Clinical Chemistry 57:11
000–000 (2011)

Purified Anthocyanin Supplementation Improves Endothelial Function via NO-cGMP Activation in Hypercholesterolemic Individuals

Yanna Zhu,[1,2] Min Xia,[1,2] Yan Yang,[1,2] Fengqiong Liu,[1,2] Zhongxia Li,[1,2] Yuantao Hao,[1,3] Mantian Mi,[4] Tianru Jin,[1,2,5] and Wenhua Ling[1,2*]

BACKGROUND: Anthocyanins have been shown to improve endothelial function in animal models. However, whether these compounds have similar beneficial effects in humans is largely unknown.

METHODS: In a short-term crossover study, 12 hypercholesterolemic individuals were given oral anthocyanins (320 mg) isolated from berries or placebo. Brachial artery flow-mediated dilation (FMD) was assessed before and after the intervention. In a long-term intervention trial (12 weeks), 150 hypercholesterolemic individuals were given anthocyanins (320 mg/day, n = 75) or placebo (n = 75), after which we measured FMD, plasma cGMP, and other serum biomarkers. Another short-term intervention was conducted in the presence of NO-cGMP inhibitors in 6 people and in a rat aortic ring model (n = 8).

RESULTS: Significant increases of FMD from 8.3% (0.6%) at baseline to 11.0% (0.8%) at 1 h and 10.1% (0.9%) at 2 h were observed after short-term anthocyanin consumption, concomitantly with increases of plasma anthocyanin concentrations ($P < 0.05$). In the study participants who received long-term anthocyanin intervention, compared with the control group, we observed significant increases in the FMD (28.4% vs 2.2%), cGMP (12.6% vs −1.2%), and HDL-cholesterol concentrations, but decreases in the serum soluble vascular adhesion molecule-1 and LDL cholesterol concentrations ($P < 0.05$). The changes in the cGMP and HDL cholesterol concentrations positively correlated with FMD in the anthocyanin group ($P < 0.05$). In the presence of NO-cGMP inhibitors, the effects of anthocyanin on endothelial function were abolished in human participants and in a rat aortic ring model.

CONCLUSIONS: Anthocyanin supplementation improves endothelium-dependent vasodilation in hypercholesterolemic individuals. This effect involves activation of the NO-cGMP signaling pathway and leads to improvements in the serum lipid profile and decreased inflammation.

Blood vessel endothelial cells not only function as a barrier but also play an important role in the maintenance of vascular homeostasis *(1)*. Endothelial dysfunction is characterized by diminished production or availability of NO and by alterations in other important vasoactive molecules, such as endothelin-1 and prostacyclin, resulting in impaired endothelium-dependent vasodilation *(2)*. Many studies have demonstrated that endothelium dysfunction is an early marker of the development of atherosclerosis, which contributes to various cardiovascular disorders *(3, 4)*. Many cardiovascular risk factors are associated with the onset of endothelial dysfunction, including hypertension, hypercholesterolemia, smoking, increased age, type 2 diabetes, chronic inflammation, and oxidative stress *(5–7)*. The reduced production or availability of endothelium-derived NO is well correlated with endothelial dysfunction, which can be assessed by measuring flow-mediated dilation (FMD)[6] of the brachial artery *(8, 9)*. In addition, certain inflammatory molecules such as soluble vascular cell adhesion molecule-1 (sVCAM-1) *(10)* are among the important biomarkers of endothelial dysfunction.

A large number of epidemiological and medical anthropological investigations have shown that the

[1] Guangdong Provincial Key Laboratory of Food, Nutrition, and Health; [2] Department of Nutrition, and [3] Department of Statistics, School of Public Health, SunYat-Sen University (Northern Campus), Guangzhou, China; [4] Department of Nutrition, Third Military Medical University, Chongqing, China; [5] Department of Medicine, Physiology, and Laboratory Medicine and Pathobiology, University of Toronto, Ontario, Canada.
* Address correspondence to this author at: Department of Nutrition, School of Public Health, Sun Yat-Sen University (Northern Campus), 74 Zhongshan Rd. 2, Guangzhou, Guangdong Province, 510080, Peoples Republic of China. Fax +86-20-87330446; e-mail lingwh@mail.sysu.edu.cn.

[6] Nonstandard abbreviations: FMD, flow-mediated dilation; sVCAM-1, soluble vascular adhesion molecule-1; NOS, NO synthase; GTND, glyceryltrinitrate-induced dilatation; L-NMMA, N^G-monomethyl-L-arginine acetate; GC, guanylyl cyclase; ODQ, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one; HOMA-IR, homeostasis model assessment of insulin resistance; C, cholesterol; Dp-3g, delphinidin-3-O-β-glucosides; Cy-3g, cyanidin-3-O-β-glucosides.

Received April 20, 2011; accepted July 25, 2011.
Previously published online at DOI: 10.1373/clinchem.2011.167361

consumption of polyphenol-rich plant foods is negatively associated with the incidence of cardiovascular diseases *(11–13)*. There are thousands of different plant polyphenols, including flavanols, flavones, catechins, isoflavones, and anthocyanins, which are abundantly present in various fruits, vegetables, and beverages *(14–16)*. Investigations from our group and others have shown that anthocyanin-rich food and beverages and purified anthocyanins improve lipid profiles and inhibit the formation and progression of atherosclerosis, possibly through their antiinflammatory and antioxidative properties *(17–19)*. Theoretically, these beneficial effects should lead to the alleviation of endothelial dysfunction. Indeed, several recent studies have shown that anthocyanin-rich foods can activate endothelial NO synthase (NOS) and improve endothelial function in vitro and in animals *(20, 21)*. However, whether the direct consumption of purified anthocyanins can have a beneficial effect on endothelial function in people has not been previously assessed.

The focus of our current study was to evaluate the effects of anthocyanins purified from bilberries and blackcurrants on endothelial function in individuals with hypercholesterolemia and to explore the underlying mechanism of any observed effects.

Participants and Methods

MATERIALS AND METHODS

Anthocyanin and placebo capsules were obtained from Polyphenols AS. The total anthocyanin content was 80 mg per capsule and consisted of 17 different natural anthocyanins purified from the bilberry (*Vaccinium myrtillus*) and blackcurrant (*Ribes nigrum*) *(18)*. More detailed information of the content of treatment capsules and other materials is provided in the Supplemental Data that accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue11.

