

Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects^{1–4}

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ABSTRACT

Background: Anthocyanins have been shown to exert benefits on the lipid profile in many animal models. Whether these molecules have similar beneficial effects in humans is currently unknown.

Objective: The objective was to investigate the effects of berry-derived anthocyanin supplements on the serum lipid profile in dyslipidemic patients.

Design: A total of 120 dyslipidemic subjects (age 40–65 y) were given 160 mg anthocyanins twice daily or placebo for 12 wk in a double-blind, randomized, placebo-controlled trial.

Results: Anthocyanin consumption increased HDL-cholesterol concentrations (13.7% and 2.8% in the anthocyanin and placebo groups, respectively; $P < 0.001$) and decreased LDL-cholesterol concentrations (13.6% and -0.6% in the anthocyanin and placebo groups, respectively; $P < 0.001$). The cellular cholesterol efflux to serum increased more in the anthocyanin group than in the placebo group (20.0% and 0.2%, respectively; $P < 0.001$). Anthocyanin supplementation decreased the mass and activity of plasma cholesteryl ester transfer protein (CETP) (10.4% and 6.3% in the anthocyanin group and -3.5% and 1.1% in the placebo group, respectively; $P < 0.001$). In the anthocyanin group, the change in HDL cholesterol was negatively correlated with the change in CETP activity ($r_s = -0.330$). The change in LDL cholesterol was positively correlated with the change in CETP mass ($r_s = 0.354$). The change in cellular cholesterol efflux to serum was positively correlated with the change in HDL cholesterol ($r_s = 0.485$). In vitro, cyanidin 3-*O*- β -glucosides dose-dependently lowered CETP activity in human HepG2 cells.

Conclusions: Anthocyanin supplementation in humans improves LDL- and HDL-cholesterol concentrations and enhances the cellular cholesterol efflux to serum. These benefits may be due to the inhibition of CETP. *Am J Clin Nutr* doi: 10.3945/ajcn.2009.27814.

INTRODUCTION

It is well established that elevated triglyceride and LDL-cholesterol concentrations are risk factors for cardiovascular disease (CVD) (1). In the past several decades, many intervention studies of CVD have focused on the decrease in LDL. Recent studies have, however, consistently shown that the serum concentration of HDL cholesterol is inversely related to the risk of developing CVD, which is independent of serum LDL and triglyceride concentrations (2). Therefore, raising HDL concentrations is a potential strategy for the treatment of CVD (3). The primary function of HDL is the promotion and facilitation of

reverse cholesterol transport (RCT) (3, 4), a process by which cholesterol is effluxed from macrophages, foam cells, and atherosclerotic plaques and is delivered back to the liver and eliminated as bile salts or biliary cholesterol. The efficiency of RCT is dependent on macrophage cholesterol efflux, lecithin cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), and selective uptake of cholesteryl esters from HDL to the liver (3). These elements or molecules are determinants of the function of HDL.

Dietary or dietary composition intervention is considered a first approach in treating and controlling CVD. Many studies have shown that phytochemicals from plant foods have appreciable beneficial roles in preventing CVD (5–7). Anthocyanins, a category of phytochemicals, are the largest group of water-soluble pigments in the plant kingdom. It has been shown that anthocyanin-rich extracts from foods have effective antioxidant properties in vitro (8, 9). In recent years, several studies have shown that these materials have a wide range of biological activities, such as antioxidant (10) and antiatherosclerotic (11, 12) activities in cellular models in vitro and in animal models in vivo. Supplementation of diets rich in anthocyanins or anthocyanin extracts resulted in a decrease in serum triglyceride, total cholesterol, and non-HDL cholesterol and an increase in serum HDL-cholesterol and apolipoprotein A-I (apo A-I) in different animal models (13, 14). Furthermore, anthocyanins showed similar beneficial properties in vitro by the promotion of cholesterol efflux from macrophages (11), which may also contribute to their beneficial effects on lipid profiles.

It has been well documented that lipid metabolic characteristics of humans are quite different from that of rodents. Hence,

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directly extrapolating the benefits of anthocyanins from animal models to develop potential clinical applications may not be feasible. We have therefore attempted to evaluate the effects of pure anthocyanins derived from berries on the serum lipid profile in dyslipidemic patients. We also used the human HepG2 cell line to investigate the mechanism by which cyanidin 3-*O*- β -glucosides (Cy-3-G) improves lipid profile.

SUBJECTS AND METHODS

Subjects and design

One hundred twenty community-dwelling dyslipidemic patients aged 40–65 y were recruited into this clinical trial between April 2007 and October 2008 by distributing advertisement leaflets in each community in Guangzhou, Guangdong, China. The subjects were considered to have dyslipidemia if they met 2 of the following 4 criteria: fasting total cholesterol concentration >200 mg/dL, fasting triglyceride concentration >150 mg/dL, fasting LDL-cholesterol concentrations >100 mg/dL, or fasting HDL-cholesterol concentrations <40 mg/dL. Exclusion criteria included a history of CVD or other severe chronic disease or use of any drugs known to affect lipid metabolism. This study was approved by the Ethics Committee of Sun Yat-Sen University, and each of the participants provided written informed consent. All procedures were in accordance with institutional guidelines and were carried out in compliance with the Helsinki Declaration.

