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RESEARCH ARTICLE

Purified berry anthocyanins but not whole berries normalize lipid parameters in mice fed an obesogenic high fat diet

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Male C57BL/6 mice received diets with either 10% of kcal from fat, or a high fat diet [45% (HF45) or 60% (HF60) kcal from fat]. Diets were prepared with or without freeze-dried powders (10%) from whole blueberries (BB), strawberries (SB), Concord grape or black raspberry. In the 2nd study, purified anthocyanins (ACNs) from SB or BB were added to the drinking water of the treatments fed the HF60 diet. In Study 1, serum triglycerides were increased by feeding the HF45 diet but were elevated further when black raspberry or BB was included in the HF45 diet. Liver total lipids and triglycerides were increased in mice fed HF45 diet and inclusion of any of the berry powders in the HF45 diet did not alter concentrations compared to HF45 controls. In the 2nd study, mice fed the HF60 diet plus purified ACNs from BB in the water had lower body weight gains and body fat than the HF60 fed. Serum cholesterol and triglyceride levels were elevated with the HF60 diet and decreased to control levels when ACNs from either SB or BB were included in the drinking water. Serum leptin levels were consistently decreased to control low fat levels in those ACN treatments in which measures of body fat were decreased. Administering purified ACNs from BB and strawberry *via* drinking water prevented the development of dyslipidemia and obesity in mice, but feeding diets containing whole berries or purple corn (PC) ACNs did not alter the development of obesity.

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1 Introduction

Anthocyanins (ACNs) are important plant pigments. They have important functions in plant physiology and are implicated to have health effects in the prevention of chronic diseases. Berries are particularly rich sources of ACN. Different berries, such as strawberries, blueberry (BB) and black raspberry (BRB), provide unique patterns of ACNs to study not only because of total concentration differences, but also because of differences in individual ACNs [1–4]. Total ACN concentrations in these freeze-dried berries were 2.9, 27.2 and 43.7 mg/g dry weight, respectively. However, the ACN composition varies considerably among the berries. BRBs are rich in cyanidin-based ACNs, BBs contain a complex mixture of delphinidin, cyanidin, peonidin, petunidin and malvidin glycosides and strawberries [5] are one of only a few berries in which pelargonidin is the primary anthocyanidin. Pelargonidin is much more stable and/or more readily absorbed *in vivo* and even though the concentration of pelargonidin-based ACNs in strawberries is

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Abbreviations: ACN, anthocyanins; **BB,** blueberry; **BRB,** black raspberry; **CG,** Concord grape; **HF45,** high fat diet with 45% kcal from fat; **HF60,** high fat diet with 60% kcal from fat; **LF,** low fat; **MCP-1,** monocyte chemoattractant protein-1; **PAI-1,** plasminogen activator inhibitor; **PC,** purple corn; **SB,** strawberry; **TNF-α,** tumor necrosis factor-alpha

low relative to ACNs in other berries, its apparent absorption may be as much as 10 times higher than other ACNs [3, 6]. The specific health effects that ACNs might have *in vivo* are not known although there are several possibilities related to obesity, cardiovascular disease and cancer [7–16].

Feeding ACNs in an extract from PC (rich in cyanidin-3-glucoside) has been demonstrated to prevent obesity in C57BL/6 mice fed a high fat (HF) compared to a HF diet with no ACNs [17]. Dietary ACNs from PC also acted to return elevated serum levels of glucose, insulin and leptin in the HF fed mice to control levels in HF + ACN fed mice. ACNs from PC when included in the diet lowered liver total lipid and triglycerides in HF fed mice compared to control HF mice fed no ACNs. In subsequent work [18], gene expression of adiponectin was up-regulated in white adipose tissue in mice fed an ACN supplemented diet although the elevation of the adiponectin gene expression by ACNs was not reflected in the serum protein level *in vivo*. These authors suggest that ACNs 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 ACN in PC, or whether other ACNs have similar effects. Also, there is no data available on the dose required. ACNs from PC were included in the diet at a level to provide 2 g/kg of cyanidin-3-glucoside equivalents [17]. No studies have been conducted with berry ACNs relative to the prevention of obesity.

ACNs from Cornelian cherry (*Cornus mas*) were also observed to ameliorate obesity and insulin resistance in C57BL6 mice fed a HF diet containing ACNs at 1 g/kg diet [19]. ACN-treated mice exhibited elevated insulin levels and the ACN treatment preserved pancreatic architecture and insulin staining relative to mice receiving no ACNs [19].

We observed in rats fed 0, 2.5, 5 or 10% of the diet as freeze-dried BB powder from weaning (Post Natal Day 21) to Post Natal Day 34 that abdominal fat pad weight, as a percentage of body weight, was significantly lower with 10% BB powder in the diet in both male and female rats fed a diet with normal fat levels (Prior and Wu, unpublished data). This suggested that BBs might impact adipose tissue formation. A similar trend due to diet was observed in gonadal fat weight although the effects were not statistically significant (Prior and Wu, unpublished data).

In our initial studies with C57BL/6J obese mice, we compared whole BB or strawberry powders *versus* ACN rich extracts from these two berries [20] in their effects in preventing obesity. We found that feeding the whole strawberry powder had some impact on obesity, but mice fed a HF fat diet containing whole BB powder had increased body weight gain and increased adiposity relative to HF fat fed controls. However, feeding of the isolated ACNs from BB and strawberry decreased weight gain and body fat. Thus, although feeding purified forms of ACNs may decrease obesity, feeding of the whole berry appears to have a negative effect that adiposity was increased.

In this study, we present additional data from feeding freeze-dried powders of BRB, and Concord grape (CG) and an extract of PC ACNs as dietary treatments. ACNs in BRB or CG represent extremely high or relatively low ACN content. Our objective was to determine whether other whole berries or fruits act similarly to BB or SB regardless of their ACN content and to determine how these different treatments influenced serum lipid profiles and cytokines related to obesity. Another objective of these experiments was to demonstrate that ACNs from berries would have a similar effect on the development of obesity as PC ACNs reported previously [17].

