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Effects of purified anthocyanin supplementation on platelet chemokines in hypocholesterolemic individuals: a randomized controlled trial

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Abstract

Background: It is becoming increasingly evident that platelet chemokines are involved in distinct aspects of atherosclerosis. The aim of this study was to examine the effects of long-term supplementation with purified anthocyanins on platelet chemokines in hypercholesterolemic individuals and to identify correlations of decreased platelet chemokine levels with serum lipid and inflammatory marker levels.

Methods: A total of 146 hypercholesterolemic individuals were recruited and treated with 320 mg of purified anthocyanins ($n = 73$) or a placebo ($n = 73$) daily for 24 weeks in this randomized, double-blind, placebo-controlled trial.

Results: Anthocyanin supplementation for 24 weeks significantly decreased the plasma CXCL7 (-12.32% vs. 4.22% , $P = 0.001$), CXCL5 (-9.95% vs. 1.93% , $P = 0.011$), CXCL8 (-6.07% vs. 0.66% , $P = 0.004$), CXCL12 (-8.11% vs. 5.43% , $P = 0.023$) and CCL2 levels (-11.63% vs. 12.84% , $P = 0.001$) compared with the placebo. Interestingly, the decreases in the CXCL7 and CCL2 levels were both positively correlated with the decreases in the serum low-density lipoprotein-cholesterol (LDL-C), high-sensitivity C-reactive protein (hsCRP) and interleukin-1 β (IL-1 β) levels after anthocyanin supplementation for 24 weeks. The decrease in the CXCL8 level was negatively correlated with the increase in the low-density lipoprotein-cholesterol (HDL-C) level and was positively correlated with the decrease in the soluble P-selectin (sP-selectin) level in the anthocyanin group. In addition, a positive correlation was observed between the decreases in the CXCL12 and tumor necrosis factor- α (TNF- α) levels after anthocyanin supplementation. However, the plasma CXCL4L1, CXCL1, macrophage migration inhibitory factor (MIF) and human plasminogen activator inhibitor 1 (PAI-1) levels did not significantly change following anthocyanin supplementation.

Conclusions: The present study supports the notion that platelet chemokines are promising targets of anthocyanins in the prevention of atherosclerosis.

Trial registration: ChiCTR-TRC-08000240. Registered: 10 December 2008.

Keywords: Anthocyanins, Platelet chemokines, Hypercholesterolemia, Inflammation, Serum lipids, CXCL7

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Background

Atherosclerosis is a type of chronic inflammatory disease resulting from disordered lipid metabolism and a maladaptive inflammatory response [1, 2]. Hypercholesterolemia has been identified as an independent risk factor for atherosclerosis [3] that is mainly attributed to the modulation of macrophage and platelet biogenesis and activity in early atherosclerotic lesions [4]. Numerous studies have demonstrated that platelet chemokines play an important role in the pathogenesis of hypercholesterolemia-induced atherosclerotic disease [5–7]. These chemokines contribute to the recruitment of circulating leukocytes and progenitor cells to the site of injured endothelium, forming a surface on which platelets are further activated to enhance inflammation [8, 9]. In addition, platelet chemokines have a possible pathogenic role in interactions among platelets, oxidized low-density lipoproteins (ox-LDLs), and peripheral leukocytes [10, 11]. The most abundant platelet-derived chemokines include CXCL4 (platelet factor 4; PF4) and CXCL7 (neutrophil-activating peptide-2; NAP-2). Several other chemokines, such as CCL5 (regulated on activation in normal T-cell expressed and secreted; RANTES), CXCL5 (epithelial neutrophil-activating peptide; ENA-78), CXCL12 (stromal cell-derived factor 1; SDF-1 α), CXCL1 (growth-regulated oncogene-alpha; GRO- α), CXCL8 (interleukin-8; IL-8), CCL2 (monocyte chemoattractant protein-1; MCP-1), CXCL4L1 (PF4alt), macrophage migration inhibitory factor (MIF) and human plasminogen activator inhibitor 1 (PAI-1), are abundantly released or expressed by platelets but have been primarily identified in other cell types [12–15]. Emerging evidence indicates that the plasma levels of some platelet chemokines, such as CXCL4, CCL5, CXCL5, CXCL1, CCL2 and CXCL8, are increased in hypercholesterolemia [16–19]. Hence, reduction of platelet chemokine levels has already been suggested as a feasible approach for preventing and treating atherosclerosis [20].

Numerous epidemiological and medical anthropological investigations have suggested that anthocyanins play a protective role against atherosclerosis [21, 22]. Anthocyanins, a category of phenolic flavonoids, are abundant in various fruits, vegetables and beverages and are commonly consumed in the human diet [23–25]. Consumption of anthocyanins beneficially influences inflammatory disorders, the lipid profile, platelet activation and obesity-related disorders [26–29]. Our previous studies have shown that purified anthocyanin supplementation reduces the levels of inflammatory biological markers and improves serum lipids and endothelial function [30–32]. We have also found that anthocyanins significantly inhibit platelet activation, thrombosis, platelet hyperactivity [33, 34] and platelet granule secretion of molecules such as CCL5, β -TG and soluble P-selectin (sP-selectin), both in vitro and in vivo [35]. However, the

effects of purified anthocyanin supplementation on most other platelet chemokines in patients with hypercholesterolemia have not yet been assessed.

