>	4.14 ± 0.18 <sup>c</sup>	1.16 ± 0.04 <sup>d</sup>	0.59 ± 0.02 <sup>d</sup>	5.01 ± 0.40 <sup>d</sup>	1.59 ± 0.08 <sup>d</sup>
blueberry (BB-HF45)	39.1 ± 1.00 <sup>e</sup>	3.70 ± 0.40 <sup>c</sup>	1.00 ± 0.02 <sup>c</sup>	0.52 ± 0.02 <sup>c</sup>	6.63 ± 0.62 <sup>e</sup>	1.60 ± 0.06 <sup>d</sup>
strawberry (SB-HF45)	34.4 ± 1.75 <sup>d</sup>	4.10 ± 0.11 <sup>c</sup>	1.13 ± 0.04 <sup>cd</sup>	0.59 ± 0.04 <sup>d</sup>	5.32 ± 0.42 <sup>d</sup>	1.51 ± 0.09 <sup>d</sup>

<sup>a</sup> Data presented as means ± SEM. Means with different letters are significantly different within a column from corresponding dietary fat control level using ANOVA and LSD comparison ( $p < 0.05$ ). <sup>b</sup> BW, body weight. <sup>c–e</sup> Traits in animals fed the high-fat diet were all significantly different from those in animals fed the low-fat diet ( $p < 0.001$ ).

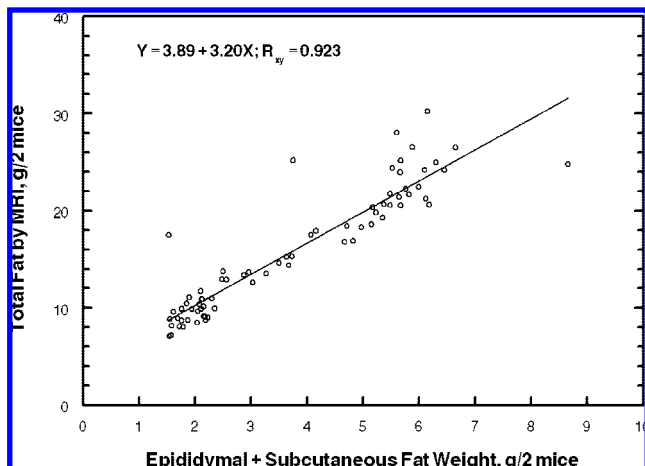
the C-HF45 mice, similar to expt 1, but the differences in gain in this experiment were not statistically significant ( $p > 0.05$ ).

Food intake data (grams or kilocalories per day per mouse) are presented in **Table 3**. Using a *t* test comparing the HF berry treatments and the C-HF in expt 1, there were no overall significant differences in food intake either on a per gram or per kilocalorie basis. The mice fed the BB-HF diet did consume significantly more ( $p < 0.05$ ) on four different weekly periods (14–21, 21–28, 42–49, and 63–70 days); however, the overall intake was not different ( $p > 0.05$ ). It is possible that increased energy intake may account for a portion of the increased weight gain observed with the BB-HF diet, but this probably does not account for the total effect on weight gain. In expt 2, the numerical differences in food intake and weight gains were smaller and not statistically different between BB-HF45 and C-HF45 (**Table 3**).

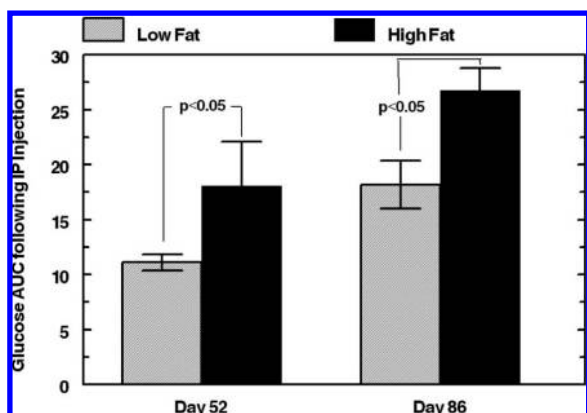
Body composition of mice was determined on days 57 and 80 in expt 1 using Echo MRI techniques. Body weight (grams) and fat or lean [percent of body weight (BW)] were not different ( $p > 0.05$ ) between any of the low-fat treatment groups (**Table**