2 Materials and methods

2.1 Standards and solvents

Standards of the 3-*O*-β-glucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (six mixed ACN 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).

2.2 ACN analysis

An Agilent 1100 series HPLC (Palo Alto, CA) equipped with an autosampler/injector and diode array detector was used to determine the concentration of ACNs as well as other phenolic acids. The ACN content of the berries or purified ACN powders were determined as described by Wu *et al.* [5].

2.3 Preparation of purified ACNs

Freeze-dried BB powder was provided by FutureCeuticals (Momence, IL) and strawberries were provided by Oregon Raspberry and Blackberry Commission that was freeze-dried and prepared by Oregon Freeze Dry (Albany, OR). Extraction of berry ACNs was described in our previous paper [20]. Briefly, berry powders were weighed 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 (Sigma-Aldrich). The resin was washed with 0.5% formic acid and subsequently the absorbed ACNs were recovered with 0.5% formic acid in methanol. The methanol eluent was subjected to vacuum evaporation again and then was extracted three times with ethyl acetate (EtOAc). After EtOAc extraction, the aqueous layer was subjected to vacuum evaporation to remove residual organic solvents. The aqueous extract was analyzed by HPLC to quantitate the ACNs present and volumes were aliquoted to individual

glass jars based upon quantity of ACNs needed for each feeding. The extract in the jars was lyophilized and stored at −70°C until feeding. An appropriate amount of deionized water was added to the jar at the time of feeding. The final composition of the ACNs in the water for BB and strawberry is presented in Table 1. Steps were taken in an attempt to end up with a high purity product, but with the BB it was not possible to remove all the chlorogenic acid, which is a major phenolic acid in BBs. Minor quantities of procyanidins may have been extracted with methanol.

2.4 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). Freeze-dried powder of CGs was provided by FutureCeuticals; BRBs were provided by Oregon Raspberry and Blackberry Commission that was freeze-dried and prepared by Oregon Freeze Dry; PC ACN extract was provided by Artemis International (Fort Wayne, IN) in the form of a powder standardized to a minimum of 11% ACNs,

Table 1. Composition (%) of anthocyanins in blueberry and strawberry extracts, black raspberry and Concord grape diets and purple corn extract used in experiments

Anthocyanin[a)]	BB	SB	BRB	CG	PC
Delphinidin-3-galactoside	7.88	–	–	–	–
Delphinidin-3-glucoside	13.44	–	–	20.16	–
Delphindin-3-arabinoside	3.97	–	–	–	–
Cyanidin-3-galactoside	2.20	–	–	–	–
Cyanidin-3-glucoside	3.76	6.01	17.94	15.79	39.46
Cyanidin-3-arabinoside	1.33	–	–	–	–
Cyanidin 3-sambubioside-5-rhamnoside	–	–	22.55	–	–
Cyanidin 3-rutinoside	–	–	57.99	–	–
Cyanidin-3-sophoroside	–	20.64	–	–	–
Petunidin-3-galactoside	4.64	–	–		–
Petunidin-3-glucoside	10.88	–	–	8.94	–
Petunidin-3-arabinoside	1.94	–	–	–	–
Peonidin-3-galactoside	2.53	–	–	–	–
Peonidin-3-glucoside	10.53	–	–	2.41	11.53
Malvidin-3-galactoside	7.94	–	–	–	–
Malvidin-3-glucoside	20.67	–	–	3.59	–
Malvidin-3-arabinoside	3.95	–	–	–	–
Delphinidin-3-(6-acetyl)glucoside	1.22	–	–	4.11	–
Cyanidin-3-(6-acetyl)-glucoside	0.39	–	–	–	–
Malvidin-3-(6-acetyl)-galactoside	0.50	–	–	6.52	–
Petunidin-3-(6-acetyl)-glucoside	0.52	–	–	–	–
Malvidin-3-(6-acetyl)-glucoside	1.73		–	–	–
Pelargonidin-3-glucoside	–	62.94	–	–	8.53
Pelargonidin-3-rutinoside	–	3.41	–	–	–
Pelargonidin-3-(malonoyl)glucoside	–	5.74	–	–	–
Pelargonidin-3-(acetoyl)glucoside	–	1.27	–	–	–
Pelargonidin 3-rutinoside	–	–	1.52	–	–
Delphinidin 3-(6"-acetyl)glucoside	–	–	–	–	–
Delphinidin 3-(6"-coumaroyl)-5-diglucoside	–	–	–	5.28	–
Cyanidin 3-(6"-acetyl)glucoside	–	–	–	–	–
Delphinidin 3-(6"-coumaroyl)glucoside	–	–	–	26.74	–
Cyanidin 3-(6"-coumaroyl)glucoside	–	–	–	6.46	
Cyanidin-3-(malonyl)glucoside	–	–	–	–	20.42
Peonidin-3-(malonyl)glucoside	–	–	–	–	6.49
Pelargonidin-3-(malonyl)glucoside	–	–	–	–	3.21
Other unidentified (18 compounds)	–	–	–	–	18.89
Total	100	100	100	100	100
Total (mg/g)	1.16[b)]	1.0[b)]	4.16[c)]	0.23[c)]	192.4[d)]

a) Anthocyanins were quantitated as glucose equivalents using the glucoside of each of the six aglycones. BB, blueberry; SB, strawberry; BRB, black raspberry; CG, Concord grape; PC, purple corn.
b) Milligram *per* milliliter deionized water.
c) Total anthocyanins *per* gram extract.
d) Milligram total anthocyanins *per* gram diet.

obtained by aqueous extraction followed by concentration and spray-drying.

