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ARTICLE

Purified Blueberry Anthocyanins and Blueberry Juice Alter Development of Obesity in Mice Fed an Obesogenic High-Fat Diet†

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Male C57BL/6J mice (25 days of age) were fed either a low-fat diet (10% kcal from fat) (LF) or a high-fat diet (45% kcal from fat) (HF45) for a period of 72 days. Blueberry juice or purified blueberry anthocyanins (0.2 or 1.0 mg/mL) in the drinking water were included in LF or HF45 treatments. Sucrose was added to the drinking water of one treatment to test if the sugars in blueberry juice would affect development of obesity. Total body weights (g) and body fat (%) were higher and body lean tissue (%) was lower in the HF45 fed mice compared to the LF fed mice after 72 days, but in mice fed HF45 diet plus blueberry juice or blueberry anthocyanins (0.2 mg/mL), body fat (%) was not different from those mice fed the LF diet. Anthocyanins (ACNs) decreased retroperitoneal and epididymal adipose tissue weights. Fasting serum glucose concentrations were higher in mice fed the HF45 diet. However, it was reduced to LF levels in mice fed the HF45 diet plus 0.2 mg of ACNs/mL in the drinking water, but not with blueberry juice. β cell function (HOMA-BCF) score was lowered with HF45 feeding but returned to normal levels in mice fed the HF45 diet plus purified ACNs (0.2 mg/mL). Serum leptin was elevated in mice fed HF45 diet, and feeding either blueberry juice or purified ACNs (0.2 mg/mL) decreased serum leptin levels relative to HF45 control. Sucrose in drinking water, when consumption was restricted to the volume of juice consumed, produced lower serum leptin and insulin levels, leptin/fat, and retroperitoneal and total fat (% BW). Blueberry juice was not as effective as the low dose of anthocyanins in the drinking water in preventing obesity. Additional studies are needed to determine factors responsible for the differing responses of blueberry juice and whole blueberry in preventing the development of obesity.

KEYWORDS: Obesity; leptin; blueberry; anthocyanins

INTRODUCTION

Attention has recently focused on foods that may be beneficial in preventing diet-induced obesity and possibly reduce the risk of diabetes and heart disease. Anthocyanins (ACNs), which are especially high in berries and some other foods, have been shown to have an antiobesity effect in vivo (*1*−*6*). Most of these studies used a concentrated extract of the ACNs from food sources other than berries. However, in our studies of ACNs in berries, when whole powdered blueberries or strawberries were fed and compared to purified ACNs, the whole berries were not effective in preventing obesity (*2*, *5*) and in some cases tended to promote obesity in the context of a high-fat diet but not with a low-fat diet. Whole freeze-dried powders of Concord grapes and black raspberries were also ineffective in preventing obesity (*2*). Whole strawberries did not promote obesity when fed in a high-fat diet, and some measures indicated possible antiobesity effects (*2*, *5*). This observation that purified ACNs but not the whole berry slowed the development of obesity (*2*, *5*) was unexpected and raised several questions regarding the whole food versus isolated ACNs.

Recent studies have also reported either increased obesity (*7*) or equivalent adiposity (*8*) with feeding whole blueberry powder at 2 or 4% of a high-fat diet. From earlier work, ACNs extracted from purple corn were observed to prevent obesity (*1*, *4*). In a similar mouse model of obesity, ACNs from the Cornelian cherry were demonstrated to have antiobesity effects (*3*). An aqueous extract of *Hibiscus sabdariffa* calyces containing 0.28 mg of total ACNs/mg of extract at a dose of 33.6 mg of ACNs/kg/day for 60 days was also shown to have antiobesity effects in an obese animal model induced by the oral administration of monosodium glutamate (*9*). ACNs in black soybean seed coats have also been shown to have an antiobesity effect, which can reverse the effects

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Table 1. Anthocyanin Composition of Freeze-Dried Whole Blueberry (BB) Powder, Blueberry Juice, and Purified Blueberry Anthocyanins

peak	compound name	MW[a]	BB powder (μg/g of diet)	BB juice (μg/mL)	purified BB ACNs[b] (μg/mL)
1	delphinidin 3-galactoside	465	310	154	56
2	delphinidin 3-glucoside	465	365	223	72
3	cyanidin 3-galactoside	449	55	35	45
4	delphinidin 3-arabinoside	435	173	89	34
5	cyanidin 3-glucoside	449	60	52	57
6	petunidin 3-galactoside	479	141	86	45
7	cyanidin 3-arabinoside	419	31	22	26
8	petunidin 3-glucoside	479	214	171	75
9	peonidin 3-galactoside	463	40	32	16
10	petunidin 3-arabinoside	449	63	42	22
11	peonidin 3-glucoside	463	98	91	40
12	malvidin 3-galactoside	493	164	160	112
13	malvidin 3-glucoside	493	262	271	176
14	malvidin 3-arabinoside	463	81	73	53
15	cyanidin 3-(malonyl)glucoside	535	4	3	3
16	cyanidin 3-(6″-acetyl)galactoside	491	0	0	0
17	malvidin + acetyl + hexose	535	9	7	4
18	petunidin + pentose	449	0	0	0
19	delphinidin 3-(malonyl)glucoside	551	113	86	28
20	malvidin 3-(malonyl)glucoside	579	0	0	0
21	delphinidin 3-(6″-acetyl)glucoside	507	3	2	0
22	peonidin 3-(6″-acetyl)galactoside	505	7	6	2
23	cyanidin 3-(6″-acetyl)glucoside	491	20	20	18
24	malvidin 3-(6″-acetyl)galactoside	535	32	34	21
25	petunidin 3-(6″-acetyl)glucoside	521	58	57	22
26	peonidin 3-(6″-acetyl)glucoside	505	34	40	11
27	malvidin 3-(6″-acetyl)glucoside	535	98	131	61
	total		2432	1887	1000

[a] Molecular weight. [b] Concentrations of anthocyanins in solution fed to mice on treatments 4 and 9 receiving 1 mg of total anthocyanins/mL of water.