Therefore, we designed this randomized, double-blind, placebo-controlled, 24-week trial to investigate the effects of long-term supplementation with purified anthocyanins on platelet chemokines in subjects with hypercholesterolemia. We also assessed whether decreased platelet chemokine levels were correlated with changes in serum lipids and inflammatory biological markers after treatment.

Methods

Subjects

A total of 150 hypercholesterolemic individuals were recruited for this trial between November 2008 and December 2010 in Guangzhou, Guangdong, China. The participants were recruited from physical examination centers at three hospitals and ranged in age from 40 to 65 years old, as previously described [32, 35]. Briefly, the clinical inclusion criterion for participation in the trial was a fasting total cholesterol level of between 5.17 mmol/L and 8.01 mmol/L (approximately 200 mg/dL to 310 mg/dL). Individuals with a history of cardiovascular disease (CVD), hypertension, diabetes mellitus, thyroid disorder, smoking or the use of any drug that could influence the measurement of lipid parameters, inflammatory markers, or chemokines were excluded from the study. The study was approved by the ethics committee of Sun Yat-sen University (the protocol number is No. 200802; the clinical trial number is ChiCTR-TRC-08000240), and written informed consent was obtained from all participants.

Study design

This randomized, double-blind, placebo-controlled trial was carried out for 24 weeks. Briefly, the participants were randomly assigned to either an anthocyanin group ($n = 75$; 31 males and 44 females) or a placebo group ($n = 75$; 32 males and 43 females). During the trial period, the participants were instructed to consume two anthocyanin capsules or placebo capsules twice daily for 24 weeks. The anthocyanin capsules provided a total daily intake of 320 mg of anthocyanins. The participants were told not to change their dietary habits and not to consume any anthocyanin-rich foods or any other medications that might affect the serum anthocyanin level over the following 24 weeks. Fasting blood samples (≥ 10 h) were collected at the beginning and at weeks 12 and 24 of the trial.

Materials and reagents

The anthocyanin and placebo capsules were obtained from Polyphenols AS (Sandnes, Norway). CXCL7 human ELISA kits were purchased from Abcam (Cambridge,

UK), and human CXCL8 and CCL2 ELISA kits were purchased from eBioscience (San Diego, CA, USA). In addition, MILLIPLEX Multi-analyte Profiling (MAP) Human Cytokine/Chemokine Kits for human CXCL12, CXCL5 and PAI-1 were purchased from Millipore (Billerica, MA, USA). Further, human CXCL1 and MIF ELISA kits were obtained from R&D Systems (Minneapolis, MN, USA) and human CXCL4L1 ELISA kits were obtained from LifeSpan Biosciences (Denver, CO, USA).

Biochemical analysis

Fasting blood samples were collected at baseline and weeks 12 and 24. Blood samples were drawn into heparin anticoagulant tubes for all study participants early in the morning after they had fasted overnight for 12 h, and the samples were centrifuged at $1500 \times g$ for 15 min at 4°C within 2 h to separate the plasma. Then, the plasma was stored at -80°C until testing. The concentrations of serum lipids, including total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), were measured enzymatically using an automatic analyzer. The serum high-sensitivity C-reactive protein (hsCRP) levels were determined using a Hitachi 911 automated assay analyzer and the immunoturbidimetric method. The serum tumornecrosis factor- α (TNF- α) level was measured using a commercially available ELISA kit obtained from R&D Systems (Minneapolis, MN, USA). Further, the plasma interleukin- 1β (IL- 1β) and sP-selectin levels were measured with commercial ELISA kits obtained from eBioscience (San Diego, CA, USA) as previously described [32, 35].

Analysis of platelet chemokine levels

Platelet chemokines levels in the plasma were quantified using commercial ELISA kits or MILLIPLEX MAP Human Cytokine/Chemokine Kits. The samples used in ELISA were diluted based on the pre-experiment concentrations and were run in duplicate in accordance with the manufacturer's instructions. The recombinant products and standard solutions provided in the kits were used as positive controls. The optical density of each well was determined at 450 nm using a plate reader within 30 min. Average absorbance values were calculated for each set of duplicate standards and samples. The data were analyzed based on the standard curve values. Multiplex immune assays were performed according to the manufacturer's instructions.

Statistical analysis

The platelet chemokine data were expressed as the mean \pm SD or as the mean and 95% confidence interval (CI). The baseline characteristics of the two groups were compared using unpaired Student's *t*-test for continuous data.