PARTICIPANTS

A total of 150 hypercholesterolemic individuals age 40–65 years were recruited into this clinical trial between November 2008 and December 2010 from physical examination centers in 3 hospitals in Guangzhou, Guangdong, China. Women and men represented 58% and 42% of this cohort, respectively. Participants selected for inclusion in the study had a fasting total cholesterol concentration between 200 and 310 mg/dL (5.2 and 8.0 mmol/L). Exclusion criteria included a history of cardiovascular disease, diabetes mellitus, hypertension, thyroid disorders, smoking, or the use of any drugs that could influence the measurement of lipid parameters, inflammatory markers, or vasomotion. This study was approved by the ethics committee of Sun Yat-Sen University, and signed informed consent was obtained from all participants. All protocols adhered to institutional guidelines as well as the Helsinki Declaration.

STUDY DESIGN

For the short-term study, 12 individuals with hypercholesterolemia after overnight fasting were randomized in a crossover design with a 7-day washout, and consumed either anthocyanins (320 mg) or placebo capsules. During a 4-h (8 AM to 12 PM) intervention, the participants consumed no food but drank water twice. Blood samples were obtained before and at 1, 2, and 4 h after the intake of the capsules to determine the plasma concentrations of anthocyanin and cGMP. FMD measurements were made each time blood samples were obtained.

For the long-term study, eligible participants were randomized in a double-blind, placebo-controlled, parallel, 12-week trial and assigned to either the anthocyanin group (n = 75; 31 males and 44 females) or the placebo group (n = 75; 32 males and 43 females). During the trial period, the participants were instructed to consume 2 anthocyanin capsules or placebo capsules twice daily (30 min after breakfast and supper). They were also asked to maintain their habitual diet and lifestyle. The anthocyanin capsules (80 mg anthocyanins per capsule, 4 per day) provided a total daily intake of 320 mg anthocyanins. Each of the participants attended a follow-up session every 4 weeks. During these visits, the adherence to the protocol was assessed by recalling the empty packages and obtaining related information. Meanwhile, the capsules were dispensed, and the body weight, blood pressure, and circumferences of the waist and hip of each participant were measured. In addition, overnight fasting blood samples were taken in the morning at baseline and at week 12 to measure the lipid and glucose profiles as well as the concentrations of sVCAM-1 and cGMP. Brachial artery endothelial function was also evaluated in the morning by use of an ultrasound based method. Notably, 12 hours before and during the examination periods of endothelial function, the participants did not eat or drink anything but water. Moreover, a 3-day 24-h dietary recall was conducted at baseline and at week 12 to ascertain whether the nutrient and energy intake of the participants changed during the study.

OUTCOME MEASURES

Assessment of endothelium-dependent brachial artery FMD. Endothelial function as determined by the FMD, endothelium-independent glyceryltrinitrate-induced dilation (GTND), and blood flow was measured noninvasively in the right brachial artery by use of a high-

frequency ultrasound scanning machine (Sonos 4500; Phillips Medical Systems) and a high-resolution (7.5 MHz) linear array transducer in accordance with published guidelines *(22)*. More detailed information regarding this method is available in the online Supplemental Data.

Plasma anthocyanins. The plasma anthocyanin concentrations were determined by HPLC in accordance with the method of Matsumoto et al. *(23)* with slight modifications. For more detailed information regarding their detection see the description in the online Supplemental Data.

Effect of NOS inhibition on anthocyanin-induced vascular dilation. Twelve hours before and during the experimental periods, the participants did not consume any foods and drank water only. On day 1, 6 individuals were intravenously injected with normal saline solution at 8 AM, and then administered anthocyanin (320 mg) capsules 30 min later. On day 3, the same individuals were intravenously infused with N^G-monomethyl-L-arginine acetate (L-NMMA), an NOS inhibitor, at 8 AM, and again consumed anthocyanin capsules 30 min later. The FMD, blood flow, blood pressure, and heart rates were measured at 8 AM and at 9:30 AM on day 1 and 3. The infusion rate of L-NMMA was set at 1 $mg \cdot kg^{-1} \cdot min^{-1}$ for the first 3 min followed by a 0.2 $mg \cdot kg^{-1} \cdot min^{-1}$ maintenance dose.

Ex vivo aortic ring experiments. Ex vivo aortic ring assays were performed in accordance with the previously described methods of Nakamura et al. *(24)*. Briefly, male Sprague–Dawley rats (180–220 g) were killed and the thoracic aortas were then isolated and cut into rings. The rings were then mounted in an organ bath filled with Krebs solution. After equilibration for 90 min, purified anthocyanins, L-NMMA, or a guanylate cyclase (GC) inhibitor, 1H- [1, 2, 4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), were added to the bath and their effects on vasorelaxation were observed. Results from the contractile experiments were expressed as a percentage of the level of precontraction. More detailed information regarding the experiment is provided in the online Supplemental Data.

Other parameters. Assessments of the serum sVCAM-1, lipid, glucose, and plasma cGMP concentrations are described in the online Supplemental Data.

STATISTICAL ANALYSIS

All statistical analyses were performed by use of SPSS for Windows software (version 17.0, 2008; IBM-SPSS). We used the Kolmogorov–Smirnov test to assess the normality of distributions. Nonnormal variables were logarithmically transformed for statistical analyses. Variables that followed a normal distribution were expressed as means (SD) or means (SE). Transformed data, including the concentrations of triacylglycerols and insulin and a homeostasis model assessment of insulin resistance (HOMA-IR), are presented as geometric means (upper and lower quartiles). The percentage change was calculated as follows: [(value at week 12 − value at baseline)/value at baseline] × 100. The average percentage change was displayed as the mean (95% CI). Statistical significance was set at $P < 0.05$.

We used the unpaired Student *t*-test to evaluate the differences in the variables between the 2 treatment groups at baseline and the vascular relaxant effects of anthocyanin in vitro. The effects of the intervention on anthropometric characteristics, dietary intake, lipid and glucose profile, sVCAM-1, and vascular function were assessed by using repeated-measures ANOVA, with a Bonferroni corrected posthoc *t*-test to assess differences between time points. The plasma anthocyanin concentrations and the effects of an NOS inhibitor on anthocyanin-induced vascular dilation in study participants were evaluated by using paired Student *t*-tests. Pearson correlation coefficients (*r*) were used to determine the association between the changes in the plasma cGMP and HDL-cholesterol (HDL-C) concentrations and the changes in FMD in the 12-week study.