Table 2. Composition of Modified AIN-93G Diets Fed to Mice

ingredient	low-fat diet (10% kcal of fat) (LF)[a]	high-fat diet (45% kcal of fat) (HF45)[b]	blueberry (45% kcal of fat) (HF45+BB)
casein, 80 mesh	200	200	196.5
L-cystine	3	3	3
corn starch	315	72.8	32.8
maltodextrin 10	35	100	100
sucrose	350	172.8	130.5
cellulose, BW200	50	50	50
soybean oil	25	25	24
lard	20	177.5	177.5
Mineral Mix S10026	10	10	10
dicalcium phosphate	13	13	13
calcium carbonate	5.5	5.5	5.5
potassium citrate, 1 H_2O	16.5	16.5	16.5
Vitamin Mix V10001	10	10	10
choline bitartrate	2	2	2
blueberry powder	0	0	100[c]
total	1055	858	871
g %			
protein	17.0	20.9	20.6
carbohydrate	66.3	40.3	39.7
fat	4.3	23.6	23.2
fiber	4.7	5.8	6.4
kcal			
protein	716	716	717
carbohydrate	2800	1382	1382
fat	405	1823	1823
total	3921	3921	3921
kcal %			
protein	18.3	18.3	18.3
carbohydrate	71.4	35.3	35.2
fat	10.3	46.5	46.5
total	100.0	100.0	100.0

[a] Diet fed to mice on treatments 1−4 except treatment 2 included blueberry juice in place of drinking water (LF+BBJ), treatment 3 included purified ACNs in the drinking water (0.2 mg/mL) (LF+ACNs), and treatment 4 included purified ACNs in the drinking water (1.0 mg/mL). [b] HF45 diet fed to mice on treatments 5−9 except treatment 6 included blueberry juice in place of drinking water (HF45+BBJ), treatment 7 included purified ACNs in the drinking water 0.2 mg/mL) (HF45+ACNs), treatment 8 included sucrose in drinking water (88 mg/mL) (HF45+S), and treatment 9 included purified ACNs in the drinking water (1.0 mg/mL) (HF45+ACNs-1). [c] See **Table 1** for the composition of anthocyanins in the diet.

of a high-fat diet on body weight, adipose tissue weight, and serum lipid contents (*10*). Weight gain was also significantly lowered in rats fed a high-fat diet in which isolated black soybean ACNs or whole black soybeans were fed at comparable levels of ACNs, compared to rats fed a high-fat diet alone. This is the only study other than ours that fed the whole food compared to extracted ACNs.

The mechanisms whereby ACNs exert antiobesity effects are not clear. Sasaki and co-workers (*6*) suggested that cyanidin 3-glucoside ameliorates hyperglycemia and insulin sensitivity via the reduction of retinol binding protein 4 (RBP4) expression in white adipose tissue in type 2 diabetic mice. This effect was also accompanied by down-regulation of the inflammatory adipocytokines (monocyte chemoattractant protein-1 and tumor necrosis factor-α (TNF-α)) in the white adipose tissue of the cyanidin-3-glucoside group. Mice fed a high-fat diet were observed to have up-regulated inflammatory genes in adipose tissue, which was attenuated with whole blueberry powder in the diet (4%) even though adiposity was not altered (*8*). Cyanidin-3-glucoside and other ACNs extracted from purple corn suppressed the mRNA levels of enzymes involved in fatty acid and triacylglycerol synthesis and lowered the sterol regulatory element binding protein-1 mRNA level in white adipose tissue of mice fed a high-fat diet (*1*).

Studies to date have not provided any explanation for why the purified ACNs are effective in preventing obesity but the whole berry powder containing similar amounts and types of ACNs are ineffective. One option may be that there are other factors in the whole berry powder that counteract in some way the effects of purified ACNs. Juices made from berries provide another form in which ACNs are consumed in the diet. Processing of blueberries into juice results in a significant loss of the total ACNs in the berry (*11*), but juice still provides a readily available source of ACNs that have been separated from other components in the whole berry. The main objective of the current study was to determine if consumption of blueberry juice was as effective in preventing obesity as purified ACNs provided in the drinking water.

Table 3. Body Weights, Cumulative Weight Gains, Body Composition, and Food Intake in Male C57BL/6J Mice Fed a Low-Fat (LF) or High-Fat (HF45) Diet with Blueberry Juice or Purified Anthocyanins (0.2 mg/mL) in the Drinking Water[a]

	treatment			treatment					
item	LF	LF + BBJ	LF + ACN (0.2 mg/mL)	HF45	HF45 + BBJ	HF4 5+ ACN (0.2 mg/mL)	fat	ACN	fat × ACN
initial body wt (g)	18.1 ± 0.4	17.5 ± 0.4	17.9 ± 0.4	18.5 ± 0.4	18.1 ± 0.4	18.5 ± 0.4	NS	NS	NS
body wt, day 51	26.2 ± 1.0 c	27.0 ± 0.8 c	27.0 ± 0.8 c	31.6 ± 0.8 b	27.4 ± 0.9 bc	28.6 ± 0.8 bc	<0.001	NS	0.016
cumulative gain, day 51	8.1 ± 0.7 c	9.6 ± 0.6 c	9.1 ± 0.6 c	13.2 ± 0.6 b	9.3 ± 0.7 c	10.1 ± 0.6 c	<0.001	NS	<0.001
body composition data, day 51									
lean %, day 51[b]	63.4 ± 4.4	62.7 ± 3.8	67.0 ± 3.8	57.7 ± 3.8	53.8 ± 3.8	62.0 ± 3.8	0.059	NS	NS
fat %, day 51[b]	21.5 ± 2.2 c	23.1 ± 1.9 c	20.6 ± 1.9 c	29.7 ± 1.9 *b	22.5 ± 1.9 c	23.7 ± 1.9 bc	0.039	NS	NS
body wt (g), day 72	30.0 ± 1.3 c	29.4 ± 1.1 c	29.4 ± 1.1 c	34.3 ± 1.1 b	31.2 ± 1.2 bc	31.7 ± 1.1 bc	0.004	NS	NS
cumulative gain (g) day 72	11.9 ± 1.1 c	11.9 ± 0.9 c	11.5 ± 0.9 c	15.8 ± 0.9 b	13.1 ± 1.0 bc	13.2 ± 0.9 bc	0.005	NS	NS
body composition data, day 72									
lean %, day 72[b]	59.4 ± 1.8	60.2 ± 1.6	63.8 ± 1.6	54.2 ± 1.6 *	57.3 ± 1.7	58.7 ± 1.7 *	0.002	NS	NS
fat %, day 72[b]	26.2 ± 1.6 c	24.8 ± 1.4 c	21.4 ± 1.4 d	32.2 ± 1.4 *b	29.6 ± 1.5 b	23.9 ± 1.5 c	<0.001	<0.001	NS