2.4.1 Experiment 1

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. Mice were randomized by weight and assigned to one of ten dietary treatments (Table 2): (i) Control low fat (10% calories from fat, LF); (ii) LF + 10% freeze-dried CG powder; (iii) LF + 10% BRB powder; (iv) LF + 10% freeze-dried BB powder; (v) LF + 10% freeze-dried strawberry powder; (vi) Control high fat (45% calories from fat; HF45); (vii) HF45 + 10% freeze-dried CG powder; (viii) HF45 + 10% BRB powder; (ix) HF45 + 10% freeze-dried BB powder (BB-HF45); and (x) HF45 + 10% freeze-dried strawberry powder (SB-HF45). Composition of the BB and strawberry diets have been described previously [20]. ACNs found in BBs and strawberries and the BRB and CG diets are presented in Table 1. There were 12 animals *per* treatment with three mice housed *per* cage. Weekly weights and estimates of food intake were obtained. Body composition was determined on days 57 and 80 by echo MRI techniques. On day 85 of the experiment, nonfasted animals were killed and serum, liver, kidney and adipose tissue (subcutaneous, epididymal, mesenteric, retroperitoneal and interscapular) were collected, weighed and frozen for later analysis.

2.4.2 Experiment 2

Male C57BL/6J mice (25 days of age) were assigned at random to treatments such that there were nine animals *per* treatment. Mice were housed three *per* cage. The treatments included: (i) Control low fat (10% calories from fat) (C-LF) (same as Expt. 1); (ii) High fat diet (60% calories from fat) (HF60) (Table 3); (iii) HF60 (Table 3) containing a concentrated source of ACNs from PC in which the entire diet was pelleted; (iv) Same diet as treatment 3 but the entire diet was fed in powder form; (v) Diet 2 (HF60) plus purified ACNs from BB provided in the drinking water; (vi) Diet 2 (HF60) plus purified ACNs from strawberry provided in the drinking water. All diets except treatment 4 were pelleted. Mice were weighed weekly. Fresh water containing ACNs was provided every other day. Volume of water consumed was determined every other day. A stability test of purified ACNs from BB in deionized water was performed and the mean recovery of 13 BB ACNs in deionized water (pH 3.38) was $94.4 \pm 4.4\%$ after 48 h at room temperature.

2.5 Serum glucose and lipid analysis

Glucose, triglycerides and total cholesterol were analyzed in serum from mice that were not fasted using specific reagents from Synermed (Westfield, IN) and followed the protocol provided by manufacturer.

2.6 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.* [21] and the amounts of triglycerides and total cholesterol were analyzed using specific reagents indicated from Synermed.

2.7 Insulin and cytokine analysis

Serum cytokines (leptin, resistin, monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor (tPAI-1), tumor necrosis factor-alpha, (TNF-α) and IL-6) were analyzed using the Luminex xMAP technology with Linco® LINCOplex® multiplex immunodetection kits and reagents (Millipore, Billerica, MA). Cytokines were quantitated with Bio-Plex Manager software (Bio-Rad Laboratories). Serum insulin was analyzed using a rat/mouse insulin ELISA kit (EZRMI-13K) from Linco Research (St. Charles, MO).

2.8 Statistical analysis

Data were analyzed using analysis of variance (ANOVA) with a *post-hoc* comparison (Holm-Sidak method) using SigmaStat for Windows, Ver. 3.5 (San Jose, CA).

3 Results

3.1 Experiment 1

Body weight gains were increased in mice fed the HF45 diet compared to the controls fed a LF diet (Fig. 1). Overall average caloric intake was higher (~1.4 kcal/day) in mice fed the HF45 diets compared to the LF diets (Table 4). However, feeding of BRB ($p < 0.05$) or CG powders in the HF45 diet increased weight gain above the control HF45 diet (Fig. 1). This does not appear to be due to changes in food or energy intake in the HF treatments (Table 4) as g or kcal of food consumed was not altered by CG or BRB powder in the HF45 diet. Inclusion of the BRB or CG powders in the LF diet did not alter weight gain significantly relative to the control LF diet. Body weight gains of mice fed the strawberry and BB treatments have been presented previously [20] and growth rate of mice fed strawberry powder was not altered in the HF45 diet treatment but BB powder increased growth rate in one study but not significantly in the second study using a HF60 diet.

Table 2. Composition of low fat diets (10% kcal from fat) and high fat diets (45% kcal from fat) used in Experiment 1[a)]

%	D12450B (C-LF)		Concord grape		Black raspberry		D12451 (C-HF45)		Concord grape		Black raspberry	
	g	kcal	g	kcal	g	kcal	g	kcal	g	kcal	g	kcal
Diet												
Protein	19.2	20	18.9	20	18.9	20	23.7	20	23.1	20	23.1	20
Carbohydrate	67.3	70	67.9	70	67.9	70	41.4	35	42.8	35	42.8	35
Fat	4.3	10	4.2	10	4.2	10	23.6	45	23.1	45	23.1	45
Total	90.8	100	91.0	100	91.0	100	88.7	100	89.0	100	89.0	100
Anthocyanins (mg/kg)	0.0	–	508.8	–	4060	–	0.0	–	622.9	–	4971.0	–
Anthocyanins (mg/kcal)	–	–	0.13	–	1.08	–	–	–	0.13	–	1.08	–
Procyanidins (mg/kg)	–	–	–	–	163.8	–	–	–	–	–	200.5	–
Procyanidins (mg/kcal)	–	–	–	–	0.043	–	–	–	–	–	0.043	–
kcal/g	3.85	–	3.77	–	3.77	–	4.73	–	4.62	–	4.62	–
Ingredient												
Casein, 80 Mesh	200	800	200	800	200	800	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12	23	12	3	12	3	12
Corn Starch	315	1260	275	1100	275	1100	72.8	291	32.8	131	32.8	131
Maltodextrin 10	35	140	35	140	35	140	100	400	100	400	100	400
Sucrose	350	1400	310	1240	310	1240	172.8	691	132.8	531	132.8	531
Cellulose, BW200	50	0	50	0	50	0	50	0	50	0	50	0
Soybean oil	25	225	25	225	25	225	25	225	25	225	25	225
Lard	20	180	20	180	20	180	177.5	1598	177.5	1598	177.5	1598
Mineral Mix S10026	10	0	10	0	10	0	10	0	10	0	10	0
DiCalcium phosphate	13	0	13	0	13	0	13	0	13	0	13	0
Calcium carbonate	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0
Potassium citrate, 1 H_2O	16.5	0	16.5	0	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40	10	40	10	40	10	40	10	40
Choline bitartrate	2	0	2	0	2	0	2	0	2	0	2	0
Concord grape powder	0	0	100	320	0	0	0	0	100	320	0	0
Black raspberry	0	0	0	0	100	320	0	0	0	0	100	320
FD&C Yellow Dye no. 5	0.05	0	0	0	0	0	0.05	0	0	0	0	0
Total	1055.05	4057	1075	4057	1075	4057	858.15	4057	878.1	4057	878.1	4057

a) Composition of diets containing blueberry and strawberry powders have been described previously [20].