Results

ANTHROPOMETRIC CHARACTERISTICS AND DIETARY INTAKE

As illustrated in Fig. 1, 4 participants were withdrawn because of their intake of lipid-lowering drugs or because they had moved away from the study area. Thus 146 hypercholesterolemic individuals finished this study (n = 73 in the anthocyanin group, 31 men and 42 women; n = 73 in the placebo group, 30 men and 43 women). The distribution of anthropometric characteristics and mean daily nutrient intake was uniform between the 2 groups at baseline and after the 12-week intervention (Table 1). Anthocyanin treatment for 12 weeks, however, decreased systolic blood pressure significantly compared with the baseline value [126.2 (14.9) vs 119.5 (12.5) mmHg; $P < 0.05$]. No adverse effects were reported by any of the participants consuming anthocyanins or placebo throughout the intervention period.

SHORT-TERM EFFECTS OF ANTHOCYANINS ON FMD

In the anthocyanin group, the plasma delphinidin-3-O-β-glucosides (Dp-3g) and cyanidin-3-O-β-glucosides (Cy-3g) concentrations were examined before and within 4 hours of taking the capsule. We found that the concentrations of Dp-3g and Cy-3g were highest 1 h after the capsule was taken [9.3 (1.3) ng/mL and 5.5 (1.2) ng/mL, respectively] (Fig. 2A) and the plasma

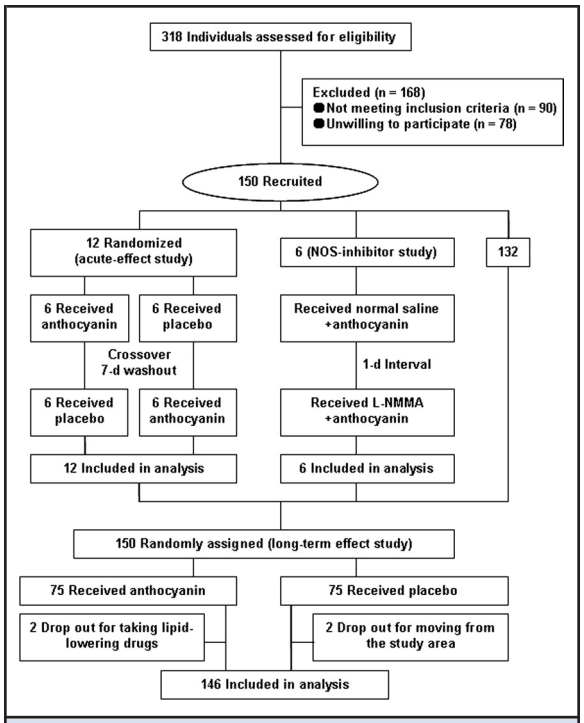

Fig. 1. Flowchart illustrating the procedures used in the clinical trial.

cGMP concentrations had increased as well (Fig. 2B). Interestingly, plasma cGMP concentrations also were highest at 1 h [143.9 (4.6) pmol/mL] after the anthocyanin capsule was taken, concomitant with the increased concentrations of Dp-3g and Cy-3g. Furthermore, anthocyanin ingestion significantly improved FMD from 8.3% (0.6%) at baseline to 11.0% (0.8%) at 1 h and 10.1% (0.9%) at 2 h ($P < 0.05$; Fig. 2C).

LONG-TERM EFFECTS OF ANTHOCYANIN SUPPLEMENTATION ON cGMP CONCENTRATIONS AND ENDOTHELIAL FUNCTION

As shown in Table 2, after the 12-week intervention, no anthocyanins or anthocyanin metabolic products were found in the overnight fasting blood samples in either the anthocyanin or placebo groups. In addition, no significant differences in the cGMP concentrations, brachial diameter, baseline and hyperemic blood flow, FMD, and GTND between the 2 groups at baseline were observed. There were significant increases in the cGMP concentrations [123.4 (21.6) pmol/mL at baseline vs 138.8 (22.5) pmol/mL at week 12; $P = 0.003$], and FMD [8.04% (1.82%) vs 10.91% (2.06%); $P < 0.001$] after the12-week anthocyanin supplementation. There were no significant changes in the cGMP concentrations or the FMD in the placebo group. When mean values of the 2 variables were compared between the anthocyanin and the placebo groups, we observed significant changes in the cGMP concentrations [−1.2% (95% CI, −4.6% to 2.2%) vs 12.6% (95% CI, 8.6%–16.5%), $P = 0.031$], and FMD [28.4% (95% CI, 13.2%–43.6%) vs 2.2% (95% CI, −3.6% to 8.0%), $P = 0.006$]. No significant mean differences in the brachial diameter, baseline and hyperemic blood flow, and GTND were observed between the 2 groups after the 12-week intervention ($P = 0.583$, Table 2).

The increase in cGMP after the 12-week anthocyanin intervention was found to be positively correlated with the change in FMD ($r = 0.428$; $P < 0.001$) (Fig. 3A). No such effect was evident in the placebo group (Fig. 3B).

LONG-TERM EFFECTS OF ANTHOCYANIN SUPPLEMENTATION ON SERUM INFLAMMATORY MOLECULES AND THE LIPID PROFILE

The concentrations of serum inflammatory molecules, lipids, glucose, and insulin at baseline and 12 weeks after the intervention are summarized in Table 2. There were no significant differences in these parameters between the anthocyanin and the control groups at baseline. Anthocyanin treatments for 12 weeks led to appreciable decreases ($P = 0.023$) of serum sVCAM-1 concentrations [542.9 (103.6) ng/mL at baseline vs 481.0 (91.8) ng/mL at 12 weeks], compared with the placebo treatment [544.2 (107.8) ng/mL vs 546.3 (106.9) ng/mL]. In the anthocyanin group, the concentration of serum HDL-C was found to be significantly increased from 47.2 (8.9) mg/dL [1.22 (0.23) mmol/L] at baseline to 53.0 (8.5) mg/dL [1.37 (0.22) mmol/L] at week 12 ($P = 0.010$), accompanied by a significant reduction in the LDL-C concentrations from 130.0 (22.4) mg/dL [3.36 (0.58) mmol/L] to 116.5 (15.9) mg/dL [3.01 (0.41) mmol/L] ($P = 0.015$). Moreover, significant differences were found in the 2 variables between the anthocyanin and the placebo groups after the12-week treatment period ($P < 0.05$ in both cases). The changes in the HDL-C concentrations also positively correlated ($r = 0.309$; $P = 0.008$) with the FMD changes in the anthocyanin group (Fig. 3C). No such correlations were found in the control group (Fig. 3D). The intervention, however, did not cause significant changes in the concentrations of total cholesterol, triglyceride, apolipoprotein AI, apolipoprotein B, glucose, insulin, or HOMA-IR index between the 2 groups (Table 2).