**4**). All of the HF45 treatments had increased weight and body fat (percent of BW) and decreased percent lean tissue compared to the LF10% treatments (**Table 4**). The BB-HF45 treatment had significantly higher percent body fat and trends ( $p < 0.10$ ) toward increased body weight and decreased percent lean on day 57 compared to C-HF45 (**Table 4**). On day 80 of the experiment, the BB-HF45 treatment had significantly higher percent body fat compared to the C-HF45 treatment ( $p < 0.05$ ; *t* test) (**Table 4**). At the end of the experiment, the liver weight (percent of BW) was higher in BB-LF10% compared to C-LF10% ( $p < 0.05$ ) (**Table 5**). Mice fed the BB-HF45 diet had significantly lower percent kidney and percent heart weights and significantly higher epididymal fat weight (percent of BW) compared to the C-HF45 treatment (**Table 5**).

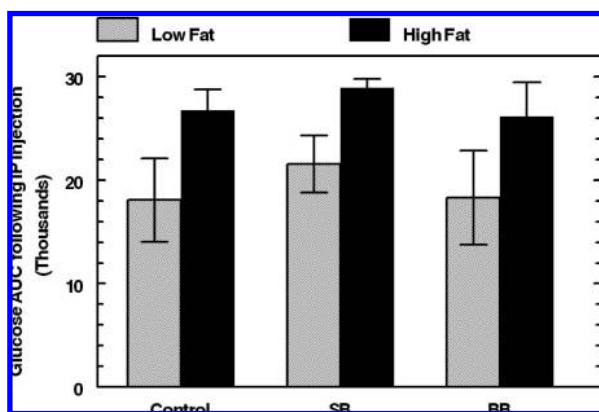
The relationship between the sum of the weights of the epididymal and subcutaneous fat and total body fat determined by MRI from mice in expt 1 is presented in **Figure 2**. A linear relationship was described by the equation  $Y = 3.89 + 3.20X$ ,  $R_{xy} = 0.923$ , where  $Y$  = total body fat (g/2 mice) and  $X$  = epididymal plus subcutaneous fat weight (g/2 mice). The strong



**Figure 2.** Relationship between weighed amount of epididymal plus subcutaneous fat on the terminal day of the experiment compared to total body fat as estimated by Echo MRI in mice 2 days earlier (day 83) of experiment 1.



**Figure 3.** Glucose area-under-curve (mg/dL · min) at days 52 and 86 in mice fed a low- or high-fat (HF45) diet following an intraperitoneal injection of glucose (2 mg/kg) in experiment 1. Data are presented as means ± SEM, *N* = 4 mice per group.



**Figure 4.** Effect of dietary fat level and blueberry (BB) or strawberry (SB) in the diet on the plasma glucose area-under-curve (mg/dL · min) following an intraperitoneal (IP) glucose tolerance test. Data are presented as means ± SEM, *N* = 4 mice per group.

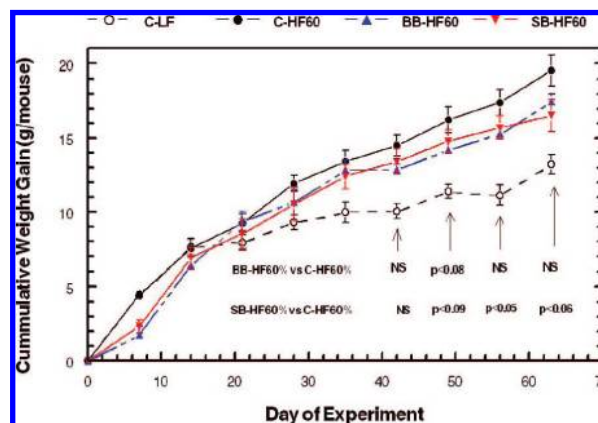
relationship indicates that a good estimation of total body fat was obtained with the MRI technique.

An intraperitoneal glucose tolerance test was performed on days 52 and 86 of expt 1 in a subgroup of mice on the C-LF10, HF45, SB-HF45, and BB-HF45 diets using a glucose dose of 2 g/kg of BW. The area under the plasma glucose curve (AUC)