[a] Values are presented as mean ± SEM. LF, low-fat diet (10% kcal from fat); HF45, high-fat diet (45% kcal from fat); ACN concentration = 0.2 mg/mL total ACNs in drinking water. Body composition data were not obtained for treatments 4 and 9, which received 1.0 mg of ACNs/mL in the drinking water. Means without a common letter differ among HF45 treatments ($p \leq 0.05$, ANOVA). Means with an asterisk (*) in the HF diet group differ significantly from the comparable low-fat group. [b] Body weights were taken after a 12 h fast.

MATERIALS AND METHODS

Chemicals and Reagents. Standards of the 3-*O*-β-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 wild blueberry powder was provided by FutureCeuticals Inc. (Momence, IL). The extraction of ACNs was as described previously (*2*, *5*). Blueberry powder was weighed (500 g) 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, Germany) to remove methanol. The concentrated extracts were loaded onto an Amberlite XAD-7 resin column (Sigma-Aldrich, St. Louis, MO). The resin was washed with 0.5% formic acid in water, and subsequently the absorbed ACNs 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 diode array detector to determine the concentration of ACNs as well as other phenolic acids. Total volume of the final blueberry extract was measured, distributed appropriately, and lyophilized to yield dry purified ACN powders. The ACN content of the purified powders was determined as described previously (*12*). The compositions of the ACNs in the blueberry extract, blueberry juice, and whole freeze-dried blueberry powder are presented in **Table 1**.

Animals and Diets. All experimental animal protocols were approved by the Animal Care and Use Committee of the Arkansas Children's Hospital Research Institute. Purified diets were prepared by Research Diets (New Brunswick, NJ), and the compositions are presented in **Table 2**. Blueberry juice from wild blueberries was provided by Van Dyk's Health Juice Products Ltd. (Kentville, NB, Canada).

Male C57BL/6J mice (25 days of age) were assigned at random to treatment such that there were nine animals per treatment. Mice were housed three per cage. The treatments included (1) low-fat diet (10% kcal from fat) (LF); (2) LF + blueberry juice (LF-J) in place of drinking water; (3) LF + purified blueberry ACNs in the drinking water (0.2 mg/mL) (LF-A0.2); (4) LF + purified blueberry ACNs in drinking water (1.0 mg/mL); (5) high-fat diet (45% kcal from fat) (HF45); (6) HF45 diet + BBJ in place of drinking water (HF-J); (7) HF45 diet + ACNs in drinking water (0.2 mg/mL) (HF-A0.2); (8) HF45 + purified blueberry ACNs in drinking water (1.0 mg/mL); (9) HF45 diet + sucrose in drinking water at a level calculated to be equivalent to the sugars in the blueberry juice (88 mg/mL) (HF-S); and (10) HF45 diet containing 11.5% whole blueberry powder on dry weight basis. All diets were prepared by Research Diets (New Brunswick, NJ) and fed in pelleted form. Fresh deionized water containing purified ACNs was provided every other day, and the volume consumed was recorded daily. Preliminary studies indicated that the ACNs were stable in water over a 48 h period (*2*). Consumption of sucrose water in treatment 8 was limited to the volume of juice consumed by the mice in treatment 5, which was necessary to equalize the higher energy intake from liquids consumed. Weekly weights and estimates of feed intake were obtained. Whole body composition (fat and lean tissues) was determined using nuclear magnetic resonance technology with an EchoMRI Analyzer system by Echo Medical Systems (model EchoMRI-900; http://www.echomri.com/echo_rats.html) (Houston, TX) on days 51 and 72. The EchoMRI-900 system provides measurements of whole body composition parameters: total body fat, lean mass, and total body water in live mice without the need for anesthesia or sedation in about 1 min. On days 75−79 of the experiment, the animals were sacrificed after euthanization with isofluorane, and serum, heart, liver, kidney, and adipose tissue (epididymal and retroperitoneal) were collected, weighed, frozen under liquid N_2, and stored at −70 °C until further analyses.

Cytokine and Insulin Analysis. Serum cytokines [leptin, ACTH (adrenocorticotropic hormone), osteocalcin, RANKL (receptor activator for nuclear factor κ B ligand)] were analyzed using the Luminex xMAP technology with Linco LINCOplex multiplex immunodetection kits and reagents (Millipore Corp., Billerica, MA). Cytokines were quantitated with Bio-Plex Manager software (Bio-Rad Laboratories, Inc.). Serum insulin was analyzed using a commercially available rat/mouse insulin ELISA kit (EZRMI-13K) from Linco Research, Inc. (St. Charles, MO).