EFFECTS OF NOS INHIBITION ON ANTHOCYANIN-INDUCED VASCULAR DILATION

It is well known that NO plays an important role in endothelium-dependent vasorelaxation *(25)*. We hence investigated the role of NO in mediating anthocyanin-stimulated FMD in a subgroup of our participants (n = 6), who received an intravenous infusion of the NOS inhibitor L-NMMA. We found that an in-

Table 1. Anthropometric characteristics and daily nutrient intake of the participants at baseline and at weeks 8 and 12 of the anthocyanin trial.[a]

	Placebo group (n = 73)			Anthocyanin group (n = 73)			
	Baseline	8 Week	12 Week	Baseline	8 Week	12 Week	P^b
Anthropometric features							
Weight, kg	70.1 (9.8)	70.2 (10.0)	69.7 (9.9)	68.9 (8.8)	68.2 (8.6)	67.8 (8.9)	0.322
BMI, kg/m^2	26.8 (2.0)	27.0 (2.2)	26.7 (2.1)	26.4 (2.1)	26.1 (1.9)	25.9 (2.0)	0.102
Waist circumference, cm	89.6 (7.9)	89.9 (7.4)	89.5 (8.2)	88.6 (6.4)	87.7 (6.2)	87.4 (6.4)	0.174
Hip circumference, cm	100.3 (6.3)	100.5 (6.4)	100.3 (6.5)	100.0 (5.0)	99.5 (4.8)	99.1 (5.2)	0.368
Waist/hip ratio	0.89 (0.06)	0.89 (0.05)	0.89 (0.06)	0.89 (0.05)	0.88 (0.05)	0.88 (0.05)	0.329
Systolic blood pressure, mmHg	124.3 (16.0)	120.1 (15.8)	123.8 (15.0)	126.2 (14.9)	122.8 (15.5)	119.5 (12.5)[c]	0.245
Diastolic blood pressure, mmHg	82.8 (10.5)	81.4 (10.4)	81.2 (9.1)	84.7 (10.7)	83.2 (10.5)	82.8 (9.6)	0.290
Nutrients							
Energy, kcal/day	2163.6 (124.2)	2191.3 (120.4)	2168.2 (116.1)	2185.4 (132.5)	2213.1 (141.6)	2199.2 (123.7)	0.648
Protein							
g/day	84.8 (10.8)	83.1 (9.9)	83.6 (9.4)	85.7 (10.5)	84.1 (9.2)	84.5 (9.8)	0.712
% Energy	18.5 (3.1)	18.3 (2.5)	18.4 (2.7)	18.6 (2.9)	18.4 (2.1)	18.5 (3.1)	0.745
Total fat							
g/day	82.4 (18.2)	80.1 (17.2)	81.2 (16.3)	80.2 (15.8)	83.3 (16.7)	83.7 (17.6)	0.264
% Energy	26.5 (3.5)	26.1 (3.2)	26.3 (3.7)	26.4 (3.2)	27.1 (3.6)	27.4 (4.0)	0.489
Total carbohydrate							
g/day	258.6 (34.2)	260.2 (37.4)	258.8 (40.5)	262.1 (43.8)	260.6 (36.7)	263.2 (42.5)	0.163
% Energy	55.2 (4.7)	55.6 (5.2)	55.3 (4.6)	55.0 (5.3)	54.7 (4.2)	55.3 (4.8)	0.816
Cholesterol, mg/day	339.4 (43.2)	343.0 (39.6)	340.1 (38.4)	341.3 (40.2)	339.4 (41.5)	342.5 (39.3)	0.325
Fiber, g/day	20.5 (4.4)	21.2 (4.1)	20.8 (4.2)	20.6 (5.0)	20.7 (4.9)	20.6 (4.5)	0.642

[a] Data are the means (SD). No significant differences were found for any variable between the 2 groups at baseline via the unpaired Student *t*-test.
[b] The intervention had no significant effects on anthropometric characteristics and the daily intake of nutrients as determined by repeated-measures ANOVA.
[c] $P < 0.05$ vs baseline, assessed by repeated-measures ANOVA with a Bonferroni corrected *t*-test.

fusion of anthocyanin improved the FMD compared with the infusion of saline [11.0 (3.4)% vs 8.4 (1.9)%, $P < 0.05$], whereas no changes in the baseline and hyperemic blood flow, blood pressure, or heart rate were observed. However, the beneficial effects of anthocyanin on FMD were significantly blocked by the presence of L-NMMA [anthocyanin infusion on day 1 vs L-NMMA plus anthocyanin infusion on day 3: 11.0% (3.4%) vs 3.2% (0.8%); $P < 0.001$]. Moreover, differences in the baseline and hyperemic blood flow and blood pressure between the anthocyanin and L-NMMA plus anthocyanin intervention were also observed ($P < 0.05$; see online Supplemental Table 2).

VASCULAR RELAXANT EFFECTS OF ANTHOCYANIN IN VITRO

We further examined the role of the NO-cGMP pathway in mediating the effect of anthocyanins on vascular dilation in vitro by using a well-established rat aortic ring model *(24)*. The relaxant effects of anthocyanins upon phenylephrine-precontracted aortic rings with a functional endothelium were found to be significantly augmented when the cumulative anthocyanin concentration reached 5 μg/mL ($P < 0.001$; Fig. 4). The maximal relaxation was 80.1% (18.6%) (n = 8) when the cumulative concentration of anthocyanins reached 50 μg/mL. This effect was not observed in the aortic rings without functional endothelia (Fig. 4). More importantly, the relaxation effect of anthocyanins was not observed in the presence of NOS inhibitor L-NMMA (Fig. 4) or GC inhibitor ODQ (see online Supplemental Data Fig. 1).