Table 3. Composition high fat diets (60% kcal from fat) with and without purple corn anthocyanins (PC ACN) used in Experiment 2

%	High Fat (C-HF60)		High Fat + PC ACN	
	g	kcal	g	kcal
Diet				
Protein	26.2	20	26.0	20
Carbohydrate	26.3	20	26.1	20
Fat	34.9	60	34.5	60
Total	–	100	–	100
kcal/g	5.24	–	5.19	–
Ingredient				
Casein, 80 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn starch	0		0	131
Maltodextrin 10	125	500	125	500
Sucrose	68.8	275.2	68.8	275.2
Cellulose, BW200	50	0	50	0
Soybean oil	25	225	25	225
Lard	245	2205	245	2205
Mineral Mix S10026	10	0	10	0
DiCalcium phosphate	13	0	13	0
Calcium carbonate	5.5	0	5.5	0
Potassium citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline bitartrate	2	0	2	0
Purple corn extract	0	0	8.1	0
FD&C Blue Dye no. 1	0.05	0	0	0
Total	858.15	4057	781.95	4057

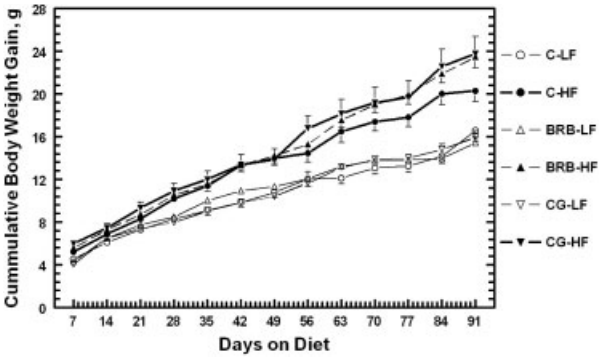

Figure 1. Cumulative body weight gain in C57BL6J mice fed a LF (10% kcal from fat) or HF (45% kcal from fat) containing BRBs or CG dried powder (Experiment 1).

Mice fed the LF diet with CG powder had increased % body fat and decreased % body lean compared to LF fed controls ($p<0.05$) (Table 5) on day 80 but not on day 57 of the experiment. Liver and kidney weights (% of body weight) were increased in mice fed the LF diet containing CG relative to LF controls. Mice fed the HF45 diet containing BRB powder had increased % body fat and decreased % body lean ($p<0.10$) and increased final body weight ($p<0.05$) and decreased % kidney weight ($p<0.05$) compared to control HF45 fed mice (Table 5). For all weight and composition traits measured on day 91 (final day of experiment) (Table 5), the HF45 fed mice were significantly different from the mice fed a LF diet ($p<0.001$) using 2-way analysis of variance.

In mice fed the LF and HF45 diets, serum triglycerides were significantly increased relative to the respective control with the feeding of diets containing the BB and BRB treatments (Table 5). Overall, there was a significant increase in serum triglycerides in the HF45 diets; however, the individual within dietary treatment effects were not significant between the corresponding HF45 and LF diets. Serum cholesterol levels were not significantly different between the respective control and any of the treatments within either the LF or HF45 diets (Table 5). Serum glucose levels were not affected by diet or fat level. Serum insulin was elevated in mice fed the CG and BB HF45 diets relative to the respective LF diet. Relative to mice fed the respective LF diets, serum leptin levels were increased in mice fed the HF45 control and HF CG diets and leptin was higher in mice fed the HF45 CG diet compared to the control HF45 diet ($p<0.01$) (Table 6). Serum resistin was elevated in mice fed the HF45 diet containing strawberry or BB relative the respective LF diet. Serum resistin was also higher in mice fed the HF45 strawberry diet relative to the control HF45 diet.

Table 4. Total food (g/day/mouse) and caloric intake (kcal/day/mouse) of C57BL6 mice fed a high or low fat diet with or without black raspberries, Concord grape, blueberry or strawberry powders[a] (Experiment 1)

Treatment	Food intake		Anthocyanin concentrations and intake	
	g/day	kcal/day	mg/g diet	mg/day
Low fat, black raspberry	2.52±0.05	9.20±0.19	3.24	8.18
High fat, black raspberry	2.43±0.04	10.86±0.41	4.16	10.13
Low fat, Concord grape	2.67±0.06	9.72±0.21	0.29	0.77
High fat, Concord grape	2.47±0.07	11.01±0.32	0.23	0.57
Low fat, blueberry[b]	2.56±0.05	–	1.27	3.25
High fat, blueberry[b]	2.71±0.15	–	1.38	3.74
Low fat, strawberry[b]	2.46±0.03	–	0.21	0.52
High fat. strawberry[b]	2.40±0.06	–	0.24	0.58
Low fat, control	2.61±0.03	9.70±0.12	0	0
High fat, control	2.38±0.11	10.88±0.52	0	0

a) Data presented as Mean ± SEM of four observations *per* group with three mice comprising each observation.
b) Data from [20].