This study was a randomized, double-blind, placebo-controlled trial. Eligible patients were randomly assigned by age to either the anthocyanin group ($n = 60$; 21 men and 39 women) or the placebo group ($n = 60$; 21 men and 39 women). The total duration of this trial was 12 wk, during which time the subjects were asked to maintain their habitual diet and lifestyle. The anthocyanin group was instructed to consume two 80-mg anthocyanin capsules (Medox; Polyphenols AS, Sandnes, Norway) twice daily (30 min after breakfast and supper) for a total intake of 320 mg anthocyanins/d. The placebo group took 2 placebo capsules twice daily. Each of the participants attended our facility every 4 wk after taking the capsules. During the visit, the adherence of the subjects to the protocol was assessed by recalling the remaining capsules and obtaining the related information and reinforced if necessary. Meanwhile, the capsules were dispensed, and body weight, blood pressure, and waist and hip circumferences were measured. At baseline and at the end of the trial, all patients were required to fast overnight to enable blood sample collection the next morning. The subjects were asked to complete a 3-d (including 2 workdays and 1 weekend) record of their food intake, which was analyzed by using CDGSS3.0 software to estimate nutrient intake at baseline and at 12 wk. A questionnaire survey was undertaken for each subject at baseline and contained questions regarding dietary habits (including how much and how often the participants consumed meat, milk, eggs, and vegetables, etc, and if any vitamin or mineral supplements were commonly taken).

Materials

The anthocyanin and placebo capsules were identically packaged. The total anthocyanin content was 80 mg/capsule, which consisted of 17 different natural purified anthocyanins

from bilberry (*Vaccinium myrtillus*) and black currant (*Ribes nigrum*). The relative content of each anthocyanin was as follows: 33.0% of 3-*O*- β -glucosides, 3-*O*- β -galactosides, and 3-*O*- β -arabinosides of cyanidin; 58.0% of 3-*O*- β -glucosides, 3-*O*- β -galactosides, and 3-*O*- β -arabinosides of delphinidin; 2.5% of 3-*O*- β -glucosides, 3-*O*- β -galactosides, and 3-*O*- β -arabinosides of petunidin; 2.5% of 3-*O*- β -glucosides, 3-*O*- β -galactosides, and 3-*O*- β -arabinosides of peonidin; 3.0% of 3-*O*- β -glucosides, 3-*O*- β -galactosides, and 3-*O*- β -arabinosides of malvidin; and 1.0% of 3-*O*-rutinoside of cyanidin and delphinidin. In addition to the anthocyanins mentioned above, the anthocyanin capsules also contained pullulan, maltodextrin, and citric acid (which occupied for 4% per capsule and was helpful to maintain stability of anthocyanins), whereas the placebo capsules only contained pullulan and maltodextrin.

The dose of anthocyanins was determined based on our previous animal study (15) and a recent human study (16). Supplementation of 130 mg anthocyanins $\cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ contained in black rice anthocyanin extract was shown to significantly improve the lipid profile in mice (15). The effective dose of anthocyanins was determined to be in the range of 100.5–335.0 mg/d for a 70-kg human being when it was extrapolated to the humans by the method of specific surface area. In the human study by Karlsten et al (16), daily intake of 320 mg anthocyanins was found to be safe and effective in improving the inflammatory response.

Experimental determinations

Red and white blood cell counts and the hemoglobin concentration were determined with a Blood Cell Analyzer (MEK-6318K; Nihon Kohden Corporation, Tokyo, Japan). The serum total protein concentration was measured by using the biuret method. The serum albumin concentration was detected by the dye binding method. Aspartate transaminase and alanine transaminase activities were analyzed by using the kinetic method recommended by International Federation of Clinical Chemists. Urea nitrogen and creatinine concentrations were measured by using the trinitrophenol method.

Total cholesterol and triglyceride concentrations were measured by using the peroxidase-antiperoxidase method. HDL-cholesterol and LDL-cholesterol concentrations were measured by using the clearance method. Apo A-I and apo B concentrations were measured by immunoturbidimetry. The glucose concentration was analyzed by using the glucose oxidase method.

The plasma CETP and LCAT masses were determined by enzyme-linked immunosorbent assay (Daiichi Pure Chemicals Co, Tokyo, Japan) according to the manufacturer's instructions. Plasma CETP activity was assayed by using fluorometric assay kit (BioVision Inc, Mountain View, CA) with the Multimode Microplate Reader (Infinite F200; TECAN, Männedorf, Switzerland). The fluorometric method detects total activities independent of serum lipoproteins. The CETP activity assay is based on the fluorescence of the self-quenched donor molecule being absorbed by the acceptor molecule (17). CETP activity in the samples can separate the donor from the acceptor so that fluorescence becomes observable and measurable. Plasma LCAT activity was assayed by using a colorimetric method with autsubstrate in the presence of dipalmitoyl lecithin (Sigma-Aldrich, St Louis, MO) and assessed as the decrease in

unesterified cholesterol (18). The unesterified cholesterol content was determined by a combined enzymatic method with the use of cholesterol oxidase and peroxidase (Sigma-Aldrich, St Louis, MO for both).