Discussion

Several previous studies have revealed that the consumption of anthocyanin-rich foods and beverages im-

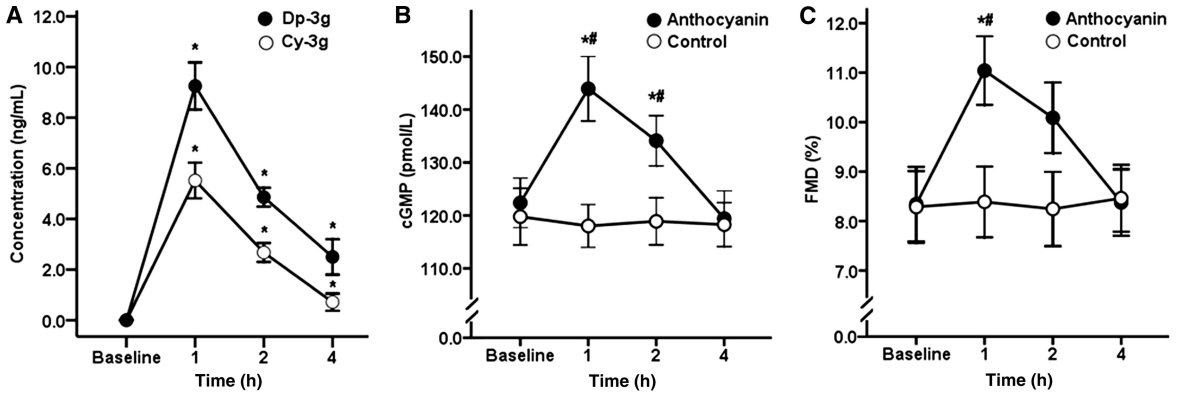

Fig. 2. Concentrations of plasma anthocyanins (A), cGMP (B), and FMD (C) before and after capsule ingestion (n = 12).
Values are the means (SE). * $P < 0.05$, significantly different from the baseline (paired Student *t*-tests); # $P < 0.05$, significantly different from the respective time point in the control group (repeated-measures ANOVA with a Bonferroni corrected posthoc *t*-test).

proves endothelium-dependent vasodilation *(20, 26, 27)*. Chaves et al. have demonstrated, for example, that a standardized grape product has vasoprotective effects in humans *(26)*. Poreba and colleagues have also reported beneficial effects of chokeberry juice on endothelial function in men with mild hypercholesterolemia *(27)*. In vitro, anthocyanin improves vascular function by enhancing the activity of endothelial NOS and soluble guanylyl cyclase *(26, 27)*. We thus speculated that this effect would be partially mediated by the activation of NO-cGMP signaling in humans. However, anthocyanin-rich foods and beverages also contain thousands of other types of polyphenols, an unknown number of which may exert beneficial effects on endothelial function *(28)*. An evaluation of whether pure anthocyanins could improve endothelial function in human study participants was therefore necessary.

To investigate whether dietary anthocyanins exert direct effects on endothelium-dependent vasodilation in the current study, we first conducted a short-term intervention trial in hypercholesterolemic individuals. The results showed that a maximal plasma concentration of Dp-3g and Cy-3g was reached at 1 h after dietary anthocyanin supplementation, and was associated with the highest achieved levels of FMD and plasma cGMP in these participants. We thus concluded that purified anthocyanins improve endothelial function. On the basis of the results obtained from the short-term intervention study, we explored whether pure anthocyanins would have a sustained beneficial effect on endothelial function via a long-term intervention. In our cohort of hypercholesterolemic individuals who were administered anthocyanin supplementation for 12 weeks, we observed dramatically improved endothelial function, as determined by FMD measurement, whereas we observed no significant differences in GTND, suggesting that anthocyanins do not affect NO-independent vasodilation. Moreover, long-term anthocyanin supplementation led to a significant increase in the plasma concentration of cGMP, a surrogate of NO bioactivity.

Vascular dilation is directly stimulated by NO, which is released from the vascular endothelial cells. NO is synthesized from L-arginine by an endothelial enzyme (NOS) *(29)*, and its activity can be specifically blocked by arginine analogs, such as L-NMMA, which serve as useful tools in studying the biological distribution and the function of NO *(30)*. The vasodilator activity of NO is attributed to its diffusion to the vascular smooth muscle cells and the activation of soluble GC, leading to the production of cGMP and cGMP-mediated vasodilation *(31, 32)*. Because the half-life of NO is extremely short and the methods for NO measurement must be further developed *(28)*, we used the circulating cGMP concentration as the index of NO activity and thus as an indirect marker of endothelium-dependent vasodilation *(33)*. We found that increased plasma cGMP concentrations correlated with improved FMD in hypercholesterolemic individuals treated by anthocyanin supplementation. Furthermore, in the presence of an NOS inhibitor (L-NMMA) or a GC inhibitor (ODQ), the effects of anthocyanin on endothelium-dependent vasodilation were abolished in humans and in a rat aortic ring model. These findings are consistent with the data shown in previous reports. Andriambeloson et al. demonstrated that delphinidin, a monomer of anthocyanin, evokes an 89% endothelium-dependent vasorelaxation *(34)*. Nakamura et al. have shown that a blackcurrant concentrate

Table 2. Comparison of endothelial function, vascular inflammation, and the lipid profile at baseline and after the 12-week intervention.[a]