Table 5. Effect of dietary fat and berry treatment on body and tissue weights as a percentage of body weight (Experiment 1)[a]

	LF diet, 10% kcal from fat			HF diet, 45% kcal from fat[b]			Fat	Berry	FxB
	Control	Black raspberry	Concord grape	Control	Black raspberry	Concord grape			
Initial weight (g)							NS	NS	NS
Day 57									
Weight (g)	25.0±0.7	25.4±0.3	25.2±0.5	28.8±1.2	29.1±0.5	30.3±1.1	<0.001	NS	NS
Fat (% BW)	15.1±1.3	16.4±1.3	16.9±1.3	23.0±1.3	25.6±1.3	25.8±1.3	<0.001	NS	NS
Lean (% BW)	69.9±1.6	70.1±1.6	68.7±1.6	64.9±1.6	61.2±1.6	60.3±1.6	<0.001	NS	NS
Day 80									
Weight (g)	27.0±0.8	27.2±0.2	28.7±0.7	32.6±1.4	33.9±0.7	35.0±1.1	<0.001	NS	NS
Fat (% BW)	18.0±1.3	17.7±1.3	22.3±1.3[c]	27.5±1.3	31.1±1.3[c]	30.5±1.3	<0.001	0.034	NS
Lean (% BW)	64.7±1.2	65.1±1.2	61.0±1.2[d]	58.3±1.2	54.6±1.2[c]	53.9±1.2[d]	<0.001	0.007	NS
Day 91, Final									
Final weight (g)	28.4±1.1	28.9±1.1	28.9±1.1	33.4±1.1	36.4±1.0[d]	36.8±1.1	<0.001	NS	NS
Liver Wt (% BW)	4.45±0.19	4.26±0.19	4.78±0.19[d]	4.14±0.18	3.93±0.19	4.28±0.19	0.014	NS	NS
Kidney Wt (% BW)	1.20±0.12	1.56±0.12	1.28±0.12[d]	1.16±0.11	1.05±0.11[d]	1.11±0.12	0.014	NS	NS
Heart Wt (% BW)	0.68±0.02	0.68±0.02	0.68±0.02	0.59±0.02	0.55±0.02	0.57±0.02	<0.001	NS	NS
Epididymal Wt (% BW)	2.75±0.36	2.87±0.36	3.06±0.36	5.01±0.35	5.89±0.36	4.60±0.36	<0.001	NS	NS
Subcutaneous Wt (% BW)	0.88±0.07	0.90±0.07	0.96±0.07	1.59±0.06	1.67±0.06	1.54±0.07	<0.001	NS	NS

BW, body weight.
a) Data presented as mean±SEM. Data on blueberry and strawberry treatments has been presented previously [20].
b) Traits in mice fed the high fat diet were all significantly different from those in animals fed the low fat diet ($p<0.001$) using 2-way analysis of variance.
c) Treatment mean differs ($p<0.10$) from control within level of fat in the diet using *t*-test.
d) Treatment mean differs ($p<0.05$) from control within level of fat in the diet using *t*-test.

Total liver lipids were elevated in all treatments containing a HF45 diet relative to the respective LF diet (Table 6). Liver triglycerides were higher in mice fed the HF45 diet containing CG, BRB, BB, or strawberry relative to the respective LF diets. Liver cholesterol levels were not altered due to level of fat or any of the berry treatments.

3.2 Experiment 2

In experiment 2, mice fed the HF60 diet had elevated serum triglycerides, cholesterol and leptin relative to the control LF diet (Table 7). Mice fed the HF60 diet and given SB or BB ACNs in the drinking water had levels of serum triglycerides, cholesterol and leptin that were not significantly

Table 6. Effect of berries or Concord grapes in low (10% kcal from fat) or high (45% kcal from fat) fat diets on serum glucose, cholesterol and triglycerides, liver lipids and serum insulin and leptin in BL57/6J mice (Experiment 1)

		Control	Concord grape	Black raspberry	Blueberry	Strawberry	Fat[e)]	Berry[e)]	BxF[e)]
Serum									
Triglyceride (mg/dL)	LF	90.7 ± 5.5[a)]	91.2 ± 5.9[a)]	126.2 ± 7.1[b)]	130.3 ± 12.9[b)]	126.7 ± 10.4[a, b)]			
	HF	114.0 ± 6.3[a)]	114.2 ± 8.0[a, b)]	181.0 ± 10.3[c, d)]	153.0 ± 8.5[c, d)]	127.0 ± 4.5[a, b, c)]			
Mean		102.4[a)]	102.7[a)]	153.6[c, d)]	141.7[b, c)]	126.9[b)]	<0.001	<0.001	0.10
cholesterol (mg/dL)	LF	116.5 ± 4.6	127.0 ± 7.4	111.3 ± 12.4	113.8 ± 9.5	124.3 ± 2.6			
	HF	155.6 ± 5.8[a, b)]	176.5 ± 10.4b[b)]	184.3 ± 7.8[b)]	176.6 ± 9.6[a, b)]	139.1 ± 13.3[a)]			
Mean		136.0[a)]	151.8[a, b, c)]	147.8[b, c)]	145.2[b, c)]	131.7[a, b)]	0.001	0.008	0.023
glucose (mg/dL)	LF	278.4 ± 42.9	206.6 ± 16.4	270.8 ± 27.6	247.2 ± 11.7	269.0 ± 42.9			
	HF	225.2 ± 13.8	272.9 ± 10.2	265.3 ± 20.7	277.3 ± 26.1	290.0 ± 30.3	NS	NS	NS
Insulin (pg/mL)	LF	1059 ± 289[a, c)]	1426 ± 362[a)]	956 ± 391[a, c)]	811 ± 362	1002 ± 391			
	HF	1727 ± 276	3140 ± 362*	1756 ± 442	2299 ± 362*	1877 ± 391	<0.001	0.064	NS
Leptin (pg/mL)	LF	5476 ± 3091	5856 ± 3875	4507 ± 4186	4527 ± 3875	3387 ± 4186			
	HF	16 831 ± 2960 [a)]*	30 551 ± 3875[b)]*	11 746 ± 3875[a)]	0833 ± 3875[a)]	10 423 ± 4186[a)]	<0.001	0.033	NS
Resistin (pg/mL)	LF	973 ± 155	ND	ND	707 ± 131	666 ± 141			
	HF	890 ± 141[a)]	ND	ND	1222 ± 131[a, b)]*	1561 ± 173[b)]*	<0.001	NS	0.011
Liver									
Total lipid (mg/g)	LF	34.3 ± 4.4	22.7 ± 4.4	22.7 ± 4.4	24.7 ± 4.4	27.3 ± 4.4			
	HF	38.0 ± 4.4*	39.3 ± 4.4*	36.7 ± 4.4*	48.3 ± 4.4*	42.7 ± 4.4*	<0.001	NS	NS
Triglycerides (mg/g)	LF	17.9 ± 4.0	15.3 ± 4.0	10.9 ± 4.0	12.9 ± 4.0	13.2 ± 4.0			
	HF	21.0 ± 4.0	26.8 ± 4.0*	23.8 ± 4.0*	33.3 ± 4.0*	30.6 ± 4.0*	<0.001	NS	NS
Cholesterol (mg/g)	LF	1.03 ± 0.09	0.91 ± 0.09	1.05 ± 0.09	1.02 ± 0.09	1.10 ± 0.09			
	HF	1.06 ± 0.09	0.96 ± 0.09	1.06 ± 0.09	1.09 ± 0.09	1.14 ± 0.09	NS	NS	NS