Further, repeated-measures ANCOVA was conducted to determine the effects of anthocyanin supplementation on platelet chemokines in the hypercholesterolemic subjects. The percentage changes in the platelet chemokine levels were calculated as follows: (value at week 24 – value at baseline)/value at baseline \times 100. The average percentage change was expressed as the mean (95% CI). Differences in the average percentage changes in the platelet chemokine levels between the two groups were evaluated using unpaired Student's *t*-test. Comparisons of the absolute changes in the platelet chemokine levels after the 24-week intervention within the groups were conducted using paired Student's *t*-test. In addition, Pearson correlation coefficients (*r*) were calculated to assess the associations between the decreased plasma chemokine concentrations and the changes in the plasma lipid and inflammatory maker levels over the 24-week study period. Differences were considered significant at a $P < 0.05$. All statistical analyses were performed using IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA).

Results

Baseline characteristics of the subjects

A total of 4 participants withdrew from this trial, and 146 subjects ultimately completed it ($n = 73$ in the anthocyanin group; and $n = 73$ in the placebo group). The distributions of age, the anthropometric characteristics and the mean daily intake of nutrients were uniform between the two groups, as previously described (Table 1) [32]. No subjects reported any adverse events resulting from the treatment throughout the intervention period.

Effects of anthocyanin supplementation on platelet chemokines

The plasma platelet chemokine concentrations at baseline and at weeks 12 and 24 after the intervention are shown in Table 2. No significant differences in these concentrations were observed between the two groups at baseline. At week 12, only the plasma CXCL5 ($P = 0.021$) and CXCL8 concentrations ($P = 0.015$) were significantly decreased by anthocyanins compared with the baseline concentrations. At week 24, anthocyanin supplementation resulted in significant decreases in the plasma CXCL7 [–12.32% (95% CI, –19.25 to –5.00%), $P = 0.001$], CXCL5 [–9.95% (95% CI, –15.84 to –3.56%), $P = 0.011$], CXCL8 [–6.07% (95% CI, –8.15 to –4.23%), $P = 0.004$], CXCL12 [–8.11% (95% CI, –15.98 to –0.30%), $P = 0.023$] and CCL2 concentrations [–11.63% (95% CI, –18.16 to –4.85%), $P = 0.001$] compared with the placebo. Although the plasma CXCL4L1, CXCL1, MIF and PAI-1 concentrations also exhibited trends toward slight decreases during the treatment period, no significant changes were detected between the two groups.

Table 1 Anthropometric characteristics and daily nutrient intake of the participants

	Placebo (n = 73)			Anthocyanins (n = 73)			P-value ^a
	Baseline	12 wk	24 wk	Baseline	12wk	24 wk	
Weight (kg)	70.1 ± 9.8	69.7 ± 9.9	69.5 ± 9.6	68.9 ± 8.8	67.8 ± 8.9	66.5 ± 8.1	0.342
BMI (kg/m ²)	26.8 ± 2.0	26.7 ± 2.1	26.8 ± 2.2	26.4 ± 2.1	25.9 ± 2.0	26.1 ± 2.0	0.063
Waist circumference (cm)	89.6 ± 7.9	89.5 ± 8.2	89.2 ± 8.3	88.6 ± 6.4	87.4 ± 6.4	87.2 ± 6.5	0.226
Hip circumference (cm)	100.3 ± 6.3	100.3 ± 6.5	101.4 ± 6.4	100.0 ± 5.0	99.1 ± 5.2	98.3 ± 4.6	0.308
Waist/hip ratio	0.89 ± 0.06	0.89 ± 0.06	0.88 ± 0.05	0.89 ± 0.05	0.88 ± 0.05	0.88 ± 0.05	0.624
Systolic BP (mmHg)	124.3 ± 16.0	123.8 ± 15.0	120.7 ± 15.1	126.2 ± 14.9	119.5 ± 12.5	119.4 ± 12.6	0.135
Diastolic BP (mmHg)	82.8 ± 10.5	81.2 ± 9.1	81.1 ± 9.8	84.7 ± 10.7	82.8 ± 9.6	82.6 ± 9.3	0.219
Energy (kcal/d)	2163.6 ± 124.2	2168.2 ± 116.1	2154.4 ± 114.2	2185.4 ± 132.5	2199.2 ± 123.7	2171.6 ± 121.8	0.684
Protein (g/d)	84.8 ± 10.8	83.6 ± 9.4	81.4 ± 9.5	85.7 ± 10.5	84.5 ± 9.8	86.5 ± 10.9	0.702
% of energy	18.5 ± 3.1	18.4 ± 2.7	17.3 ± 3.0	18.6 ± 2.9	18.5 ± 3.1	19.1 ± 3.2	0.746
Total fat (g/d)	82.4 ± 18.2	80.1 ± 17.2	80.2 ± 16.8	80.2 ± 15.8	83.7 ± 17.6	84.9 ± 18.1	0.282
% of energy	26.5 ± 3.5	26.3 ± 3.2	24.3 ± 4.0	26.4 ± 3.2	27.4 ± 4.0	27.6 ± 4.3	0.493
Total carbohydrates (g/d)	258.6 ± 34.2	258.8 ± 40.5	261.0 ± 39.7	262.1 ± 43.8	263.2 ± 42.5	260.4 ± 40.3	0.161
% of energy	55.2 ± 4.7	55.3 ± 4.6	56.9 ± 4.5	55.0 ± 5.3	55.3 ± 4.8	54.2 ± 4.2	0.824
Cholesterol (mg/d)	339.4 ± 43.2	340.1 ± 38.4	331.3 ± 40.4	341.3 ± 40.2	342.5 ± 39.3	340.7 ± 43.6	0.327
Fiber (g/d)	20.5 ± 4.4	20.8 ± 4.2	19.2 ± 4.0	20.6 ± 5.0	20.6 ± 4.5	21.3 ± 4.7	0.644