	Placebo group (n = 73)			Anthocyanin group (n = 73)			
	Baseline	12 week	Mean change, % (95% CI)[b]	Baseline	12 week	Mean change, % (95% CI)[b]	*P*[c]
Anthocyanins, ng/mL	None	None	None	None	None	—	—
cGMP, pmol/mL	124.9 (22.3)[d]	123.6 (21.2)	−1.2 (−4.6 to 2.2)	123.4 (21.6)	138.8 (22.5)[e]	12.6 (8.6 to 16.5)	0.031
Brachial diameter, mm	4.08 (0.72)	4.06 (0.61)	−0.6 (−1.6 to 0.4)	4.02 (0.59)	4.05 (0.53)	0.7 (0.3 to 1.1)	0.452
Baseline blood flow, mL/min	138.9 (54.5)	142.0 (52.3)	2.0 (−5.6 to 9.6)	127.3 (49.2)	138.7 (51.0)	6.5 (−2.8 to 15.7)	0.304
Hyperemic blood flow, mL/min	367.6 (130.4)	374.2 (134.6)	1.6 (−4.3 to 7.5)	336.8 (123.4)	362.5 (127.2)	7.8 (−1.2 to 16.8)	0.219
FMD, %	8.32 (2.04)	8.53 (1.96)	2.2 (−3.6 to 8.0)	8.04 (1.82)	10.91 (2.06)[e]	28.4 (13.2 to 43.6)	0.006
GTND, %	18.0 (5.6)	18.4 (5.9)	2.4 (−0.6 to 5.4)	18.3 (5.0)	18.8 (5.2)	2.7 (−0.2 to 5.6)	0.583
sVCAM-1, ng/mL	544.2 (107.8)	546.3 (106.9)	0.4 (−3.1 to 5.0)	542.9 (103.6)	481.0 (91.8)[e]	−11.6 (−14.8 to −8.6)	0.023
HDL-C, mg/dL	48.0 (8.1)	47.2 (9.7)	−1.6 (−4.8 to 1.6)	47.2 (8.9)	53.0 (8.5)[e]	12.8 (7.4 to 18.2)	0.028
LDL-C, mg/dL	127.3 (18.2)	126.9 (18.2)	−0.5 (−6.0 to 1.3)	130.0 (22.4)	116.5 (15.9)[e]	−10.0 (−13.8 to −6.2)	0.045
Triacylglycerol, mg/dL	213.5 (130.2 to 239.2)[f]	205.6 (123.2 to 235.7)	−3.4 (−9.6 to 2.8)	217.1 (135.6 to 242.8)	208.2 (121.4 to 231.2)	−4.1 (−10.2 to 1.8)	0.469
Total cholesterol, mg/dL	250.8 (32.5)	241.1 (33.3)	−3.5 (−6.4 to −0.6)	249.6 (39.5)	239.2 (31.7)	−4.6 (−8.4 to −0.8)	0.385
Apolipoprotein AI, mg/dL	133 (14.6)	130 (16.2)	−1.6 (−4.4 to 1.2)	130 (16.9)	133 (18.0)	1.8 (−1.8 to 5.4)	0.467
Apolipoprotein B, mg/dL	121 (26.0)	124 (27.1)	3.2 (−2.4 to 8.8)	126 (28.4)	122 (28.9)	−1.9 (−7.1 to 3.4)	0.254
Glucose, mg/dL	95.4 (29.2)	95.2 (28.3)	−0.2 (−3.2 to 2.9)	93.4 (29.9)	95.0 (28.6)	3.0 (−1.0 to 6.9)	0.620
Insulin, mU/L	10.5 (6.4 to 13.8)	11.1 (7.8 to 15.4)	5.8 (−1.3 to 12.9)	10.9 (6.9 to 14.0)	11.2 (8.0 to 15.6)	3.6 (−4.5 to 11.7)	0.671
HOMA-IR	2.50 (1.51 to 3.18)	2.64 (1.62 to 3.43)	6.4 (−4.1 to 16.9)	2.59 (1.53 to 3.29)	2.65 (1.67 to 3.40)	3.3 (−6.3 to 12.9)	0.714

[a] No significant differences were found for any variable between the 2 groups at baseline via the unpaired Student *t*-test.
[b] Calculated as [(value at 12 − value at baseline)/value at baseline] × 100.
[c] The effects of the intervention on these variables were tested by repeated-measures ANOVA.
[d] Mean (SD).
[e] $P < 0.05$ vs baseline, assessed by paired Student *t*-tests.
[f] Geometric mean; upper and lower quartiles are shown in parentheses (all such values).

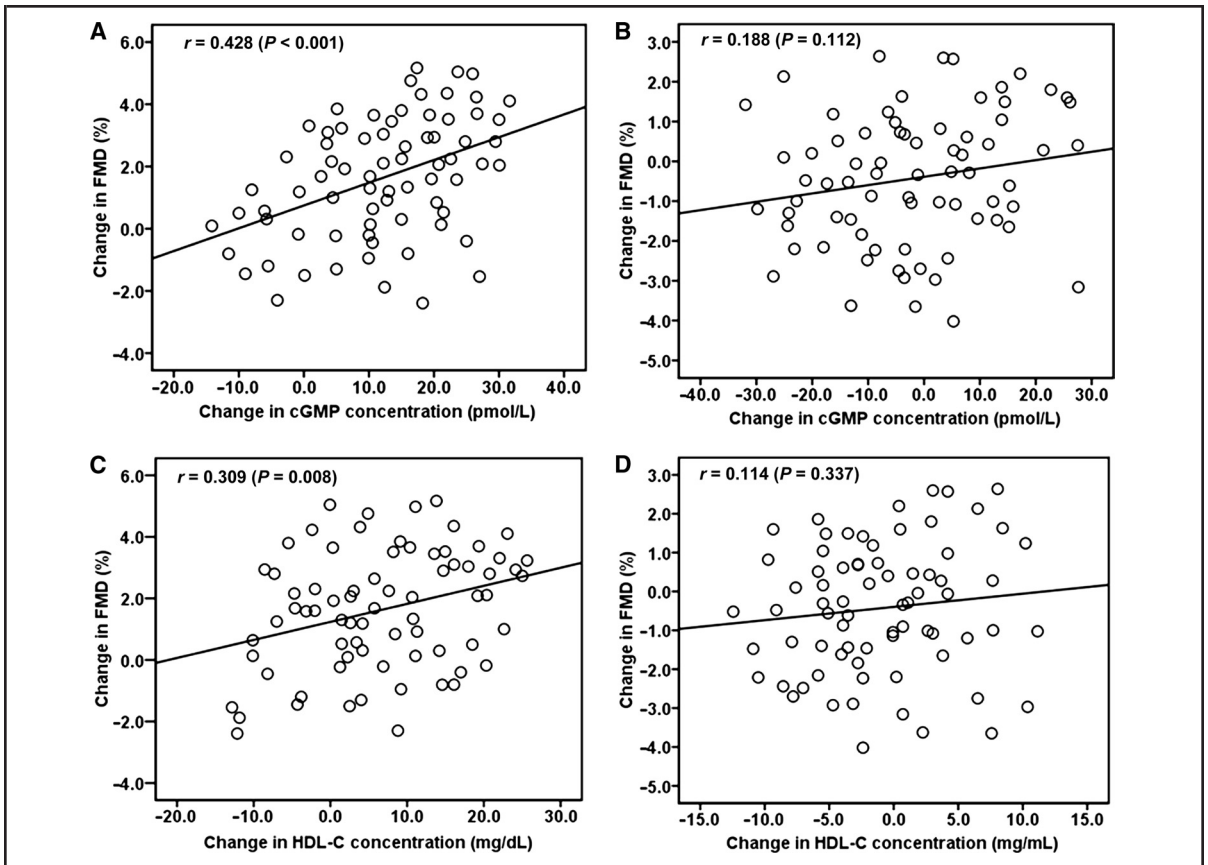

Fig. 3. Correlation analyses between the changes in the plasma cGMP and serum HDL-C concentrations and the changes in FMD in the anthocyanin (A, C) and control (B, D) groups after the 12-week intervention (n = 73 in each group).