a-d) Mean ± SEM within a row without a common superscript are significantly different ($p < 0.01$).
e) Level of statistical significance for main effects of fat (F) and berry (B) and interaction of fat × berry (B × F) in 2-way analysis of variance.
*) Significantly different than corresponding LF dietary treatment ($p < 0.05$).

Table 7. Effect of purified anthocyanins from blueberry (BB) or strawberry (SB) provided in the drinking water and anthocyanins from purple corn (PC) provided in a pelleted (PE) or powdered (P) diet to BL57/6J mice fed a high fat diet (HF60) (60% kcal from fat) on serum cholesterol, triglycerides, insulin, leptin, and other cytokines and liver total lipids, triglycerides and cholesterol ($n = 4$ or 5 mice/treatment) (Experiment 2)

	Control-LF	HF60	HF60+BB	HF60 + SB	HF60 – PC-PE	HF60 – P
Serum						
Triglyceride (mg/dL)	86±11[a)]	155±19[b)]	84±8[a)]	77±17[a)]	186±7[b)]	213±15[b)]
Cholesterol (mg/dL)	64±6[a)]	119±10[b)]	84±12[a, b)]	82±5[a, b)]	123±5[b)]	132±10[b)]
Insulin (pg/mL)	1512±319	2218±931	1315±360	1457±361	1315±360	ND
Leptin (pg/mL)	3603±377[a)]	10818±2068[b)]	5627±1108[a)]	7306±447[a, b)]	9486±1555[b)]	ND
Resistin (pg/mL)	1508±97	1717±163	1379±98	1458±55	1658±169	ND
MCP-1 (pg/mL)	37.6±3.7[a)]	22.9±2.7[b)]	13.9±1.7[b, c)]	20.5±9.0[b)]	19.4±3.7[b)]	ND
tPAI-1 (pg/mL)	1966±225	2141±295	1766±160	2055±220	2263±165	ND
TNFα (pg/mL)	4.7±0.4	4.9±0.3	3.8±0.3	3.7±0.2	4.2±0.2	ND
IL-6 (pg/mL)	11.8±3.9	8.2±1.2	7.1±0.8	6.1±0.6	5.4±0.7	ND
Liver						
Total lipid (mg/g)	38.0±2.6	41.6±6.9	26.4±1.9	30.0±3.0	26.0±2.8	25.2±1.6
Triglycerides (mg/g)	12.4±3.0	16.7±4.4	8.0±2.7	11.3±1.9	11.0±2.1	8.7±1.2
Cholesterol (mg/g)	1.47±0.12[a, b)]	1.89±0.12[b)]	1.21±0.07[a)]	1.53±0.07[a, b)]	0.82±0.08[a)]	1.16±0.07[a)]

a-c) Mean±SEM within a row without a common superscript are significantly different ($p<0.05$).
ND, not determined.

Table 8. Average food and energy and water intake and final weights in mice fed a control low fat diet (LF10) or high fat (60% calories from fat, HF60) or HF diet plus an extract from purple corn (PC) fed in a pelleted (PE) or powdered (P) form (Experiment 2)

Item	Control LF	Control HF60	PC-PE HF60	PC-P HF60
Food intake (g/d/mouse)	2.82±0.03[a)]	2.29±0.02[b)]	2.32±0.05[b)]	2.40±0.07[b)]
kcal intake (kcal/d/mouse)	10.9±0.1[a)]	12.0±0.1[b)]	12.0±0.3 [b)]	12.4±0.2[b)]
Total anthocyanin intake (mg/d/mouse)	–	–	4.64±0.1	4.78±0.09
Final weight (g)	24.5 ±0.3[a)]	29.9 ±0.9[b)]	29.4 ±1.1[b)]	30.8 ±1.2[b)]
Liver weight (% BW)	4.6±0.1[a)]	3.7±0.1[b)]	3.4±0.1[b)]	3.7±0.2[b)]
Heart weight (% BW)	0.78±0.04	0.76±0.06	0.75±0.06	0.76±0.03

a, b) Mean±SEM with different superscripts differ ($p<0.05$).

different from the control LF diet (Table 7). Total liver lipids and triglycerides were not altered significantly by dietary treatment, and mice fed the BB and SB ACN treatments with the HF60% diet were not significantly different from mice fed the control LF diet (Table 7).