Serum Glucose and Lipid Analysis. Serum glucose (IR070), triglycerides (IR140), and cholesterol (IR060) were analyzed using specific reagents indicated from Synermed (Westfield, IN) using a BMG Fluorstar microplate reader (BMG Labtech GmbH, Offenburg/Germany).

Insulin Resistance and β Cell Function. The degree of insulin resistance was estimated by a homeostasis assessment model (HOMA-IR) which was calculated by the formula:

$$\text{HOMA-IR} = [\text{plasma glucose (mmol/L)} \times \text{serum insulin (mU/L)}]/22.5$$

β cell function was assessed by the β cell homeostasis assessment (HOMA-BCF) score:

$$\text{HOMA-BCF} = [20 \times \text{serum insulin (mU/L)}]/[\text{plasma glucose (mmol/L)} - 3.5]$$

Insulin values were expressed in international units (1 IU = 0.04167 mg) (*13*, *14*).

Hepatic Triglyceride and Total Cholesterol Analysis. Samples from the liver of each mouse were homogenized, and total lipids of the liver homogenates were extracted with a mixture of chloroform and methanol according to the method of Folch et al. (*15*); the amounts of triglycerides (IR140) and total cholesterol (IR060) were determined as described above.

Statistical Analysis. Data from treatments 1−6 were analyzed using a two-way [dietary anthocyanins (none, juice, or purified ACNs) and fat level (CF10 vs HF45) as factors] analysis of variance (ANOVA) with a post hoc comparison using Sigmastat for Windows, ver. 3.5 (San Jose, CA). For the analysis of food or liquid intakes where three mice were housed per cage, the data were treated as one mean value per cage and for presentation, the mean was divided by 3 to express the data on a "per mouse" basis. Treatments 8−10 plus treatment 5 were analyzed using a simple one-way analysis of variance.

RESULTS

Animals were randomized to treatments at the beginning of the study such that initial weights were not different among treatments (**Table 3**). At days 51 and 70, body weight and cumulative weight gains were higher in mice fed the HF45 diet compared to the LF diet (**Table 3**; **Figure 1**). In the mice fed HF45 diet with blueberry juice or purified ACNs (0.2 mg/mL), body weight gains were reduced but not significantly different from the mice fed LF diet. The percent body fat, using MRI data, was also higher in the HF45 fed mice compared to the LF mice, but in those mice receiving blueberry juice or blueberry ACNs, the percent body fat was decreased relative to the HF45 fed mice and was not significantly different from those fed the LF diet at day 51 (**Table 3**). At day 72, a similar pattern was observed, except that body lean percent was significantly lower in treatments with the HF45 diet compared to the LF group. Body fat percent was reduced in mice fed purified ACNs in the drinking water compared to the HF45 diet (**Table 3**). However, cumulative weight gain and percent body fat in mice fed the HF45 diet plus BB juice were not significantly different ($p > 0.05$) from those in mice fed the HF45 diet (**Table 3**).

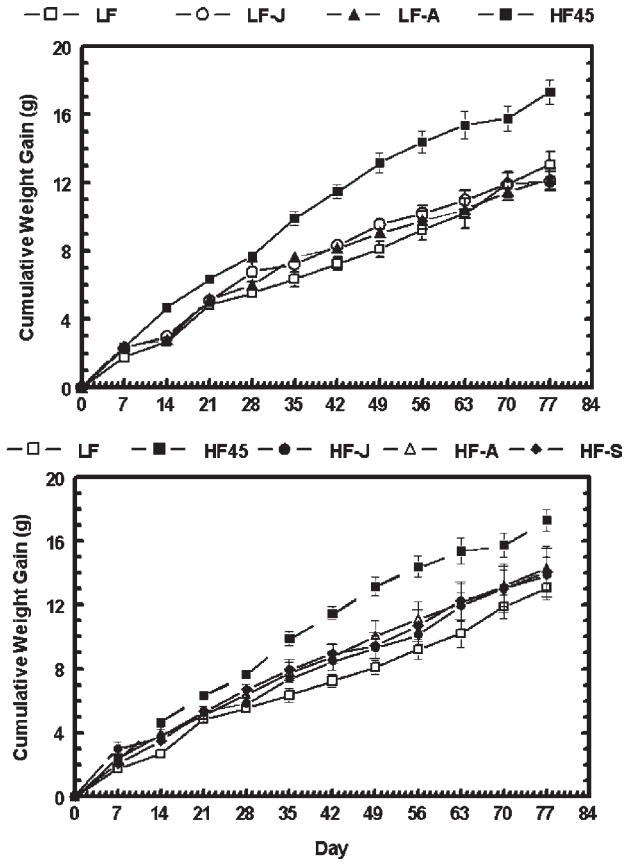

Figure 1. Cumulative body weight gains in male C57BL/J6 mice fed a low-fat (LF) or high-fat (HF) (45% kcal from fat) diet with or without blueberry juice (J) or anthocyanins (A) (0.2 mg/mL) in place of drinking water or sucrose (S) in place of drinking water. Intake of sucrose was limited to the volume of juice consumed by mice fed the high-fat diet plus juice.

Food intake (g/mouse/day) was slightly higher in mice fed the LF diet compared to the HF45 diet (2.87 ± 0.6 vs 2.74 ± 0.28), but caloric intake (kcal/mouse/day) was higher with the HF45 diet (12.5 ± 0.22 vs 9.4 ± 0.24) (**Table 4**). Liquid consumption by mice on the LF + BBJ treatment was highest and on the HF45 + ACNs (1.0 mg/mL) was lowest (**Table 4**). Anthocyanin consumption was lowest in mice fed 0.2 mg/mL in the drinking water and highest in those given blueberry juice or blueberry powder in the diet (**Table 4**). Calculated sugar/monosaccharide intake was higher in mice consuming blueberry juice, sucrose, or blueberry powder (treatments 2, 9, 10) (**Table 4**).