The data were evaluated by using Pearson correlation coefficients (*r*).

with a high content of anthocyanins produced an approximately 80% relaxation in norepinephrine precontracted rat aortic rings, and that the response was inhibited after addition of L-NMMA or ODQ *(24)*. We hence suggest that the improvement of endothelium-dependent vasodilation in individuals with hypercholesterolemia by anthocyanins occurs mainly through the activation of the NO-cGMP signaling pathway.

The endothelial dysfunction seen in hypercholesterolemia is among the early events in the development of atherosclerosis, and is attributable to various cardiovascular abnormalities. In hypercholesterolemic individuals, endothelial function can be impaired by dyslipidemia and mild chronic inflammation, represented as increased total cholesterol or LDL-C concentrations and increased expression of sVCAM-1 *(8, 10, 11)*. In the present study, both dyslipidemia and inflammation among the hypercholesterolemic participants were found to be ameliorated by anthocyanin supplementation for 12 weeks. These lipid profile and inflammatory response changes were consistent with the findings of our previously reported investigations, in which anthocyanin supplementation in dyslipidemic individuals increased the serum HDL-C concentration and decreased the serum LDL-C concentration *(18)*, and an anthocyanin-rich black rice outlayer fraction was found to significantly reduce the plasma concentrations of inflammatory molecules, including sCD40 ligand, sVCAM-1, and high-sensitivity C-reactive protein, in individuals with coronary artery diseases *(35)*. Other investigators have shown also that berry consumption can lead to an increase in the HDL-C concentration in individuals showing cardiovascular risk factors *(36)* and that anthocyanin-rich red wine consumption produces a decrease in the serum VCAM-1 (−17%) and ICAM-1 (−9%) concentrations in healthy individuals *(19)*. Because dietary anthocyanin supplementation was shown in our analyses to im-

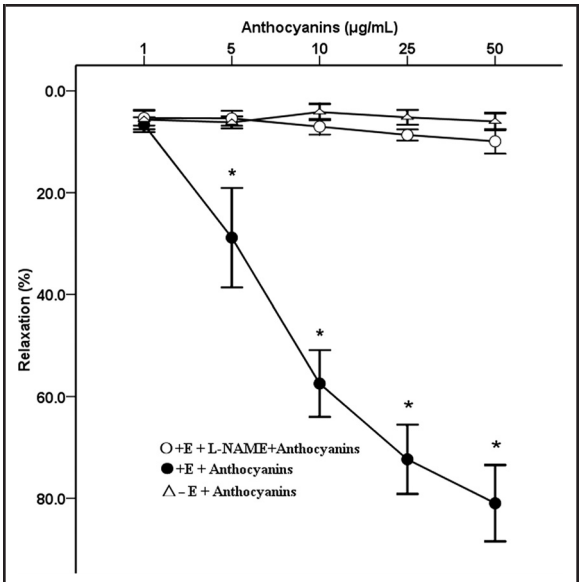

Fig. 4. Concentration–response curve for anthocyanin-induced relaxation in phenylephrine-precontracted rat thoracic aortic rings (n = 8).

Values are the means (SD). $*P < 0.001$, significantly different from the tests conducted in the presence of NOS inhibitor L-NMMA. The data were assessed via unpaired Student *t*-tests. +E, rat thoracic aortic rings with a functional endothelium; −E, those without a functional endothelium.

prove not only endothelium-dependent vasodilation but also the lipid and inflammatory biomarker profiles, we suggest that the amelioration of the lipid profile and inflammation underlie the improvement of endothelium-dependent vasodilation by anthocyanin supplementation.

It should be pointed out that the bioavailability of anthocyanins in humans and animal models is accompanied by extensive conjugation and metabolism *(37)*; moreover, the intact anthocyanins and the metabolic products can be found in the blood and urine after the administration of anthocyanins *(38, 39)*. These findings indicate that anthocyanin metabolites are likely to exert biological effects. In a previous study we have shown that protocatechuic acid, a major metabolite of the anthocyanins, inhibits monocyte adhesion and reduces atherosclerosis in apolipoprotein E–deficient mice *(40)*. In the present study, the results of the short-term anthocyanin intervention showed that the increases in the FMD and cGMP concentrations were concomitant with increased plasma anthocyanin (Dp-3g and Cy-3g) concentrations. This finding indicates that intact anthocyanins are one of the components responsible for the improvement in FMD. Although the present data show that in the long-term intervention participants neither intact anthocyanins nor metabolic products were detected in fasting blood samples, owing to their rapid metabolism and elimination *(37)*, this did not exclude the possibility that they existed in the tissues or organs nor that anthocyanin metabolites might also contribute to the improvement of FMD.

In conclusion, we have demonstrated for the first time that dietary anthocyanin supplementation improves endothelium-dependent vasodilation through the activation of the NO-cGMP signaling pathway in hypercholesterolemic individuals. Additional investigations are needed to assess the causative relationship between the beneficial effect of anthocyanins on the lipid profile and the reduction in inflammatory molecule release.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: National Natural Science Foundation of China (30730079) and the Supporting Program of the "Eleventh Five-Year Plan" for Science and Technology Research of China (2008BAI58B06).
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We thank Mr. Renyou Gan from Sun Yat-Sen University for blood sample and data collection. We are also grateful to the participants, as well as doctors and nurses involved in this study.

References

1. Vita JA, Keaney JF Jr. Endothelial function: a barometer for cardiovascular risk? Circulation 2002;106:640–2.
2. Verma S, Anderson TJ. Fundamentals of endothelial function for the clinical cardiologist. Circulation 2002;105:546–9.
3. Lavi S, Yang EH, Prasad A, Mathew V, Barsness GW, Rihal CS, et al. The interaction between coronary endothelial dysfunction, local oxidative stress, and endogenous nitric oxide in humans. Hypertension 2008;51:127–33.
4. Sitia S, Tomasoni L, Atzeni F, Ambrosio G, Cord-