The capacity of serum to promote cholesterol efflux from J774 mouse macrophage cells in culture was assayed by using a previously validated method (19–21). J774 cells were plated in 24-well plates at 3×10^5 cells/well and incubated for 24 h. The cellular cholesterol was then labeled by exposing the cells for a further 24 h to 1 μCi [^3H]cholesterol/mL (Perkin Elmer, Boston, MA). To allow for equilibration of the label in cellular cholesterol, the monolayers were incubated for an additional 12 h in fetal bovine serum-free culture medium. To ensure that all of the radiolabeled cholesterol was present as free cholesterol, the acyl coenzyme A-cholesterol acyltransferase inhibitor S58-035 compound (2 $\mu\text{g/mL}$, Sigma-Aldrich, St Louis, MO) was also added to the medium during the labeling period and at all subsequent stages of the experiment. Serum samples at a 1% dilution in efflux medium or the cholesterol acceptor-free medium as the control were incubated with the labeled cells for 4 h at 37°C. Aliquots of medium and cell monolayers were then harvested. Radioactivity levels in the lysates and media were determined in a liquid scintillation counter (Packard, Ramsey, MN). Cholesterol efflux was calculated as cpm in the medium as a percentage of the total cpm (medium + cellular cpm). The cholesterol efflux to serum was calculated as the total cholesterol efflux in the presence of serum minus the basal cholesterol efflux measured in the absence of serum (control).

Human HepG2 cells were seeded in 6-well plates, $\approx 3.5 \times 10^5$ cells/well, in RPMI 1640 supplemented with 10% fetal bovine serum. After 24 h of incubation, cells were switched into the serum free medium, incubated with different doses of Cy-3-G (provided by Polyphenols AS) for 24 h. The media were harvested for the mass and activity of CETP assays. Before CETP activity was determined, the samples were brought to 0.05% NaN_3 , 0.5 IU/mL aprotinin (Merck, Germany), and 1 mmol EDTA/L (pH 7.4) and centrifuged to remove cell debris. To investigate whether Cy-3-G has the potential ability of autofluorescence, the fluorescence intensity of HepG2 culture media was measured under the same conditions as CETP activity measurement without using the CETP activity assay kit. No autofluorescence of Cy-3-G were observed. All determinations were detected in triplicate wells for 3 experiments. All values

were expressed relative to the untreated control, set as 1. For all laboratory methods, intra- and interassay CVs ranged from 1.6% to 4.8% and from 1.9% to 5.6%, respectively.

Statistical analysis

All statistical analyses were performed by using SPSS for Windows software (version 17.0; SPSS Inc, Chicago, IL). Normal distributions were tested with the Kolmogorov-Smirnov test. Variables deviated from normality and were therefore logarithmically transformed for statistical analyses. Variables that followed normal distribution were expressed as means \pm SDs or means and 95% CIs. Transformed data were presented as geometric means and upper and lower quartiles. The percentage change was calculated as follows: (value at 12 wk – value at baseline)/value at baseline \times 100. Statistical significance was set at $P < 0.05$.

Differences in these variables between the 2 treatment groups at baseline were evaluated by using Student’s *t* test for independent samples. One-factor analysis of covariance with the 12-wk value as the dependent variable and with the baseline value as the covariate was used to compare the difference in the effects of the anthocyanins and placebo on the blood markers. Repeated-measures analysis of covariance with the 12-wk value as dependent variable, with time as the repeating factor, and the baseline value as covariate was used to compare the difference of the effects of the treatment on the anthropometric characteristics. Differences in the mass and activity of CETP in HepG2 cell-conditioned media between different treatment doses of Cy-3-G were evaluated by using one-factor analysis of variance. Then, the differences in the mass and activity of CETP in HepG2 cells conditioned media between 2 groups were analyzed by Tukey tests. Spearman’s rank correlation coefficients (r_s) were calculated to evaluate relations between the changes in LDL cholesterol, HDL cholesterol, the mass and activity of CETP, and the cholesterol efflux to serum.