Weights of heart, liver, and kidneys were not altered by dietary treatment (**Table 5**); however, when expressed as a percentage of body weight, liver and heart were smaller in the HF45-fed mice compared to the LF-fed mice ($p < 0.009$). Epididymal and retroperitoneal fat (g and % of body weight) were higher in the HF45-fed mice compared to mice fed the LF diet ($p < 0.001$). Weights of epididymal and retroperitoneal fat were decreased in mice fed ACNs in both the LF and HF45 diets compared to mice fed no ACNs (**Table 5**). Amounts of epididymal and retroperitoneal fat in mice fed the blueberry juice were intermediate between the control HF45 fed mice and the HF45 + ACN (0.2 mg/mL) fed mice. Fasting serum glucose concentrations were higher in mice fed the HF45 diet and were lowered to LF levels in mice fed the HF45 diet plus 0.2 mg of purified ACNs/mL in the drinking water (**Table 6**). β cell function, as assessed by the HOMA-BCF score, was lowered with HF45 feeding but was returned to normal levels in mice fed the HF45 diet plus purified ACNs (0.2 mg/mL) (**Table 6**). Serum leptin was elevated in mice fed the HF45 diet and feeding either blueberry juice or purified ACNs (0.2 mg/mL)

Table 4. Liquid, Food, Calorie, Anthocyanin, Fat, and Sugar Intake by Male C57BL/6J Mice Fed a Low-Fat (LF) or High-Fat (HF45) Diet Consuming Blueberry Juice, Purified Anthocyanins (0.2 or 1.0 mg/mL) in the Drinking Water, or Water as the Liquid Source[a]

treatment	liquid intake (mL/mouse/day)	food intake (g/mouse/day)	energy intake (kcal/mouse/day)	anthocyanin (mg/mouse/day)	fat intake (mg/mouse/day)	sugar/monosaccharide intake (mg/mouse/day)
(1) LF	ND	2.87 ± 0.61 bc	9.37 ± 0.24 b		123.2 ± 3.17 d	951.3 ± 24.5 b
(2) LF + BBJ	3.88 ± 0.18 b	2.76 ± 0.33 bc	10.7 ± 0.32 b	7.32 ± 0.34	118.5 ± 3.43 d	1265.8 ± 26.5 b
(3) LF + ACNs 0.2	2.87 ± 0.77 bc	2.96 ± 0.87 b	9.69 ± 0.73 b	0.57 ± 0.15	127.4 ± 9.55 d	983.5 ± 73.7 b
(4) LF + ACNs 1.0	3.42 ± 0.04 bc	3.28 ± 0.62 b	10.8 ± 0.52 b	3.42 ± 0.04	142.4 ± 6.77 d	966.8 ± 88.4 b
(5) HF45	2.28 ± 0.12 bc	2.74 ± 0.28 c	12.5 ± 0.22 c		526.7 ± 9.08 bc	552.5 ± 9.52 d
(6) HF45 + BBJ	2.79 ± 0.43 bc	2.43 ± 0.56 c	12.2 ± 0.80 c	5.26 ± 0.81	460.2 ± 27.3 c	738.7 ± 28.6 c
(7) HF45 + ACNs 0.2	2.43 ± 0.16 bc	2.63 ± 0.22 c	12.0 ± 0.15 bc	0.49 ± 0.03	504.5 ± 6.22 bc	529.2 ± 6.52 d
(8) HF45 + ACNs 1.0	1.79 ± 0.15 d	2.66 ± 0.44 c	12.3 ± 0.48 c	1.79 ± 0.15	515.0 ± 20.2 bc	540.2 ± 21.1 d
(9) HF45 + S	2.81 ± 0.33 bc	2.84 ± 0.48 bc	12.9 ± 0.51 c		543.8 ± 21.4 bc	705.2 ± 27.8 c
(10) HF45 + BB	ND	2.74 ± 0.44 bc	13.8 ± 0.68 c	6.89 ± 0.27	557.7 ± 22.5 b	761.9 ± 22.3 c

[a] Data presented as mean ± SEM. LF, low-fat diet (10% kcal from fat); HF45, high-fat diet (45% kcal from fat). Intake data were calculated on the basis of the last 35 days of the experiment. Values in a column without a common letter differ significantly ($p < 0.05$).

Table 5. Body and Tissue Weights in Male C57BL/6J Mice Fed a Low-Fat (LF) or High-Fat (HF45) Diet with Blueberry Juice or Purified Anthocyanins (0.2 or 1.0 mg/mL) in the Drinking Water[a]