The data are expressed as the mean ± SD

No significant differences in any variable were observed between the two groups at baseline, as determined using unpaired Student's *t*-test

^aThe intervention had no significant effects on daily nutrient intake, as determined by repeated-measures ANOVA

Effects of anthocyanin supplementation on serum lipids

The serum lipid levels at baseline and 24 weeks are listed in Table 3, as previously described [32]. In the anthocyanin group, the HDL-C level significantly increased from 1.22 (0.23) mmol/L at baseline to 1.37 (0.22) mmol/L at week 24 ($P = 0.018$), accompanied by a significant reduction in the LDL-C level from 3.36 (0.58) mmol/L to 3.01 (0.41) mmol/L ($P = 0.038$). Moreover, significant differences in the HDL-C ($P = 0.036$) and LDL-C levels ($P = 0.030$) were detected between the two groups after 24 weeks.

Effects of anthocyanin supplementation on serum inflammatory biomarkers

The serum levels of the inflammatory molecules hsCRP, TNF- α , IL-1 β and sP-selectin in the participants at baseline and at weeks 12 and 24 are summarized in Table 4, as previously described [32, 35]. The hsCRP and IL-1 β levels were reduced by anthocyanin supplementation at weeks 12 and 24, respectively, compared with the baseline levels. Further, anthocyanin supplementation for 24 weeks led to significant decreases in the plasma hsCRP [-21.6% (95% CI, -37.5 to -5.7%)], IL-1 β [-12.8% (95% CI, -24.4 to -1.2%)], and sP-selectin levels [-5.9% (95% CI, -17.7 to 6.0%)] compared with the placebo.

Correlations between alterations in plasma platelet chemokine and serum lipid levels

After 24 weeks of anthocyanin supplementation, the changes in the plasma CCL2 ($r = 0.545$, $P < 0.001$) (Fig. 1a)

and CXCL7 levels ($r = 0.395$, $P = 0.001$) (Fig. 1b) were positively correlated with the change in the LDL-C level. In addition, the change in the CXCL8 level ($r = -0.517$, $P < 0.001$) (Fig. 1c) was negatively correlated with the change in the HDL-C level in the anthocyanin group.

Correlations between alterations in plasma platelet chemokine levels and serum inflammatory biomarker levels

After 24 weeks of anthocyanin intervention, the change in the plasma CXCL7 level exhibited a positive correlation with the changes in the hsCRP ($r = 0.479$, $P < 0.001$) (Fig. 2a) and IL-1 β levels ($r = 0.455$, $P < 0.001$) (Fig. 2b). Interestingly, the change in the CCL2 level was also positively correlated with the changes in the hsCRP ($r = 0.399$, $P < 0.001$) (Fig. 2c) and IL-1 β levels ($r = 0.474$, $P < 0.001$) (Fig. 2d). Furthermore, the change in the plasma CXCL12 level was significantly correlated with the change in the TNF- α level ($r = 0.464$, $P < 0.001$) (Fig. 2e). In addition, a positive correlation was detected between the changes in the CXCL8 and sP-selectin levels ($r = 0.448$, $P < 0.001$) (Fig. 2f) after anthocyanin intervention for 24 weeks.

Discussion

Platelet chemokines are involved in inflammatory reactions, immune responses, and other aspects of the development of atherosclerosis [36]. In the present study, we first demonstrated that the plasma levels of the platelet