RESULTS

Anthropometric characteristics and dietary intake of the dyslipidemic patients

No differences were observed in age (55.1 ± 5.4 and 55.3 ± 5.0 y for the placebo and anthocyanin groups, respectively) or in

TABLE 1
Anthropometric characteristics of the dyslipidemic patients at baseline and during the 12-wk intervention by group¹

	Placebo (n = 60)				Anthocyanin (n = 60)				P ²
	Baseline	4 wk	8 wk	12 wk	Baseline	4 wk	8 wk	12 wk	
Weight (kg)	66.1 \pm 10.1	66.5 \pm 10.1	66.0 \pm 10.0	66.4 \pm 10.3	63.7 \pm 9.7	65.0 \pm 9.7	64.1 \pm 9.7	63.8 \pm 9.5	0.327
BMI (kg/m ²)	26.7 \pm 4.0	27.4 \pm 4.2	27.5 \pm 4.1	26.8 \pm 3.9	25.5 \pm 3.1	26.2 \pm 3.1	25.8 \pm 3.0	25.5 \pm 2.9	0.063
Waist circumference (cm)	89.7 \pm 9.6	89.9 \pm 10.2	89.8 \pm 10.6	89.5 \pm 10.8	87.1 \pm 7.1	87.1 \pm 7.1	86.8 \pm 6.4	86.4 \pm 7.2	0.159
Hip circumference (cm)	98.9 \pm 6.4	99.4 \pm 6.6	98.8 \pm 6.8	98.5 \pm 6.2	97.5 \pm 6.7	97.8 \pm 6.0	97.1 \pm 6.0	97.5 \pm 6.0	0.306
Waist-to-hip ratio	0.91 \pm 0.06	0.88 \pm 0.06	0.91 \pm 0.06	0.91 \pm 0.08	0.90 \pm 0.06	0.89 \pm 0.06	0.89 \pm 0.05	0.88 \pm 0.06	0.598
Systolic blood pressure (mm Hg)	129.1 \pm 19.0	124.2 \pm 17.0	123.7 \pm 16.5	124.8 \pm 17.0	126.5 \pm 17.8	124.4 \pm 17.3	121.7 \pm 17.0	125.3 \pm 20.0	0.888
Diastolic blood pressure (mm Hg)	82.4 \pm 10.6	80.5 \pm 9.4	77.7 \pm 10.6	81.6 \pm 10.6	82.7 \pm 10.0	79.0 \pm 9.9	79.8 \pm 11.7	82.7 \pm 11.1	0.343

¹ All values are means \pm SDs. There were no significant differences between the 2 groups at baseline for any variable by the independent-samples *t* test.
² The intervention had no significant effect on anthropometric characteristic by repeated-measures ANCOVA with the baseline value as covariate.

TABLE 2
Mean daily intake of nutrients by the subjects at baseline and at 12 wk by group¹

	Placebo (n = 60)		Anthocyanin (n = 60)		P ²
	Baseline	12 wk	Baseline	12 wk	
Energy (kcal/d)	2168 ± 98	2199 ± 133	2145 ± 95	2189 ± 111	0.726
Protein (g/d)	88.8 ± 5.1	87.0 ± 4.3	89.8 ± 6.0	86.9 ± 4.5	0.648
(% of energy)	17.5 ± 2.2	17.2 ± 3.2	17.5 ± 2.0	17.3 ± 3.0	0.846
Total carbohydrate (g/d)	269 ± 18	270 ± 22	272 ± 19	267 ± 21	0.098
(% of energy)	51.8 ± 1.4	51.4 ± 2.8	51.8 ± 1.4	51.6 ± 2.7	0.809
Total fat (g/d)	85.0 ± 2.9	83.0 ± 4.5	85.3 ± 3.2	82.6 ± 3.9	0.363
(% of energy)	31.9 ± 1.5	31.1 ± 1.6	31.7 ± 1.4	31.2 ± 1.6	0.516
Cholesterol (mg/d)	325 ± 17	326 ± 19	327 ± 21	325 ± 21	0.295
Fiber (g/d)	22.0 ± 22.3	22.3 ± 2.4	22.0 ± 2.1	22.1 ± 2.1	0.669

¹ All values are means ± SDs. There were no significant differences between the 2 groups at baseline for any variable by the independent-samples *t* test.

² The intervention had no significant effect on mean daily intake of nutrients by one-factor ANCOVA with the baseline value as covariance.

anthropometric markers (**Table 1**) between the 2 study groups. The distribution of age, sex, and blood lipid profile was uniform between the 2 groups. Seventeen subjects in the placebo group and 18 subjects in the anthocyanin group had high blood pressure. One patient had type 2 diabetes in each of the 2 groups. There were also no significant differences in daily mean energy and nutrient intakes (**Table 2**), or dietary habits (data not shown) between the 2 groups. No subjects reported any adverse events resulting from the consumption of either the anthocyanin or placebo capsules throughout the whole trial period.

Compliance

All subjects randomly assigned to the 2 intervention groups completed the study. According to the count of the recalled capsules at every visit, compliance was very good. The rates of capsule intake were 99.2% and 99.4% in the placebo and anthocyanin groups, respectively.

Effects of the consumption of anthocyanin on hematologic measures and liver enzyme markers

Anthocyanin consumption had no significant effects on red and white blood cell counts; hemoglobin, total protein, albumin, urea nitrogen, and creatinine concentrations; or aspartate and alanine transaminase activities (**Table 3**).