iano C, Catapano A, et al. From endothelial dysfunction to atherosclerosis. Autoimmun Rev 2010;9:830–4.
5. Ross R. Atherosclerosis–an inflammatory disease. N Engl J Med 1999;340:115–26.
6. Payne JA, Reckelhoff JF, Khalil RA. Role of oxidative stress in age-related reduction of NO-cGMP-mediated vascular relaxation in SHR. Am J Physiol Regul Integr Comp Physiol 2003;285: R542–51.
7. Wilkinson IB, Cockcroft JR. Cholesterol, endothelial function and cardiovascular disease. Curr Opin Lipidol 1998;9:237–42.
8. Cooke JP, Tsao PS. Is NO an endogenous antiatherogenic molecule? Arterioscler Thromb 1994; 14:653–5.
9. Faulx MD, Wright AT, Hoit BD. Detection of endothelial dysfunction with brachial artery ultrasound scanning. Am Heart J 2003;145:943–51.
10. De Caterina R, Basta G, Lazzerini G, Dell'Omo G, Petrucci R, Morale M, et al. Soluble vascular cell adhesion molecule-1 as a biohumoral correlate of atherosclerosis. Arterioscler Thromb Vasc Biol 1997;17:2646–54.
11. Arts IC, Hollman PC. Polyphenols and disease risk in epidemiologic studies. Am J Clin Nutr 2005;81: 317S–25S.
12. Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong CP, Nettleton JA, Jacobs DR Jr. Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. Am J Clin Nutr 2007;85:895–909.
13. Heiss C, Jahn S, Taylor M, Real WM, Angeli FS, Wong ML, et al. Improvement of endothelial function with dietary flavanols is associated with mobilization of circulating angiogenic cells in patients with coronary artery disease. J Am Coll Cardiol 2010;56:218–24.
14. Chun OK, Chung SJ, Song WO. Estimated dietary flavonoid intake and major food sources of U.S. Adults. J Nutr 2007;137:1244–52.
15. Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior RL. Concentrations of anthocyanins in common foods in the united states and estimation of normal consumption. J Agric Food Chem 2006;54:4069–75.
16. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79:727–47.
17. Dalgard C, Nielsen F, Morrow JD, Enghusen-Poulsen H, Jonung T, Horder M, de Maat MP. Supplementation with orange and blackcurrant juice, but not vitamin E, improves inflammatory markers in patients with peripheral arterial disease. Br J Nutr 2009;101:263–9.
18. Qin Y, Xia M, Ma J, Hao Y, Liu J, Mou H, et al. Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects. Am J Clin Nutr 2009;90:485–92.
19. Estruch R, Sacanella E, Badia E, Antunez E, Nicolas JM, Fernandez-Sola J, et al. Different effects of red wine and gin consumption on inflammatory biomarkers of atherosclerosis: a prospective randomized crossover trial. Effects of wine on inflammatory markers. Atherosclerosis 2004;175: 117–23.
20. Iwasaki-Kurashige K, Loyaga-Rendon RY, Matsumoto H, Tokunaga T, Azuma H. Possible mediators involved in decreasing peripheral vascular resistance with blackcurrant concentrate (BC) in hind-limb perfusion model of the rat. Vascul Pharmacol 2006;44:215–23.
21. Agewall S, Wright S, Doughty RN, Whalley GA, Duxbury M, Sharpe N. Does a glass of red wine improve endothelial function? Eur Heart J 2000; 21:74–8.
22. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. J Am Coll Cardiol 2002;39:257–65.
23. Matsumoto H, Inaba H, Kishi M, Tominaga S, Hirayama M, Tsuda T. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. J Agric Food Chem 2001;49:1546–51.
24. Nakamura Y, Matsumoto H, Todoki K. Endothelium-dependent vasorelaxation induced by black currant concentrate in rat thoracic aorta. Jpn J Pharmacol 2002;89:29–35.
25. Landmesser U, Hornig B, Drexler H. Endothelial function: a critical determinant in atherosclerosis? Circulation 2004;109:II27–33.
26. Chaves AA, Joshi MS, Coyle CM, Brady JE, Dech SJ, Schanbacher BL, et al. Vasoprotective endothelial effects of a standardized grape product in humans. Vascul Pharmacol 2009;50:20–6.
27. Poreba R, Skoczynska A, Gac P, Poreba M, Jedrychowska I, Affelska-Jercha A, et al. Drinking of chokeberry juice from the ecological farm Dziecilowo and distensibility of brachial artery in men with mild hypercholesterolemia. Ann Agric Environ Med 2009;16:305–8.
28. Kelm M, Schrader J. Control of coronary vascular tone by nitric oxide. Circ Res 1990;66:1561–75.
29. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation 2006;113:1708–14.
30. Gray GA, Schott C, Julou-Schaeffer G, Fleming I, Parratt JR, Stoclet JC. The effect of inhibitors of the L-arginine/nitric oxide pathway on endotoxin-induced loss of vascular responsiveness in anaesthetized rats. Br J Pharmacol 1991;103:1218–24.
31. Condorelli P, George SC. In vivo control of soluble guanylate cyclase activation by nitric oxide: a kinetic analysis. Biophys J 2001;80:2110–9.
32. Collins P, Griffith TM, Henderson AH, Lewis MJ. Endothelium-derived relaxing factor alters calcium fluxes in rabbit aorta: a cyclic guanosine monophosphate-mediated effect. J Physiol 1986; 381:427–37.
33. Kielstein JT, Impraim B, Simmel S, Bode-Boger SM, Tsikas D, Frolich JC, et al. Cardiovascular effects of systemic nitric oxide synthase inhibition with asymmetrical dimethylarginine in humans. Circulation 2004;109:172–7.
34. Andriambeloson E, Magnier C, Haan-Archipoff G, Lobstein A, Anton R, Beretz A, et al. Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. J Nutr 1998;128:2324–33.
35. Wang Q, Han P, Zhang M, Xia M, Zhu H, Ma J, et al. Supplementation of black rice pigment fraction improves antioxidant and anti-inflammatory status in patients with coronary heart disease. Asia Pac J Clin Nutr 2007;16(Suppl 1):295–301.
36. Erlund I, Koli R, Alfthan G, Marniemi J, Puukka P, Mustonen P, et al. Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol. Am J Clin Nutr 2008;87: 323–31.
37. McGhie TK, Walton MC. The bioavailability and absorption of anthocyanins: towards a better understanding. Mol Nutr Food Res 2007;51:702–13.
38. Kay CD, Mazza G, Holub BJ, Wang J. Anthocyanin metabolites in human urine and serum. Br J Nutr 2004;91:933–42.
39. Talavera S, Felgines C, Texier O, Besson C, Gil-Izquierdo A, Lamaison JL, Remesy C. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. J Agric Food Chem 2005;53:3902–8.
40. Wang D, Wei X, Yan X, Jin T, Ling W. Protocatechuic acid, a metabolite of anthocyanins, inhibits monocyte adhesion and reduces atherosclerosis in apolipoprotein E-deficient mice. J Agric Food Chem 2010;58:12722–8.