Table 2 Changes in the platelet chemokine levels in the participants

	Placebo (n = 73)				Anthocyanins (n = 73)				P-value ^b
	Baseline	12 wk	24 wk	Mean change,% (95% CI) ^a	Baseline	12 wk	24 wk	Mean change,% (95% CI) ^a	
CXCL7 (ng/ml)	167.63 ± 38.78	165.82 ± 37.83	166.20 ± 31.92	4.22 (-2.33 to 11.05)	170.52 ± 44.86	157.76 ± 43.21	138.83 ± 30.49 ^c	-12.32 (-19.25 to -5.00) ^d	0.011
CXCL5 (pg/ml)	159.43 ± 29.35	157.85 ± 31.80	157.32 ± 29.74	1.93 (-4.18 to 9.28)	160.40 ± 36.26	150.78 ± 28.98 ^c	137.61 ± 26.14 ^c	-9.95 (-15.84 to -3.56) ^d	0.006
CXCL8 (pg/ml)	22.97 ± 2.73	22.91 ± 2.51	22.97 ± 4.24	0.66 (-2.99 to 4.88)	22.93 ± 1.96	22.32 ± 1.61 ^c	21.40 ± 1.07 ^c	-6.07 (-8.15 to -4.23) ^d	0.016
CXCL12 (ng/mL)	2.16 ± 0.53	2.15 ± 0.51	2.14 ± 0.46	5.43 (-2.03 to 14.16)	2.19 ± 0.56	2.11 ± 0.34	1.86 ± 0.47 ^c	-8.11 (-15.98 to -0.30) ^d	0.020
CCL2 (pg/mL)	504.99 ± 165.34	502.68 ± 157.33	515.76 ± 132.04	12.84 (1.05 to 27.23)	502.97 ± 139.91	476.96 ± 113.74	414.61 ± 87.14 ^c	-11.63 (-18.16 to -4.85) ^d	0.014
CXCL4L1 (pg/ml)	124.29 ± 88.87	134.00 ± 69.82	135.16 ± 75.07	0.08 (-0.21 to 0.35)	126.52 ± 67.05	126.44 ± 75.21	112.27 ± 86.78	-0.80 (-2.07 to 0.06)	0.769
CXCL1 (ng/ml)	20.96 ± 3.15	21.94 ± 2.38	22.92 ± 3.67	0.07 (-0.06 to 0.19)	21.30 ± 4.71	21.00 ± 3.37	21.07 ± 1.79	-0.02 (-0.24 to 0.16)	0.529
PAI-1 (ng/ml)	101.52 ± 41.81	106.13 ± 48.55	108.84 ± 14.31	0.08 (-0.10 to 0.29)	104.07 ± 52.84	107.16 ± 36.38	99.62 ± 42.35	-0.44 (-1.34 to 0.20)	0.888
MIF (ng/ml)	32.91 ± 10.02	30.46 ± 9.54	29.51 ± 14.58	-9.90 (-25.96 to 7.13)	32.69 ± 13.94	31.26 ± 8.53	26.93 ± 10.56	2.65 (-32.90 to 39.14)	0.801

The data are expressed as the mean ± SD

No significant differences in any variable were observed between the two groups at baseline, as determined using unpaired Student's *t*-test

^aCalculated as (value at week 24 - value at baseline)/value at baseline × 100

^bThe effects of the intervention on these variables were evaluated by repeated-measures ANOVA

^c*P* < 0.05 vs. baseline, as assessed by paired Student's *t*-test

^d*P* < 0.05 vs. percentage changes in the placebo group, as assessed by unpaired Student's *t*-test

Table 3 Changes in the lipid profiles of the participants

	Placebo (n = 73)			Anthocyanins (n = 73)			P-value ^b
	Baseline	24 wk	Mean change, % (95% CI) ^a	Baseline	24 wk	Mean change, % (95% CI) ^a	
Total cholesterol (mmol/L)	6.48 ± 0.84	6.25 ± 0.83	-3.6 (-7.8 to 0.6)	6.45 ± 1.02	6.18 ± 0.82	-2.9 (-6.3 to 0.5)	0.556
HDL-cholesterol (mmol/L)	1.24 ± 0.21	1.23 ± 0.20	-0.9 (-5.2 to 3.4)	1.22 ± 0.23	1.37 ± 0.22 ^c	14.0 (7.9 to 20.2) ^d	0.036
LDL-cholesterol (mmol/L)	3.29 ± 0.47	3.30 ± 0.52	0.3 (-2.9 to 3.5)	3.36 ± 0.58	3.01 ± 0.41 ^c	-10.4 (-14.8 to 6.0) ^d	0.030
Triacylglycerol (mmol/L)	2.41 (1.47 to 2.70)	2.34 (1.35 to 2.62)	-3.2 (-7.6 to 1.2)	2.45 (1.53 to 2.74)	2.35 (1.37 to 2.61)	-4.8 (-9.8 to 0.2)	0.462

The data are expressed as the mean ± SD

No significant differences in any variable were observed between the two groups at baseline, as determined using unpaired Student's *t*-test

^a Calculated as (value at week 24 - value at baseline)/value at baseline × 100

^b The effects of the intervention on these variables were evaluated by repeated-measures ANOVA

^c *P* < 0.05 vs. baseline, as assessed by paired Student's *t*-test

^d *P* < 0.05 vs. percentage changes in the placebo group, as assessed by unpaired Student's *t*-test

chemokines CXCL7, CXCL5, CXCL8, CXCL12 and CCL2 were significantly decreased in hypercholesterolemic subjects after anthocyanin supplementation for 24 weeks. Furthermore, we found that the decreased levels of some platelet chemokines after anthocyanin treatment were closely correlated with the serum lipid and inflammatory molecule levels. These results indicated that anthocyanins exerted beneficial effects on the platelet chemokine levels, serum lipids and inflammatory factors, thereby inhibiting atherosclerosis.