Effects of anthocyanin consumption on the fasting serum lipid profile and glucose concentrations

Serum lipid, lipoprotein, apolipoprotein, and glucose concentrations at baseline and at the 12-wk time points in this trial are summarized in **Table 4**. The baseline concentrations of these lipids and glucose did not differ significantly between the 2 study groups. The serum HDL-cholesterol concentration increased significantly more in the anthocyanin group than in the placebo group [13.7% (95% CI: 10.4%, 16.9%) and 2.8% (95% CI: -1.6%, 7.2%), respectively; *P* < 0.001] after intervention.

TABLE 3
Blood chemistry results, hematologic measures, and liver enzyme values for the dyslipidemic patients at baseline and at 12 wk by group

	Placebo (n = 60)		Anthocyanin (n = 60)		P ¹
	Baseline	12 wk	Baseline	12 wk	
Red blood cell (×10 ¹² /L)	4.39 ± 0.42 ²	4.49 ± 0.37	4.27 ± 0.40	4.39 ± 0.31	0.657
White blood cell (×10 ⁹ /L)	5.59 ± 0.96	5.75 ± 1.25	5.31 ± 0.89	5.48 ± 0.94	0.860
Hemoglobin (g/L)	130.7 (123.0, 137.0) ³	137.2 (130.3, 143.0)	129.1 (122.0, 137.8)	137.1 (129.3, 145.0)	0.299
Total protein (g/L)	75.7 ± 2.5	74.9 ± 3.8	74.9 ± 3.3	74.1 ± 3.1	0.115
Albumin (g/L)	43.7 ± 2.1	43.7 ± 2.0	44.0 ± 2.0	43.9 ± 2.0	0.819
Urea nitrogen (mmol/L)	5.18 ± 1.03	4.90 ± 0.85	5.41 ± 0.86	5.15 ± 0.84	0.347
Creatinine (μmol/L)	74.9 (68.8, 79.6)	72.8 (67.0, 80.6)	74.8 (66.3, 84.7)	74.6 (66.8, 83.1)	0.095
Alanine transaminase (U/L)	19.4 (15.0, 26.0)	18.8 (15.0, 26.0)	17.5 (13.0, 23.0)	17.1 (13.0, 22.0)	0.724
Aspartate transaminase (U/L)	20.4 (16.0, 25.0)	19.8 (16.0, 25.0)	20.3 (16.3, 25.0)	18.7 (16.0, 21.8)	0.167

¹ The intervention had no significant effect on any variables by one-factor ANCOVA with the baseline value as covariate. There were no significant differences between the 2 groups at baseline for any variable by the independent-samples *t* test.

² Mean ± SD (all such values).

³ Geometric mean; upper and lower quartiles in parentheses (all such values).

TABLE 4

Blood lipid profile and lipid metabolic enzymes at baseline and after consumption of anthocyanins or placebo for 12 wk¹

	Placebo (n = 60)			Anthocyanins (n = 60)			P ³
	Baseline	12 wk	Change ²	Baseline	12 wk	Change	
			%			%	
Total cholesterol (mg/dL)	224.3 ± 36.4 ⁴	222.4 ± 39.8	-0.4 (-3.6, 2.7) ⁵	226.2 ± 35.5	220.5 ± 34.0	-2.1 (-4.5, 0.3)	0.435
Triacylglycerol (mg/dL)	205.8 ± 83.0	200.4 ± 91.2	2.0 (-7.0, 11.1)	197.9 ± 87.0	189.5 ± 85.6	-0.4 (-8.2, 7.4)	0.576
LDL cholesterol (mg/dL)	158.5 ± 37.8	157.3 ± 36.6	0.6 (-4.1, 5.2)	159.2 ± 34.4	139.9 ± 35.5	-13.6 (-17.1, -10.1)	<0.001
HDL cholesterol (mg/dL)	46.1 ± 9.6	46.9 ± 10.1	2.8 (-1.6, 7.2)	45.9 ± 8.5	51.2 ± 8.7	13.7 (10.4, 16.9)	<0.001
Apolipoprotein A-I (mg/dL)	124.9 ± 19.5	126.3 ± 18.7	1.7 (-0.7, 4.0)	125.7 ± 17.4	126.5 ± 15.6	1.4 (-1.5, 4.3)	0.842
Apolipoprotein B (mg/dL)	111.9 ± 24.0	114.1 ± 23.8	3.5 (-1.0, 8.0)	110.8 ± 21.8	112.5 ± 19.5	2.8 (-0.6, 6.3)	0.773
Glucose (mmol/L)	5.68 ± 1.68	5.79 ± 2.45	1.8 (-3.2, 6.8)	5.64 ± 1.42	5.59 ± 1.58	-0.8 (-3.3, 1.6)	0.458
LCAT mass (μg/mL)	8.31 ± 2.42	8.65 ± 2.36	9.4 (-0.1, 18.9)	8.66 ± 2.29	8.94 ± 2.29	8.8 (-2.4, 20.0)	0.773
LCAT activity (nmol · L ⁻¹ · h ⁻¹)	79.5 ± 7.3	79.4 ± 6.6	0.2 (-1.8, 2.1)	80.5 ± 9.1	80.6 ± 7.6	0.6 (-1.2, 2.5)	0.440
CETP mass (μg/mL)	2.52 ± 0.60	2.52 ± 0.58	3.5 (-3.6, 10.5)	2.55 ± 0.67	2.23 ± 0.50	-10.4 (-14.1, -6.7)	<0.001
CETP activity (nmol · L ⁻¹ · h ⁻¹)	96.9 ± 9.4	95.3 ± 10.0	-1.1 (-4.0, 1.6)	96.7 ± 9.9	90.3 ± 9.4	-6.3 (-8.0, -4.6)	0.001