	treatment									
item	(1) LF	(2) LF + BBJ	(3) LF + ACN (0.2 mg/mL)	(4) LF + ACN (1.0 mg/mL)	(5) HF45	(6) HF45 + BBJ	(7) HF45 + ACN (0.2 mg/mL)	(8) HF45 + ACN (1.0 mg/mL)	fat	ACN
body wt[b]	29.0 ± 1.3 d	27.6 ± 1.0 d	27.8 ± 1.0 d	28.7 ± 1.5 d	34.0 ± 1.0 c	30.1 ± 1.2 cd	31.6 ± 1.0 cd	33.4 ± 1.7 c	<0.001	NS
heart (% BW)	0.50 ± 0.031 de	0.56 ± 0.03 cd	0.56 ± 0.03 cd	0.57 ± 0.03 c	0.44 ± 0.03 e	0.51 ± 0.03 cd	0.49 ± 0.03 e	0.43 ± 0.02 e	0.009	0.035
liver (% BW)	3.87 ± 0.16 c	3.75 ± 0.13 c	3.76 ± 0.13 c	3.86 ± 0.17 c	3.27 ± 0.13d	3.17 ± 0.15 d	3.33 ± 0.13 d	3.19 ± 0.04 d	<0.001	NS
kidneys (% BW)	1.07 ± 0.07	1.24 ± 0.06	1.19 ± 0.06	1.19 ± 0.06	0.99 ± 0.06	1.14 ± 0.07	1.11 ± 0.06	1.00 ± 0.05	NS	0.055
epididymal fat (% BW)	4.04 ± 0.51 cd	3.47 ± 0.41 de	3.05 ± 0.41 e	1.92 ± 0.51 e	5.92 ± 0.45 c	5.05 ± 0.47 c	4.22 ± 0.44 cd	5.84 ± 0.58 c	<0.001	0.018
retroperitoneal fat (% BW)	1.27 ± 0.20 c	1.10 ± 0.16 ef	0.87 ± 0.16 ef	0.50 ± 0.14 f	1.85 ± 0.16 cd	1.78 ± 0.18 cde	1.36 ± 0.16 de	2.06 ± 0.26 c	<0.001	0.027
total fat (E+R), (% BW)	5.30 ± 0.68 d	4.57 ± 0.56de	3.91 ± 0.56 de	2.42 ± 0.66 e	7.78 ± 0.56 cd	6.85 ± 0.63 cd	5.57 ± 0.56 cd	7.90 ± 0.84 c	<0.001	0.015
heart (g)	0.144 ± 0.008	0.154 ± 0.006	0.156 ± 0.006	0.163 ± 0.007	0.149 ± 0.006	0.153 ± 0.007	0.152 ± 0.006	0.140 ± 0.004	NS	NS
liver (g)	1.13 ± 0.07	1.04 ± 0.05	1.05 ± 0.05	1.097 ± 0.039	1.11 ± 0.05	0.95 ± 0.06	1.04 ± 0.05	1.067 ± 0.032	NS	NS
kidneys (g)	0.310 ± 0.017	0.337 ± 0.014	0.327 ± 0.014	0.338 ± 0.017	0.337 ± 0.014	0.342 ± 0.015	0.345 ± 0.014	0.330 ± 0.010	NS	NS
epididymal fat (g)	1.18 ± 0.19 def	0.98 ± 0.16 def	0.86 ± 0.16 f	0.55 ± 0.16 f	2.02 ± 0.16 c	1.54 ± 0.18 cd	1.37 ± 0.16 cd	2.014 ± 0.058 cd	<0.001	0.018
retroperitoneal fat (g)	0.372 ± 0.071 de	0.313 ± 0.058 ef	0.246 ± 0.058 f	0.144 ± 0.043 f	0.632 ± 0.058 c	0.552 ± 0.066 cd	0.442 ± 0.076 de	0.706 ± 0.021c	<0.001	0.043
total fat (E+R) (g)	1.55 ± 0.25 defg	1.30 ± 0.21 defg	1.11 ± 0.21 fg	0.694 ± 0.20 fg	2.65 ± 0.21 c	2.09 ± 0.23 cd	1.81 ± 0.21 de	2.72 ± 0.08 c	<0.001	0.018

[a] Data are presented as mean ± SEM. LF, low-fat diet (10% kcal from fat); HF45, high-fat diet (45% kcal from fat); ACN, 0.2 mg/mL anthocyanin content in drinking water. Means without a common letter differ, $p \leq 0.05$, ANOVA. There were no statistically significant ($p > 0.05$) interactions of dietary fat level and anthocyanin source for any of the traits listed. [b] Body weights were taken after a 12 h fast.

lowered serum leptin levels relative to the HF45 control (**Table 6**). Serum leptin level divided by the total of the epididymal plus retroperitoneal fat (g) was not affected by level of dietary fat, but ACNs in the diet either as juice or as purified ACNs lowered the leptin/fat ratio ($p < 0.053$) (**Table 6**) Serum ACTH, RANKL, insulin, and insulin resistance homeostasis assessment (HOMA-IR) were not altered by diet (**Table 6**). Fasting serum and liver cholesterol and triglycerides were not altered by level of fat or ACNs in the diet. Blood samples were collected from the mice in this study after an overnight fast, whereas in previous studies (*2*), blood samples were collected in fed animals and significant differences were observed in blood lipids due to dietary fat consumption.

Treatments containing purified ACNs (1 mg/mL) in the drinking water, 10% freeze-dried whole blueberry powder, and sucrose in the drinking water were also studied (**Table 7**). Sucrose in the drinking water where consumption was restricted to the volume of juice consumed produced lower serum leptin levels ($p < 0.01$), lower leptin/fat ($p < 0.05$), lower serum insulin, and lower retroperitoneal and total fat (% BW) ($p < 0.05$) (**Table 7**). The whole blueberry powder in the diet was not effective in altering parameters measured relative to development of obesity except for a lowered serum leptin level relative to the HF45 diet. Likewise, the higher dose of ACNs (1 mg/mL) in the water was not effective in decreasing the development of obesity (**Table 7**).

DISCUSSION

Blueberry Juice and Obesity. Blueberry juice represents a convenient and easy to standardize source of blueberry ACNs, but the juice will contain other components such as procyanidins, chlorogenic acid, and other water-soluble compounds including sugars. Blueberry cell walls are rich in uronic acids and neutral sugars and noncellulosic sugars including xylose and arabinose (*16*). Most of the cell wall constituents may be removed during the juice-making process. Nutrient analysis of the blueberry juice used in this study by the manufacturers indicated that it contained 88 mg/mL of sugars. The present study addressed the question as to whether the juice would have the same effect as the purified ACNs. Blueberry juice did not lower fasting glucose nor correct the HOMA-BCF (**Table 6**). The percent body fat and epididymal and retroperitoneal fat (% BW) for the blueberry juice fed mice was intermediate between the control HF45 treatment group and the HF45 treatment given blueberry ACNs. Consumption of ACNs (0.2 mg/mL) decreased epididymal and retroperitoneal fat in both the low- and high-fat diets. This trend for a decrease in epididymal and retroperitoneal fat (**Table 5**) is noteworthy. Tsuda and coworkers (*1*) observed a decrease in liver and white adipose fatty acid synthetase mRNA level in mice fed ACNs from purple corn in either a low-fat or high-fat diet, suggesting that ACNs may act to decrease lipid synthesis as one mechanism of preventing obesity.