Upon activation, platelets release a number of chemokines that may play important roles as first-line inflammatory mediators [37]. The most abundant platelet chemokine is CXCL7, which recruits and activates neutrophils at sites of vessel wall injury, inducing the inflammatory response within the atherosclerotic plaque [38]. CXCL5 has been shown to be abundantly produced and secreted by activated platelets, thereby attracting neutrophils, enhancing the cholesterol efflux capacity of macrophages and regulating foam cell formation [39–41]. CXCL12 plays an important role in leukocyte recruitment and neointima formation at sites of arterial injury [42]. Ahmed et al. have shown that epigallocatechin-3-gallate (EGCG) (10–50 μM), an active constituent in green tea, significantly inhibits the IL-1β-induced production of CXCL5, CCL5 and CXCL1 incubated with rheumatoid arthritis synovial fibroblasts in serum-free medium for 12 h [43]. Other polyphenols, such as wogonin [44], flavonoid naringenin [45] and quercetin [46], have been showed to inhibit CXCL5 in vitro. However, no study has examined the effects of anthocyanins on CXCL7, CXCL5 or CXCL12 to date. In the present study, we first showed that long-term supplementation with purified anthocyanins reduced the plasma levels of the platelet chemokines CXCL7, CXCL5 and CXCL12 in subjects with hypercholesterolemia. The role of anthocyanins in reducing

platelet chemokine levels may contribute to their beneficial effects in hypercholesterolemic subjects.

In hypercholesterolemia, surface-adherent platelets express CXCL8 or CCL2 to recruit leukocytes, which adhere to the denuded artery and contribute to neointimal lesion formation [47, 48]. Recently, Karlsen et al. have demonstrated that the plasma CXCL8 level is decreased in healthy adults after supplementation with purified anthocyanins (300 mg/d) for 3 weeks [49]. Further, Kuntz et al. have revealed that treatment with an anthocyanin-rich grape extract for 4 h significantly attenuates CXCL8 secretion in activated human umbilical vein endothelial cells (HUVECs) [50]. In addition, administration of an anthocyanin-rich black elderberry extract (13% anthocyanins) for 10 weeks has been shown to significantly lower the serum chemokine CCL2 level in hyperlipidemic mice compared with control mice [51]. Garcia-Alonso et al. have shown that the circulating plasma CCL2 level is decreased in healthy volunteers after treatment with 12 g anthocyanin extract from red wine for 24 h [52]. Moreover, Björk et al. have found that CCL2 secretion by human primary fat cells is suppressed following treatment with the anthocyanin cyanidin-3-glucoside (130 nM) for 48 h [53]. In contrast, another study conducted by Kuntz et al. has revealed that in healthy young female volunteers, the CXCL8 and CCL2 levels are not affected by consumption of 330 ml/d of anthocyanin-rich fruit beverages for 14 days [54]. These inconsistencies among studies may be related to differences in the dose of anthocyanins used, the duration of the intervention and the study subjects. Our study is the first to show the long-term effects of purified anthocyanins on CXCL8 and CCL2 in Chinese individuals with hypercholesterolemia. These results represent novel, potentially valuable evidence supporting the beneficial effects of anthocyanins on CXCL8 and CCL2.

Table 4 Changes in the inflammatory cytokine levels in the participants

	Placebo (n = 73)				Anthocyanin (n = 73)				P-value ^b
	Baseline	12 wk	24 wk	Mean change, % (95% CI) ^a	Baseline	12 wk	24 wk	Mean change, % (95% CI) ^a	
hsCRP (mg/L)	2.26 (0.97 to 3.72)	2.23 (1.08 to 3.76)	2.19 (0.93 to 3.82)	-2.5 (-7.0 to 2.1)	2.25 (1.06 to 4.25)	1.95 (0.92 to 2.84) ^c	1.74 (0.86 to 2.60)	-21.6 (-37.5 to -5.7) ^d	0.001
TNF- α (pg/mL)	18.0 \pm 6.0	19.1 \pm 6.7	18.5 \pm 5.4	2.8 (-3.4 to 9.1)	18.7 \pm 6.4	17.9 \pm 5.1	18.4 \pm 5.6	-1.6 (-5.6 to 3.4)	0.673
IL-1 β (pg/mL)	4.77 \pm 1.71	4.23 \pm 0.91	4.71 \pm 1.60	-1.3 (-5.3 to 2.7)	5.18 \pm 2.11	4.62 \pm 1.20 ^c	4.51 \pm 1.60 ^c	-12.8 (-24.4 to -1.2) ^d	0.019
sP-selectin (ng/mL)	149.9 \pm 42.7	139.9 \pm 39.0	139.9 \pm 39.0	-2.4 (-15.4 to 10.6)	150.9 \pm 37.0	144.4 \pm 35.3	134.6 \pm 32.8	-5.9 (-17.7 to 6.0) ^d	0.027