¹ LCAT, lecithin cholesterol acyl transferase; CETP, cholesteryl ester transfer protein. There were no significant differences between the 2 groups at baseline for any variable by the independent-samples *t* test.

² The percentage change was calculated as (value at 12 wk - value at baseline)/value at baseline × 100.

³ The effects of the intervention on these variables were tested by one-factor ANCOVA with the baseline value as covariate.

⁴ Mean ± SD (all such values).

⁵ Mean; 95% CI in parentheses (all such values).

Serum LDL cholesterol decreased by 13.6% (95% CI: 10.1%, 17.1%) in the anthocyanin group and increased by 0.6% (95% CI: -4.1%, 5.2%) in the placebo group at the end of the intervention. The change in LDL cholesterol was significantly different between the 2 groups (*P* < 0.001). However, no significant differences in total cholesterol, triglyceride, apo A-I, apo B, or glucose concentrations were observed between the 2 groups.

Effects of anthocyanin on plasma CETP and LCAT

Plasma CETP and LCAT masses and activities at baseline and at 12 wk are presented in Table 4. The CETP mass decreased by 10.4% (95% CI: 6.7%, 14.1%) in the anthocyanin group and increased by 3.5% (95% CI: -3.6%, 10.5%) in the placebo group after intervention. The change in CETP mass was significantly different between the 2 groups (*P* < 0.001). CETP activity decreased significantly more in the anthocyanin group than in the placebo group [6.3% (95% CI: 4.6%, 8.0%) and 1.1% (95% CI: -1.6%, 4.0%), respectively; *P* = 0.001]. There were no significant differences in the mass and activity of LCAT between the 2 groups at the end of the trial.

Correlation between changes in serum HDL- or LDL-cholesterol concentrations and the mass and activity of plasma CETP

The correlation of the changes of these variables at 12 wk from the baseline in the 2 intervention groups are presented in **Figure 1**. In the anthocyanin group, the change in HDL-cholesterol concentration was found to be negatively correlated with the change in CETP activity (*r_s* = -0.330, *P* = 0.010), whereas the change in LDL-cholesterol concentration was positively correlated with the change in CETP mass (*r_s* = 0.354, *P* = 0.005). Nonetheless, no correlation between these changes in the placebo group was observed.

Effects of anthocyanin consumption on cellular cholesterol efflux into serum

At baseline, there were no differences in cholesterol efflux capacity from J774 cells to the serum between the anthocyanin and placebo groups (13.2 ± 3.1% and 12.7 ± 2.8%, respectively). After intervention, the cholesterol efflux capacity increased significantly more in the anthocyanin group than in the placebo group [20.0% (95% CI: 13.4, 26.6%) and 0.2% (95% CI: -7.3, 7.6%), respectively; *P* = 0.007].

Correlation between changes in cellular cholesterol efflux and serum HDL cholesterol

In the anthocyanin group, the change in cholesterol efflux capacity from baseline to 12 wk correlated positively with the change in serum HDL-cholesterol concentration (*r_s* = 0.485, *P* = 0.008). However, no correlation between the change in cholesterol efflux capacity and the change in HDL cholesterol in the placebo group was observed. There was no correlation between the change in cholesterol efflux capacity and the change in other lipids in each study group.

Effects of Cy-3-G on the mass and activity of CETP in HepG2 cells

As presented in **Figure 2**, Cy-3-G suppressed CETP activity in a dose-dependent manner in the HepG2 cell cultures. However, CETP mass did not change significantly after Cy-3-G treatment (data not shown).