**Table 6.** Baseline Plasma Glucose, Increase in Plasma Glucose from Baseline, and Area under Curve (AUC) of Plasma Glucose Following Intraperitoneal Glucose Injection (2 g/kg of Body Weight) (Experiment 1)

item	day 52		day 86	
	low fat	high fat	low fat	high fat
Baseline Plasma Glucose, Milligrams per 100 mL				
control	228 ± 5 <sup>c</sup>	258 ± 14 <sup>d</sup>	235 ± 16 <sup>a</sup>	268 ± 9 <sup>bg</sup>
strawberry	239 ± 7	265 ± 17	240 ± 10	229 ± 10 <sup>f</sup>
blueberry	ND	ND	216 ± 23	247 ± 29
Increase in Plasma Glucose from Baseline at 90 min, Milligrams per 100 mL				
control	71 ± 15	148 ± 39 <sup>e</sup>	215 ± 89	298 ± 21 <sup>f</sup>
strawberry	134 ± 37	159 ± 32 <sup>e</sup>	219 ± 58	317 ± 41 <sup>f</sup>
blueberry	ND	ND	180 ± 45	269 ± 26
AUC, Milligrams per 100 mL · min				
control	11175 ± 783 <sup>a</sup>	18172 ± 2177 <sup>b,e</sup>	18063 ± 4047	26737 ± 2026 <sup>f</sup>
strawberry	19453 ± 539	16657 ± 3136 <sup>e</sup>	21546 ± 2758	28890 ± 893 <sup>f</sup>
blueberry	ND	ND	18299 ± 4556	26176 ± 3268

<sup>a,b</sup> Means within a day of experiment differ significantly (*p* < 0.05). <sup>c,d</sup> Means within a day of experiment differ significantly (*p* < 0.10). <sup>e,f</sup> Means between high-fat diet on day 52 and high-fat diet on day 86 are significantly different (*p* < 0.05). <sup>g,h</sup> Differences between control HF diet and strawberry HF diet differ (*p* < 0.05).



**Figure 5.** Cumulative body weight gain in C57BL/6J mice fed a low-fat (10% of kilocalories from fat) (C-LF) or high-fat (60% of kilocalories from fat) (C-HF60) diet containing strawberry anthocyanin extract (SB-HF60) or blueberry anthocyanin extract (BB-HF60) (experiment 2). Gains for C-HF60, BB-HF60, and SB-HF60 are significantly greater than that for the low-fat control (C-LF) diet (*p* < 0.001) after 34 days. Significant differences between C-HF60 and BB-HF60 or SB-HF60 are indicated in the figure. *N* = 9 mice per group.

was significantly greater (*p* < 0.05) in mice given the HF45 diet compared to the C-LF10 diet, and the AUC was also greater at day 86 compared to day 52 (**Figure 3**). Inclusion of blueberry or strawberry in the diet did not alter the glucose AUC (**Figure 4**). The net increase in plasma glucose concentrations at 90 min after the glucose tolerance test (concentration at 90 min – that at 0 min) was higher in mice fed HF45 diets compared to the C-LF10 diet (**Table 6**). Also, the net increase in plasma glucose concentrations at 90 min after the glucose tolerance test was also higher on day 86 compared to day 52. Baseline plasma glucose concentrations were lower in the C-HF10 compared to the C-HF45 (*p* < 0.10) on day 52 (**Table 6**). Baseline plasma glucose in the mice fed SB-HF45 was lower (*p* < 0.05) than that in the mice fed the C-HF45 diet on day 86 (**Table 6**).

**Experiment 2.** In expt 2, the anthocyanins from blueberry and strawberry were extracted, concentrated, and added to the drinking water. A further difference from expt 1 was that a higher level of fat in the diet was used (60% of kilocalories from fat) (HF60). Weight gains of the C-LF10, C-HF-60, BB-

**Table 7.** Body Composition Data in Mice on Day 49 (Experiment 2)<sup>a</sup>

treatment	wt, g	fat, g	fat, % BW	lean, g	lean, % BW
control, low fat (LF10)	22.63 ± 0.69 <sup>b-e</sup>	3.49 ± 0.78 <sup>b-e</sup>	15.39 ± 3.20 <sup>b-e</sup>	15.07 ± 0.62 <sup>b-e</sup>	66.60 ± 2.90 <sup>c</sup>
control, high fat (HF60)	28.4 ± 1.10 <sup>d</sup>	6.68 ± 1.05 <sup>c</sup>	23.41 ± 2.80 <sup>d</sup>	17.75 ± 0.49 <sup>c</sup>	62.50 ± 2.4 <sup>b</sup>
blueberry, (BB-HF60)	25.75 ± 1.50 <sup>c</sup>	3.66 ± 0.75 <sup>b-e</sup>	14.30 ± 3.10 <sup>b-e</sup>	17.80 ± 1.55 <sup>c</sup>	69.20 ± 3.80 <sup>cd</sup>
strawberry, (SB-HF60)	26.73 ± 0.43 <sup>c</sup>	5.38 ± 0.68 <sup>c</sup>	20.10 ± 2.80 <sup>c</sup>	17.03 ± 0.91 <sup>c</sup>	63.70 ± 2.50 <sup>bc</sup>

<sup>a</sup> Data presented as means ± SEM of four observations per treatment group with two mice combined into one observation for determination of body composition.  
<sup>b-e</sup> Means ± SEM without a common letter within a column differ ( $p < 0.05$ ).