The data are expressed as the mean \pm SD

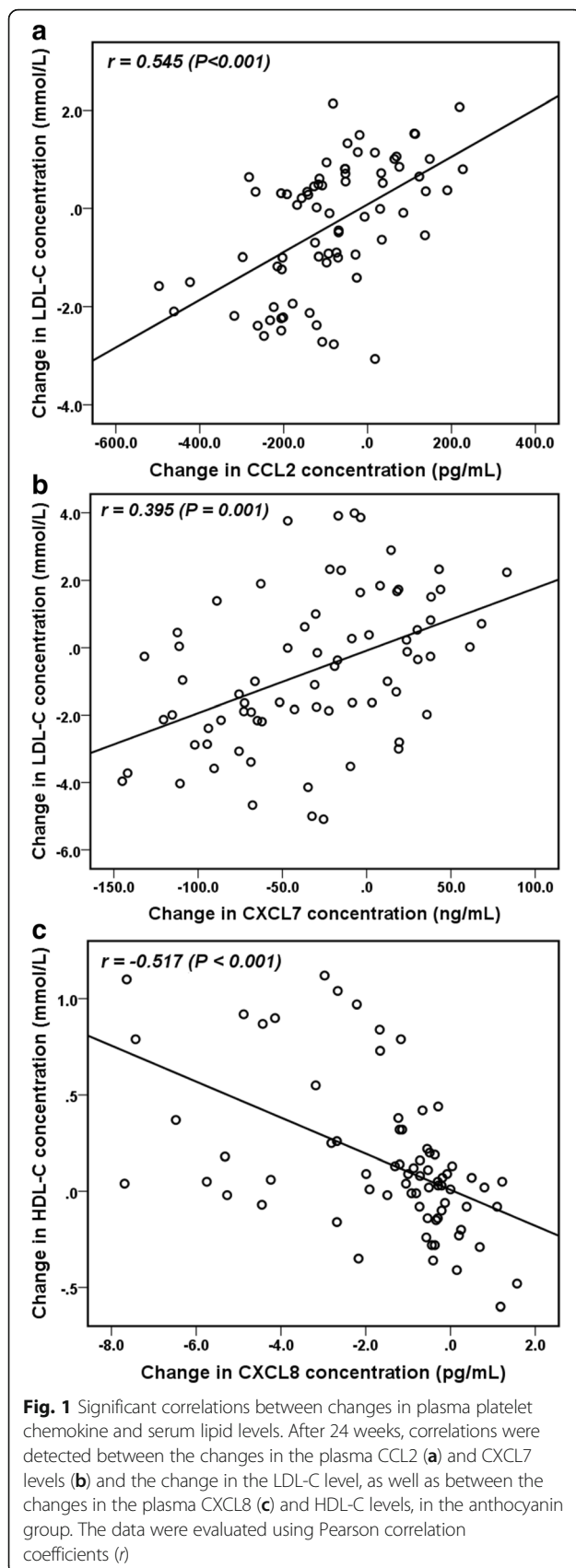
No significant differences in any variable were observed between the two groups at baseline, as determined using unpaired Student's *t*-test

^a Calculated as (value at week 24 - value at baseline)/value at baseline \times 100

^b The effects of the intervention on these variables were evaluated by repeated-measures ANOVA

^c *P* < 0.05 vs. baseline, as assessed by paired Student's *t*-test

^d *P* < 0.05 vs. percentage changes in the placebo group, as assessed by unpaired Student's *t*-test



In addition to the above mentioned platelet chemokines, platelets also secrete CXCL1, CXCL4L1, MIF and PAI-1, which are crucially involved in interactions with platelets, leukocytes and endothelial cells during the early stage of atherogenesis [55, 56]. In an ApoE*3Leiden mouse model, administration of an anthocyanin-rich extract, Mirtoselect (the original standardized bilberry extract), for 20 weeks has been shown to attenuate the high-cholesterol diet-induced increase in the CXCL1 level [57]. Similarly, Jiang et al. have found that administration of anthocyanins from purple sweet potato and purple potato (5 mg/kg) to Kunming mice for 5 weeks inhibits the increased CXCL1 mRNA expression induced by alcohol [58]. Another animal study has revealed that administration of $\geq 5\%$ Saskatoon berry powder to leptin receptor-deficient mice for 4 weeks suppresses the increase in the PAI-1 level [59]. Further, Lamy et al. have shown that delphinidin down-regulates the PAI-1 level in glioblastoma cells [60]. Broekhuizen et al. have found that the MIF and CCL2 levels are reduced following consumption of a polyphenol-rich extract (500 mg daily) for 4 weeks in subjects with clustered cardiometabolic risk factors [61]. In contrast with the above results, another study has shown that administration of an anthocyanin-rich black elderberry extract (0.17% anthocyanin, w/w) for 16 weeks does not decrease the serum PAI-1 level in diet-induced obese mice [62]. However, no studies have examined the effects of purified anthocyanins on these platelet chemokines in individuals with hypercholesterolemia. In our study, the CXCL1, CXCL4L1, MIF and PAI-1 levels were not significantly altered after anthocyanin supplementation for 24 weeks, although slight decreases were observed. Therefore, we speculate that these chemokines may not be sensitive to anthocyanins in hypercholesterolemic patients or that these patients may require a larger intervention dose or a longer intervention period to observe the significant inhibitory effects of anthocyanins.