DISCUSSION

Our previous study showed that an anthocyanin-rich extract from black rice decreased serum triglyceride, total cholesterol, and non-HDL cholesterol in the apo E-deficient mouse model (14). The present study, however, showed for the first time that in dyslipidemic patients anthocyanins derived from berries produce

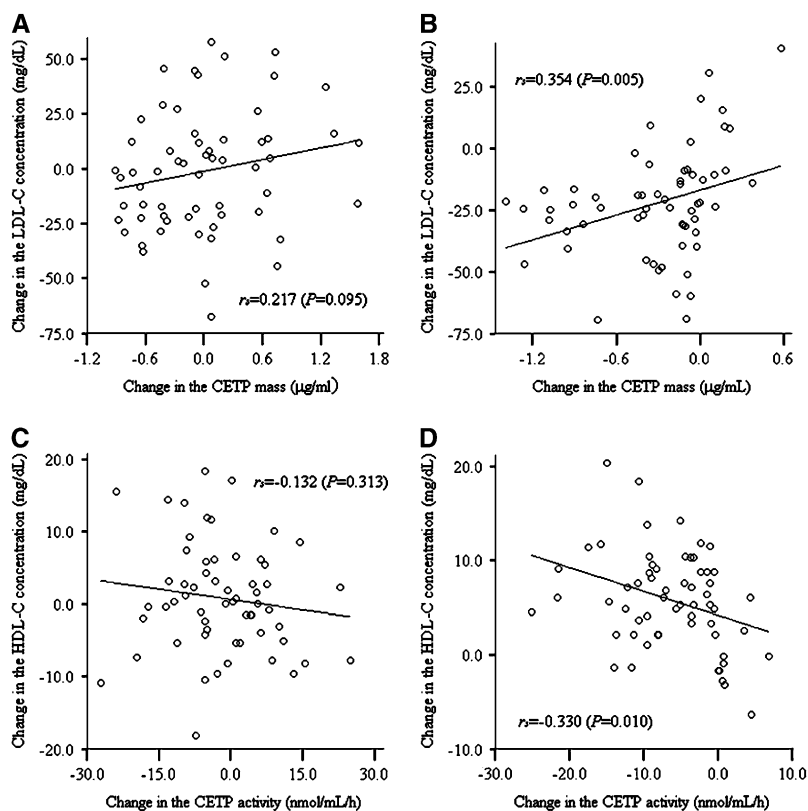


FIGURE 1. The relation between the change in serum LDL cholesterol (LDL-C) and the change in plasma cholesteryl ester transfer protein (CETP) mass in the placebo group (A) and in the anthocyanin group (B) and between the change in serum HDL cholesterol (HDL-C) and the change in plasma CETP activity in the placebo group (C) and in the anthocyanin group (D). The values deviated from normality and were analyzed by Spearman's rank correlation coefficients (r_s).

favorable effects on lipoprotein concentrations. Recently, other groups have also found that plant food rich in phytochemicals produced beneficial effects in improving the lipid profile. Hansen et al (22) found that moderate red wine consumption for 4 wk was associated with a relative increase of 11–16% in HDL cholesterol in healthy human subjects. In another report, berry consumption increased serum HDL concentrations by 5.2% (23). Zern et al (24) showed that lyophilized grape powder modestly lowered plasma LDL cholesterol and apo B and E in both pre- and postmenopausal women. These beneficial effects were considered to be attributed to anthocyanins, flavans, quercetin, myricetin, kaempferol, and resveratrol or other phytochemicals (22–24). The present study showed that pure anthocyanins resulted in a dual beneficial effect in lowering LDL-cholesterol and raising HDL-cholesterol concentrations. This prompted us to suggest that pure anthocyanins derived from berries may result in a greater reduction in CVD risk factors than wine and grape powder supplementation, which only decreases serum LDL-cholesterol or increases serum HDL cholesterol.

The observed changes in HDL and LDL are noteworthy because the anthocyanin extract is derived from berry food stuff. Changes in these risk factors, which are of the same magnitude as those observed in this study, are clinically relevant. To put this into perspective, it has been reported that the cardiovascular disease event rate is reduced by nearly 1% for each 1% reduction in LDL and by $\geq 1\%$ for each 1% increase in HDL (25). Therefore, the 13.6% decrease in LDL and 13.7% increase in HDL observed in the present study would result in a nearly

27.3% reduction in coronary heart disease risk, which is meaningful and greatly promising.

In recent years, increasing HDL-cholesterol concentrations has been considered an important strategy for preventing and treating CVD (26, 27). As mentioned earlier, the primary function of HDL is the promotion of RCT. Cholesterol efflux is the first and the most critical step of RCT. Because HDL and apo A-I are major receptors of cholesterol in the cholesterol efflux pathway, elevated HDL-cholesterol concentrations may lead to the facilitation of this process. In this study, the serum collected from the anthocyanins subject group showed enhanced efficiency of cholesterol efflux in the J774 macrophages. Although this ex vivo efflux assay for measuring cholesterol efflux to serum from cells may not represent efflux from cells under physiologic conditions, it is a useful method for investigating the capacity of cholesterol efflux in humans at the present time. Furthermore, we found that enhanced cholesterol efflux to serum was positively correlated with an increase in the HDL-cholesterol concentration in dyslipidemic patients. Because plasma concentrations of HDL do not predict RCT in mouse models (3), further investigation is required to assess whether increased HDL by anthocyanins contributes to the promotion of RCT.