**Table 8.** Final Body and Tissue Weights on Day 69 or 70 in Mice Fed Low- or High-Fat Diets with Anthocyanins from Blueberry or Anthocyanins<sup>a</sup> (Experiment 2)

item	control, 10% fat, C-LF	control, high fat, HF60	blueberry, BB-HF60	strawberry, SB-HF60
final wt, g	24.5 ± 0.35 <sup>b</sup>	29.9 ± 0.87 <sup>c</sup>	28.46 ± 0.81 <sup>c</sup>	29.34 ± 0.33 <sup>c</sup>
liver wt, % BW	4.61 ± 0.17 <sup>c</sup>	3.72 ± 0.10 <sup>b</sup>	3.68 ± 0.11 <sup>b</sup>	3.72 ± 0.09 <sup>b</sup>
kidney wt, % BW	1.38 ± 0.03 <sup>b</sup>	1.99 ± 0.51 <sup>c</sup>	1.53 ± 0.03 <sup>c</sup>	1.59 ± 0.03 <sup>c</sup>
heart wt, % BW	0.78 ± 0.04	0.76 ± 0.05	0.79 ± 0.10	0.67 ± 0.03

<sup>a</sup> Data presented as means ± SEM of eight or nine observations per treatment. <sup>b,c</sup> Means with different superscripts between low and high fat levels in diet are significant ( $p < 0.001$ ).

HF60, and SB-HF60 are presented in **Figure 5**. Weight gains of the C-LF10-fed mice were well below those of the HF60-fed mice at 28 days and beyond (**Figure 5**). By day 49 of the experiment, the cumulative gain of the SB-HF60 fed mice was lower ( $p < 0.09$ ) than that of the C-HF60-fed mice, and it remained lower throughout day 63. The cumulative gain of the BB-HF60-fed mice was lower than that of the C-HF60-fed mice at day 49 (**Figure 5**) ( $p < 0.08$ ). However, final body weights at the end of the experiment were not significantly different among the treatments using the HF60 diet (**Table 8**). No differences were observed in food intake among the HF60-fed mice on either a grams or kilocalories per day basis (**Table 3**). Mice fed strawberry anthocyanins in the water required a few days to adapt to a consistent liquid intake. The average water intake was lower than in either the BB or control mice (**Table 3**). After adaptation to the strawberry anthocyanins in the diet, the intake was about 1.6 mL per day compared to ~2.2 mL per day in the other two treatments. This resulted in a lower intake of total anthocyanins than we originally projected of ~3 mg/day. On average, blueberry anthocyanin fed mice consumed 2.8 mg of total anthocyanins per day in the water (**Table 3**).

On day 49 of the experiment, the percent fat estimated by MRI in mice fed the BB-HF60 was lower than in those fed the HF60 control diet and was not different from that of the mice fed the LF10 control diet (**Table 7**). Body weights were lower in the BB-HF60 mice compared to the control HF-60-fed mice (**Table 7**). The body weights of SB-HF60 fed mice on day 49 were intermediate between the C-HF60- and the BB-HF60-fed mice. However, by day 69/70 at the termination of the experiment, body weights in the HF60 groups had converged and were not significantly different (**Table 8**). Kidney weights as percent of BW were higher but liver weights were lower in mice fed the HF60 diets compared to mice fed the LF10% diet. Liver weight as percent of BW decreased with high-fat feeding in both experiments. Anthocyanins did not have an effect on liver, kidney, or heart tissue weights (percent of BW) (**Table 8**). However, in experiment 1, feeding of the blueberry powder increased liver weight with the low-fat diet compared to the control low-fat diet. Kidney and heart weights (percent of BW) were decreased with high-fat feeding in experiment 1 (**Table 5**). However, in experiment 2, kidney weight was increased and heart weight was not changed due to high-fat feeding.