In this study we also included treatments of 10% blueberry powder in the diet and another treatment with 1.0 mg/mL of purified ACNs in the drinking water. The blueberry powder was not effective in reducing obesity, similar to what we have observed previously (*2*, *5*). With the feeding of purified ACNs or blueberry juice, the anthocyanins may be absorbed earlier in the stomach (*17*−*19*) or small intestine (*20*), whereas absorption of anthocyanins in the whole blueberry powder may be delayed and more may reach the lower gastrointestinal tract, where production and absorption of other phenolic acids and metabolites may have an effect.

Purified ACNs provided at a level of 1.0 mg/mL of the drinking water (**Table 6**) in this study also were ineffective in preventing obesity in the high-fat-fed mouse, which is contrary to findings in our previous experiment (*2*). The reasons for this discrepancy between the two studies are not clear. One difference between the

Table 6. Fasting Serum Glucose, Lipids and Cytokines in Male C57BL/6J Mice Fed a Low-Fat (LF) or High-Fat (HF45) Diet with Blueberry Juice or Purified Anthocyanins (0.2 mg/mL) in the Drinking Water at the End of the Experiment[a]

	treatment			treatment				
item	(1) LF	(2) LF + juice	(3) LF + ACN	(5) HF45	(6) HF45 + juice	(7) HF45 + ACN	fat	ACN
serum								
glucose (mmol/L)	7.91 ± 0.72 c	7.92 ± 0.62 c	8.10 ± 0.60 c	11.67 ± 1.59 b	11.70 ± 0.79 b	7.57 ± 0.66 c	0.004	0.058
cholesterol (mmol/L)	5.41 ± 0.93	4.92 ± 0.75	5.18 ± 0.78	6.57 ± 0.49	5.82 ± 0.90	5.40 ± 0.65	NS	NS
triglycerides (mmol/L)	2.88 ± 0.52	2.87 ± 0.42	2.75 ± 0.46	3.11 ± 0.41	3.25 ± 0.34	3.70 ± 0.78	NS	NS
leptin (pg/mL)	9859 ± 3507 c	5182 ± 3202 c	4063 ± 3202 c	22856 ± 3202 b	10358 ± 3202 c	11997 ± 3202 c	0.003	0.022
leptin/fat	6357 ± 1859	4040 ± 289	3181 ± 651	7341 ± 1135	4681 ± 663	5540 ± 1532	NS	0.053
ACTH (pg/mL)	164 ± 26	209 ± 23	140 ± 23	165 ± 23	147 ± 23	167 ± 23	NS	NS
osteocalcin (pg/mL)	104049 ± 5321	95946 ± 4857	86900 ± 4857	97116 ± 4857	95036 ± 4857	86186 ± 4857	NS	0.027
RANKL (pg/mL)	213 ± 46	218 ± 42	280 ± 42	225 ± 42	197 ± 42	221 ± 42	NS	NS
insulin (pg/mL)	1146 ± 113	901 ± 112	879 ± 112	947 ± 112	819 ± 112	1056 ± 112	NS	NS
HOMA-IR	9.21 ± 2.24	7.48 ± 2.04	9.04 ± 2.24	13.0 ± 2.04	10.14 ± 2.04	8.68 ± 2.04	NS	NS
HOMA-BCF	157.4 ± 23.1 c	106.1 ± 21.1 bc	146.4 ± 23.1 c	63.1 ± 21.1 *b	52.4 ± 21.1 b	151.3 ± 21.1 c	0.012	0.012
liver								
cholesterol (mg/g)	2.07 ± 0.25	1.89 ± 0.08	2.20 ± 0.08	2.02 ± 0.06	2.01 ± 0.08	2.16 ± 0.13	NS	NS
triglycerides (mg/g)	50.7 ± 8.8	41.7 ± 2.6	44.7 ± 4.1	46.3 ± 4.0	43.5 ± 2.3	52.5 ± 7.2	NS	NS

[a] Values are mean ± SEM. LF, low-fat diet (10% kcal); HF45, high-fat diet (45% kcal); ACN, 0.2 mg/mL anthocyanin content in liquid diet; HF45 diet group means with * differ significantly from the comparable low-fat group. Means without a common letter differ among HF45 treatments, $p \leq 0.05$, ANOVA. There were no statistically significant ($p > 0.05$) interactions of dietary fat level and anthocyanin source for any of the traits listed except for serum glucose ($p < 0.035$).

Table 7. Fasting Serum Parameters, Body Weight Gain, and Adipose Tissue Weights at the End of the Experiment[a]

item	(5) HF45[b]	(8) HF45 + ACN (1 mg/mL)	(10) HF45 + BB	(9) HF45 + sucrose	$p <$
serum					
glucose (mmol/L)	11.67 ± 1.59	11.79 ± 1.50	11.20 ± 1.06	9.90 ± 0.97	0.079
cholesterol (mmol/L)	6.57 ± 0.49	5.89 ± 0.79	6.41 ± 0.92	6.06 ± 0.81	NS
triglycerides (mmol/L)	3.11 ± 0.41	2.68 ± 0.35	2.47 ± 0.34	2.51 ± 0.38	NS
leptin (pg/mL) (adipo panel)	8830 ± 2294 b	ND	5119 ± 549 c	2000 ± 432 c	0.007
leptin/fat	2826 ± 589 c	ND	2089 ± 204 c	1386 ± 261 b	0.048
insulin (pg/mL) (adipo panel)	516 ± 108 c	ND	440 ± 121 bc	364 ± 86 b	0.004
cumulative gain, day 72	15.8 ± 0.7	16.8 ± 1.1	15.7 ± 0.7	13.0 ± 1.4	0.065
epididymal fat (% BW)	5.92 ± 0.54	5.84 ± 0.58	6.20 ± 0.40	4.32 ± 0.90	0.060
retroperitoneal fat (% BW)	1.85 ± 0.16 bcd	2.07 ± 0.26 d	1.98 ± 0.13 cd	1.21 ± 0.32 b	0.042
total fat (% BW)	7.78 ± 0.68 bc	7.90 ± 0.76 bc	8.17 ± 0.49 c	5.53 ± 1.20 b	0.039