When mice fed the HF60 diet containing PC ACNs either in pellet form or as a powder, serum triglyceride and cholesterol (Table 7) concentrations were approximately doubled compared to the LF diet, but were not significantly different than the control HF60 diet. Serum levels of insulin, resistin, PAI-1, TNF-α, and IL-6 were not altered by dietary fat level or by HF60 diet containing ACNs from PC and in mice given purified ACNs from SB or BB. Serum levels of MCP-1 were lower in all HF60 diets relative to the LF diet.

Mice fed the HF60 diet plus an extract from PC had similar final body, liver and heart weights as mice fed the control HF60 diet (Table 8) and serum cholesterol, triglycerides, insulin, leptin and other cytokines and liver total lipids, triglycerides and cholesterol were not altered by feeding PC extract in the HF60 diet compared to the control HF60 diet (Table 7). Feeding the PC diet in pelleted or powdered form did not affect food intake of final weights (Table 8).

4 Discussion

High fat diets containing 45% of kcal from fat and 60% of kcal from fat have been used in these experiments. We anticipated that the HF60 diet would accelerate the development of obesity; however, we have not observed any notable differences between the two HF diets in the parameters that we have evaluated in these studies or subsequent unpublished studies. Because the HF45 diet is closer to a practical diet that would be encountered, the HF45 diet may be the preferred one to use in the future.

In these studies, C57BL/6J mice that consumed a HF diet had increased serum triglyceride, cholesterol and leptin

concentrations. When purified ACNs were provided in the drinking water along with the HF diet, serum triglycerides, cholesterol and leptin were decreased to levels similar to the LF diet (Table 7). However, feeding equivalent quantities of ACNs in the diet as a whole berry powder was not effective in correcting the dyslipidemia associated with obesity.

The finding that feeding equivalent amounts of ACNs as the whole berry powder of CG, BRB, BB and strawberry were not effective in preventing obesity whereas feeding the purified ACNs from BB and strawberry was effective raises several interesting questions. There have been a limited number of studies published on the effects of ACNs on aspects of obesity, metabolic syndrome or diabetes [8, 9, 17, 19, 22–25]. However, all these studies, with the exception of our data [20], have used an ACN rich extract of the food and not the whole food. With the ACN extracts from various sources studied including black soybean, PC, Cornelian cherry, BBs, strawberries and black rice, positive responses have been observed relative to obesity and metabolic syndrome [9, 10, 17, 19, 20, 25]. A recent study [26] looked at the effects of feeding a freeze-dried powder of tart cherries on hyperlipidemia in the Dahl Salt-Sensitive rat. Feeding the cherry powder at 1% of the diet was associated with reduced fasting glucose, reduced hyperlipidemia, reduced hyperinsulinemia and reduced fatty liver. Our studies have been the first to look at several whole powders of different berries and their effects on obesity [20]. Reasons for the differential response between whole berries and berry extracts are not clear. Possible factors, which might alter the response to ACNs in whole foods compared to purified extracts of ACNs, include: (i) Amounts consumed and rate of absorption of ACNs; (ii) Configuration (*i.e.* glycosylation or acylation) of ACNs consumed; (iii) Specific sugars, carbohydrates, lipids or other factors present in whole berries that may promote lipid synthesis; or (iv) Factors in the berry that counteract the effects of the ACNs. The quantity of ACNs consumed is probably not a factor as intakes of BB ACNs were 3.75 and 2.83 and for strawberry were 0.58 and 1.59 mg/day in the whole berry compared to purified ACNs in the drinking water, respectively (Table 4) [20]. Intakes of ACNs in the HF60 diets from BRB and CG powders were 10.1 and 0.6 mg/day (Table 4). Thus, wide ranges in concentrations have been consumed from the various ACN sources and concentrations in the whole berry powders in the diet encompassed levels consumed as purified ACNs in the drinking water. All the berry powders were included in the diet at approximately 10% of the diet (Table 2). Lower levels of the powders in the diet may alter the response.

The ACN composition is quite different from the various sources (Table 1) [5]. Strawberry has a somewhat unique ACN profile compared to any of the other powders studied in that pelargonidin-3-glucoside is the predominant ACN (Table 1). Pelargonidin treatment (3 mg/kg) of diabetic rats was shown to normalize elevated blood glucose levels and improve serum insulin levels [22]. Although these responses were rather dramatic, the injection of pelargonidin is considerably different than consumption of pelargonidin-3-glucoside in strawberries in the diet. The other ACN sources have a mixture of several ACNs with cyanidin-3-glucoside present in all of them (Table 1). PC had more than 20 different ACNs, but cyanidin-3-glucoside (39.5%), cyanidin-3-malonylglucoside (20.4%), peonidin-3-glucoside (11.5%) and peonidin-3-malonylglucoside (6.5%) accounted for ~78% of the total ACNs. BRB contains six different ACNs, but cyanidin-3-rutinoside and cyanidin-3-sambubioside-5-rhamnoside were the two predominant ACNs [27]. CG contains over 30 ACNs [27] with cyanidin and delphinidin glucosides and coumaroyl-glucosides predominating. Acylated ACNs seem not to be absorbed as well as other ACNs in the glycoside form [28]. The relatively low amounts of ACNs plus the high amount of acylated ACNs may have been partially responsible for a lack of effect of CG on obesity. Thus, based upon the available data at this point, neither amount of ACN or ACN profile seems to be a factor in explaining the differences in responses between whole berry and purified ACNs.

Although there would have been small differences in specific sugars in the diet between diets containing the berry powders and the control AIN-93 diet, those differences would have been small as the berry powders replaced sucrose in the diet (Table 2). BBs contain glucose and fructose in a ratio of ~1:1 with a total of 0.6 g/g DM. Strawberries contain a total of 0.5 g/g DM of glucose and fructose in a ratio of 0.8:1 (http://www.nal.usda.gov/fnic/foodcomp/cgi-bin/list_nut_edit.pl). Thus, these berries would have added 5–6% more sugar to the diet, but the content would still be below the sucrose levels in the control LF diet. Furthermore, we have observed that consumption of sucrose in the drinking water by mice fed a high fat diet does not promote obesity (Prior *et al.*, unpublished data).