## DISCUSSION

Obesity is recognized as a major health issue in the United States and other developed countries. Diet is one component of a multitude of complex factors that can affect this problem. The recent findings (7, 9) that anthocyanins could possibly affect the development of obesity, at least in animal models, has generated considerable interest. Many berries are rich sources of anthocyanins (10). The studies reported in this paper were developed to determine if the consumption of berries would have effects similar to those observed for extracts of anthocyanins from purple corn (7) or Cornelian cherries (9). We chose to study the whole berry because that is what is most available and consumed. Our results with the whole berries in this study with strawberries and blueberries did not produce a significant protection against dietary fat induced obesity in the mouse model. What is not known is whether there are other factors in the berries besides anthocyanins that may either positively or negatively affect the development of obesity. Another major question is whether a single anthocyanin, and if so which one, is responsible for the effects observed previously or whether several anthocyanins have similar effects. In the two previous studies, cyanidin-3-glucoside has been the anthocyanin of interest. The relative compositions of anthocyanins in the blueberry and strawberry sources used are presented in **Table 2**. Blueberry contains a complex mixture of 21 different anthocyanins, whereas strawberry has a different mixture of 7 anthocyanins. Cyanidin-3-glucoside is present in both, but definitely not as a predominant anthocyanin. Malvidin-, delphinidin-, petunidin-, and peonidin-3-glucosides predominate in blueberry, and pelargonidin-3-glucoside and cyanidin-3-sophoroside predominate in strawberry.

In the first experiment using freeze-dried powders of blueberry or strawberry, we did not observe any protection against obesity. In fact, the mice fed blueberry in the high-fat diet had increased adipose tissue mass as determined by Echo MRI compared to the control high-fat diet. We considered other factors that might be in the blueberry that may have produced this effect. One possibility considered was the amount of simple sugars present in the whole blueberry. When the diets were formulated with the freeze-dried powder, we adjusted for energy content and removed 40 g/kg each of corn starch and sucrose from the diet and substituted the blueberry freeze-dried powder, which would have provided about 31 g/kg each of glucose and

fructose. This substitution would have increased the amount of fructose some, but this does not appear to be a large enough difference to account for the increased obesity observed with blueberry feeding. Total food intake was increased and caloric intake increased by  $\sim 12\%$  in the blueberry-fed mice in the high-fat diet but not with the low-fat diet (expt 1) (Table 3). Thus, there seems to be an interaction between the level of fat in the diet and blueberry, with blueberry increasing growth and adipose tissue deposition only in the high-fat diet. In the second experiment with purified anthocyanins from blueberry, there was a decreased growth rate and decreased adipose tissue deposition. Consumption of freeze-dried strawberry powder in the high-fat diet did not increase adiposity significantly in expt 1, and consumption of the purified anthocyanins from strawberry did not produce a significant reduction in adipose tissue deposition (Table 7).

The other issue for consideration is the amount of anthocyanins consumed and whether there is a dose-dependent response. In expt 1, total anthocyanin intake was 3.75 mg/day/mouse with the BB-HF45 diet (Table 3). In the second experiment with a blueberry extract provided in the drinking water, the intake of blueberry anthocyanins was 2.82 mg/day/mouse. It is unlikely that the amount of anthocyanins consumed accounts for the differences observed.

Between the two experiments reported in this study, we have also looked at the effects of the level of fat in the diet (45 vs 60% of kilocalories from fat). Although we expected that the HF60 diet might produce obesity more rapidly than the HF45 diet, this did not appear to be the case (Figures 1B and 2). Weight gains of mice after 63 days on diet were  $18.0 \pm 0.4$  and  $19.5 \pm 1.0$  g for the HF45 and HF60 diets, respectively. Previous studies used the HF60 diet that we used in expt 2 (9) or a diet that provided 57% of kilocalories from fat (7). Thus, it appears that the level of fat within the ranges of 45–60% of kilocalories from fat is not a factor that would influence the response to anthocyanins.

Feeding the whole strawberry powder had some impact on obesity as indicated by the better glycemic control and trends toward decreased body fat and lack of any increased body weight gain. Mice fed a high-fat diet containing whole blueberry powder had increased body weight gain and increased adiposity relative to high-fat-fed controls. However, feeding of the isolated anthocyanins from blueberry and strawberry decreased weight gain and body fat, although the differences were not always statistically significant. Thus, although feeding purified forms of anthocyanins may decrease obesity, feeding of the whole berry appears to have a negative effect in terms of increasing adiposity.

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