Our major findings that anthocyanins significantly increased HDL-cholesterol and decreased LDL-cholesterol concentrations, with no effect on total cholesterol, prompted us to assess the effect of anthocyanins on the mass and activity of CETP. CETP is a plasma protein that mediates the removal of cholesteryl esters from HDL in exchange for a triglyceride molecule derived

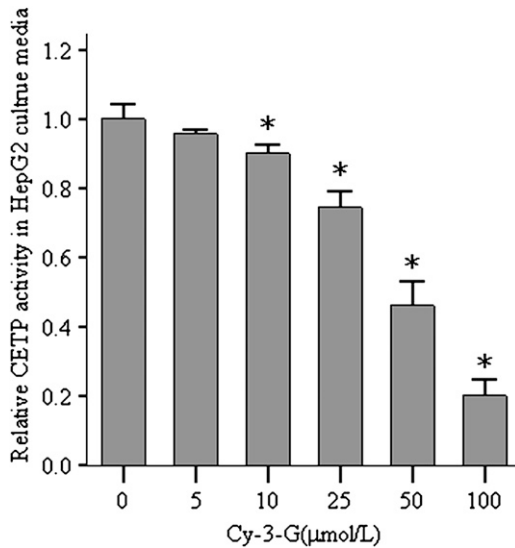


FIGURE 2. Effects of cyanidin 3-*O*- β -glucosides (Cy-3-G) on cholesteryl ester transfer protein (CETP) activity in HepG2 cell-conditioned media. Cy-3-G dose-dependently suppressed CETP activity in HepG2 culture media. All values were expressed relative to the untreated control, set as 1. Differences in CETP activity between different doses of Cy-3-G were evaluated by using one-factor ANOVA. Then, the differences in CETP activity between 2 groups were analyzed by Tukey tests. Data points represent the mean \pm SD from the triplicate wells for 3 experiments. *Significantly different from control, $P < 0.001$.

primarily from either LDL, VLDL, or chylomicrons. Several studies have indicated that CETP inhibition is a possible mechanism for the elevation of HDL cholesterol and decrease of LDL cholesterol (28). To determine the relation between CETP and the risk of CVD, torcetrapib (a CETP inhibitor) is primarily used in experiments. Brousseau et al (29) found that, in patients with low HDL-cholesterol concentrations, the use of torcetrapib markedly increased HDL-cholesterol and also decreased LDL-cholesterol concentrations. In addition, torcetrapib increases blood pressure and aldosterone concentrations through certain molecule-specific effects that are unrelated to CETP inhibition, which confounds the interpretation of the increased risk of mortality and morbidity in patients at high CVD risk and leaves hope for the development of a “clean” CETP inhibitor (30). In the present study, anthocyanins derived from berries suppressed plasma CETP without influencing blood pressure and other biomarkers, except lipoproteins. Thus, anthocyanins may be considered a category of clean CETP inhibitors.

It should be pointed out that the effects of anthocyanins on lipoproteins and CETP presented in this human study agree with the results of a recent animal study (31). Chen et al (31) reported that apple polyphenols decreased plasma CETP activity and non-HDL-cholesterol concentrations and increased plasma HDL-cholesterol concentration in Syrian hamsters. These results together with our findings in humans indicate that polyphenols from plant foods, including anthocyanins, may exert a specific role on CETP. Moreover, it was of interest that decreased plasma CETP activity negatively correlated with an increase in the HDL-cholesterol concentration and decreased CETP mass positively correlated with a decrease in the LDL-cholesterol concentration in the present study. Hepatocytes are one of the principal sources of CETP production in primates (32). We found that Cy-3-G treatment significantly decreased CETP activity in the human

HepG2 culture media in a dose-dependent manner. These observations collectively suggest that anthocyanin supplementation may ameliorate lipoproteins by decreasing serum LDL-cholesterol and increasing HDL-cholesterol concentrations in part via the inhibition of CETP target.

The present study was limited in that we did not detect anthocyanins or their metabolites in blood or urine samples from our patients. The subjects were asked to consume the capsules 0.5 h after both breakfast and supper, and urine and blood samples were collected in the morning after an overnight fast at the 12-wk time point. Thus, the participants had taken the last capsules ≥ 10 h before sample collection. A series of earlier studies have reported that the maximum time of anthocyanins in plasma is 15–60 min, and excretion is complete within 6–8 h (33). This is likely to explain why we were unable to detect these compounds in the present study.

In conclusion, our results suggest that anthocyanin supplementation in dyslipidemic patients has a beneficial effect on the lipoprotein profile, which includes a decrease in LDL-cholesterol and an increase in HDL-cholesterol concentrations. These beneficial effects may be partially mediated via the inhibition of CETP.

The authors' responsibilities were as follows—YQ, MX, JM, JL, HYM, and LC: carried out the human phase of the experiments; YQ, JL, HYM, and LC: carried out experimental determinations and cell culture; YQ and YTH: conducted the statistical evaluation of the data; YQ: contributed to summarizing and calculating the raw data and revising the manuscript; YQ, YTH, MX, and WHL: contributed to the experimental design and the manuscript; and WHL: managed the overall project and wrote the manuscript. None of the authors had a personal or financial conflict of interest.

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