We cannot rule out the possibility that there are other factors in the whole berry powders that may counteract or in some way alter the response of adipose tissue to ACNs. However, at this point we do not have any indications of what they might be. We have observed that providing BB juice in place of the drinking water seems to have similar effects as purified BB ACNs (unpublished data). Thus, it does not appear to be any soluble component retained in the juice during processing that might interfere with the action of ACNs.

It is now appreciated that adipocytes secrete a wide array of proteins that influence systemic metabolism including leptin, adiponectin, resistin and visfatin, as well as cytokines and chemokines such as TNF-α, IL-6 and MCP-1. We have measured serum levels of some of these factors. There were no differences in plasma resistin, tPAI-1, TNF-α or IL-6 between mice fed the control LF diet and those fed the HF60 diet (Table 7). However, tPAI-1 and IL-6 gene expression have been shown to be down regulated in human adipocytes treated with cyanidin-3-glucoside. However, gene expression of adiponectin was up regulated by cyanidin-3-glucoside [8]. Significant increases in serum resistin were observed with

HF45 feeding in the BB and strawberry treatments in Experiment 1 (Table 6), but no significant changes were observed in Experiment 2 with the HF60 fat level (Table 7). Resistin is a signaling molecule that is induced during adipogenesis and is specific to and secreted by white adipose tissue. Resistin levels have been shown to be increased in diet-induced obesity as well as in genetic models of obesity and insulin resistance [29]. The importance of the increase in serum resistin in the mice fed the HF45 containing strawberry powder is not clear.

Significant changes in serum glucose and insulin due to dietary fat level were not consistently observed, although insulin tended to be increased in the HF fed animals. Previous work by Tsuda *et al.* [17] demonstrated a clear increase (+60%) in serum insulin and glucose in mice fed a HF diet, whereas Jayaprakasam *et al.* [19] did not see a difference in insulin levels due to dietary fat in fasted mice, but insulin levels in mice consuming ACNs was increased more than 1000-fold.

In Experiment 2, we did not observe any diet effects on serum levels of IL-6 or TNF-α similar to Expt. 1. However, Sasaki *et al.* [9] observed that gene expression of TNF-α was decreased by 76% in white adipose tissue of cyanidin-3-glucoside fed mice. MCP-1 was reduced in serum of mice fed the HF60 diet compared to the control LF diet (Table 7). There was also a significant decrease in MCP-1 in the mice fed the HF60 diet containing BB compared to the LF control diet group (Table 7). The HF60 control was numerically lower than the HF60 BB treatment but differences were not statistically significant ($p > 0.05$). Gene expression of MCP-1 in white adipose tissue of mice fed cyanidin-3-glucoside for 5 weeks was down-regulated [9]. One might expect to observe an increase in MCP-1 between the LF and HF diet if MCP-1 levels are indicative of a chronic inflammatory condition associated with obesity. The release of adipokines by either adipocytes or adipose tissue-infiltrated macrophages may lead to a chronic subinflammatory state that could play a central role in the development of insulin resistance and the increased risk of cardiovascular disease associated with obesity [30]. Some of these factors may promote insulin sensitivity as well as others that induce insulin resistance [31, 32]. An increase in epididymal white adipose tissue TNF-α and leptin mRNA has been observed in mice fed a HF diet, which was ameliorated by feeding an ACN extract from PC [17]. In our studies, an extract of ACNs from PC when fed to mice did not prevent obesity or lower serum leptin levels compared to the control HF diet (Table 7). The reasons for the differences between studies [17] in responses to feeding ACNs from PC are not clear, but the sources and preparation and purity of the extract were likely different, which may be contributing factors.

A consistent observation in these studies has been the development of high serum leptin levels in mice fed high fat diets with increased adiposity (Tables 6 and 7) and a return to near normal levels in those berry or ACN treatments that prevented obesity (Table 7). Leptin is a product of the *ob* gene secreted predominantly by white adipocytes. Leptin secretion from adipocytes is stimulated by food consumption, insulin, leucine, glucose and glucocorticoids [33]. Most obese humans and rodents have very high plasma leptin concentrations [34]. However, this endogenous hyperleptinemia may not reduce appetite or increase energy expenditure due to a condition that has been termed ''leptin resistance.'' The development of obesity and leptin resistance in C57BL/6J mice on a high fat diet has been divided into three stages: In the early stage, mice on a HF diet gain weight but maintain a response to the anorectic effect of peripheral leptin; In the middle state, mice on a high fat diet show peripheral leptin insensitivity, expressed by changes in food intake and body weight but these mice retain the ability to respond to central leptin injection. In the final stage the mice develop central leptin resistance and do not show changes in food intake and body weight in response to intracranial leptin administration [34].

How leptin secretion by adipocytes might be controlled by ACNs is not clear. Consumption of food and particularly glucose will increase ATP in adipocytes, which will stimulate leptin secretion [33] provided the glucose is transported into the adipocyte and metabolized to provide ATP and not lactate. A rise in cAMP in adipocytes decreases leptin secretion, whereas a decrease in cAMP exerts the opposite effect [33]. Leptin mRNA levels in epididymal white adipose tissue of mice fed a HF diet containing ACNs was reduced by 43% compared to HF fed mice but was not different from the LF control fed mice [17].

In summary, the understanding of how specific factors in fruits and berries may impact the development of obesity in either a positive or negative manner is important in making dietary recommendations that might be beneficial in decreasing the obesity epidemic that is present in the U.S. and other developed countries. The findings in these studies that purified ACNs from BB and strawberry are active in preventing obesity, but consumption of the whole fruit powder is not protective and in some cases may even be detrimental were surprising. The effects of ACNs in berry powders may be dose dependent and warrants further study. Since whole fresh berry consumption is seasonal, but juices and other processed products from the berries may be consumed on a more regular basis, development of an understanding of the effects of components in various processed products and their effect on obesity is important.

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proprietary product or specific equipment, does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable. The assistance of Theo Rogers in the analysis of data from these studies is acknowledged.

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