[a] Values are mean ± SEM;. HF45, high-fat diet (45% kcal); ND, not determined. Means without a common letter differ among HF45 treatments, $p \leq 0.05$, ANOVA. [b] Data for this treatment are from the same mice presented in **Table 6**. However, a different cytokine panel was used to assay leptin and insulin, which gave different absolute values compared to data in **Table 6** for insulin and leptin.

studies was that in the previous study (*2*), we used a high-fat diet which contained 60% kcal from fat, whereas in this study we used a diet that contained 45% kcal from fat. Whether the fat level affected the response to ACNs is not known. It is noteworthy that in mice fed the LF diet, the 1.0 mg/mL dose of purified ACNs decreased quantities of epididymal and retroperitoneal fat relative to the 0.2 mg/mL dose and also the LF treatment group. However, in the HF45-fed mice receiving the 1.0 mg/mL dose of ACNs, epididymal and retroperitoneal fat levels were not decreased but were similar to or slightly higher than the HF45 mice not receiving any anthocyanins.

ACN consumption is a factor that needs further study as related to the development of obesity. The phenomenon that low doses of ACNs are effective, but high doses are not, seems to exist. However, it is not clear from the different studies exactly what those doses should be. Doses (mg/mouse/day) of purified anthocyanins of 0.5 and 0.6 (**Table 4**), 2.8 (*5*), and 2.2 (*3*) have been shown to be effective in preventing obesity in the mouse model of obesity. However, in this study 1.8 mg/mouse/day was not effective. Thus, a dose of ~2 mg/mouse/day may be close to the upper limit that is effective for purified ACNs, although an estimated intake of 6 mg/mouse/day was calculated from data by Tsuda et al (*1*). With the blueberry juice, ACN intake was 6.9 mg/mouse/day, which was efficacious, but less so than the lower does of purified ACNs. Perhaps providing a dilution of the blueberry juice instead of the full-strength juice may have been more efficacious in preventing obesity. With strawberry anthocyanins, a dose of 0.6 mg/mouse/day was efficacious (*5*). Another factor for which we have few data is the importance of the particular ACNs. Blueberry has a mixture of up to 27 different ACNs, depending upon the source of the blueberry, whereas purple corn has cyanidin-, peonidin-, and pelargonidin-based ACNs (*2*), strawberry has pelargonidin-based anthocyanins (*2*), and the Cornelian cherry has a mixture of cyanidin- and pelargoinidin-3-galactoside (*3*). There are not enough data available at this point to determine if there are different effects due to the aglycone portion of the ACNs.

Sucrose Consumption with a High Fat Diet. Sucrose was added to the drinking water in treatment 7 (88 mg of sugars/mL) to provide a control treatment for additional energy that would be consumed through the sugars in the blueberry juice. Consumption of the sucrose drink was held to the volume of blueberry juice consumed (**Table 4**). The actual caloric intake from the sucrose solution was slightly lower than from the juice as there were other sources of energy in the juice besides the sugars (**Table 4**). However, the surprising observation was that consumption of the sucrose water had an antiobesity effect, which is the opposite of what we would have predicted. Lowered weight gain and adipose tissue mass was observed, as well as lowered serum leptin and insulin as well as slightly lower fasting glucose relative to the control HF45 diet (**Table 7**). These responses are similar to that observed with

the consumption of purified ACNs (0.2 mg/mL). Zhang et al. (*21*) added 15% fructose to the drinking water of mice consuming a high-fat diet to produce obesity. However, effects of no fructose were not tested in that study. Mice consuming sucrose-sweetened water (10%) provided ad libitum along with a normal diet gained more body weight and developed glucose intolerance, hyperinsulinemia, and hypercholesterolemia (*22*). Normal female Wistar rats fed a high-sucrose−fat diet for 8 weeks demonstrated significant insulin resistance and obesity (*23*). The limited literature seems to indicate that the combination of a high-fat diet and sucrose should produce obesity and insulin resistance, which is opposite of what we observed. However, we did not allow ad libitum consumption of the fructose-containing water, which would likely have increased its consumption. Testing the effects of ad libitum consumption on the development of obesity was not our objective. What we can say from our study is that the sugars in the blueberry juice did not have the effect of increasing obesity, which is what we might have predicted.

In summary, consumption of purified ACNs in a dose of 0.2 mg/mL in the drinking water (0.49 mg/mouse/day) improved β cell function and the rate of fat deposition was decreased such that by day 72 the total fat in the body was not different from the LF-fed mice. Consumption of blueberry juice (2.8 mL/mouse/day; 5.3 mg of ACNs/mouse/day) was not quite as effective in preventing obesity in that the amount of fat deposited in the body was intermediate between the mice fed the HF45 diet and the mice fed the HF45 plus 0.2 mg/mL ACNs. Lower serum leptin concentrations were consistent observations in ACN treatments, which slowed the development of obesity.

ABBREVIATIONS USED

ACNs, anthocyanins; BB, blueberry; HF45, high-fat diet with 45% kcal from fat; HF60, high-fat diet with 60% kcal from fat; LF, low-fat diet with 10% kcal from fat; HOMA-IR, homeostasis assessment score for insulin resistance; HOMA-BCF, homeostasis assessment score for β cell function; ACTH, adrenocorticotropic hormone; RANKL, receptor activator for nuclear factor κ B ligand.

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