Hypercholesterolemia is associated with elevated levels of peripheral leukocytes, platelets and cholesterol-induced pro-inflammatory cytokines [63]. Evidence suggests that platelet chemokines are involved in interactions between ox-LDL, platelets and leukocytes, possible contributing to atherogenesis in hypercholesterolemia [64, 65]. The primary platelet chemokines CXCL4 and CCL2 have been demonstrated to co-localize with ox-LDL in atherosclerotic lesions and to facilitate ox-LDL uptake by monocytes [66, 67]. In addition, platelet chemokines are produced following stimulation of TNF- α and IL-1 β [68], which result in recruitment of leukocytes to the site of vascular injury and enhancement of inflammatory processes. Recently, some studies have revealed that anthocyanin supplementation significantly reduces the serum lipid [69] and pro-inflammatory cytokine

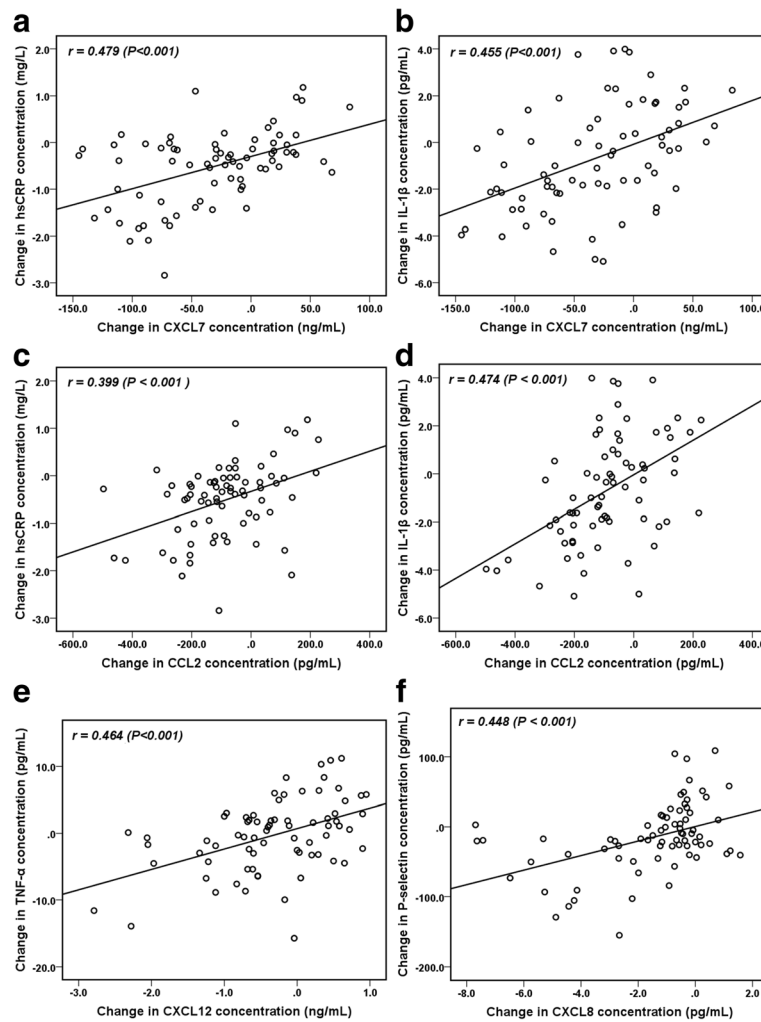


Fig. 2 Significant correlations between changes in plasma platelet chemokine levels and inflammatory biomarker levels. After 24 weeks, correlations were detected between the change in the plasma CXCL7 level and the changes in the hsCRP (**a**) and IL-1 β levels (**b**); between the change in the plasma CCL2 level and the changes in the hsCRP (**c**) and IL-1 β levels (**d**); between the changes in the plasma CXCL12 and TNF- α levels (**e**); and between the changes in the plasma CXCL8 and sP-selectin levels (**f**) in the anthocyanin group. The data were evaluated using Pearson correlation coefficients (r)

levels [70–72], consistent with the effects of anthocyanins observed in our previous studies [32, 35]. In the present study, we first revealed that the reductions in the platelet chemokine levels caused by anthocyanins were positively correlated with the changes in the inflammatory marker and blood lipid levels. In particular, the decrease in the level of CXCL7, which is only secreted by platelets, was positively correlated with the decreases in the LDL-C, hsCRP and IL-1 β levels. These findings indicate a potential mechanism by which anthocyanins exert protective effects on the cardiovascular system, achieved through the comprehensive regulation of platelet chemokines, lipid metabolism and inflammation, in which platelet chemokines may play pivotal roles. Selective interference with these functional interactions of platelet chemokines may thus enable the

development of customized treatments for specific inflammatory conditions associated with CVDs.

Chemokines interact with their target cells by binding to G protein-coupled receptors to initiate inflammatory responses [73]. Both chemokines and their receptors are the key mediators of inflammatory processes regulating leukocyte extravasation and directional migration to vascular lesions [74–76]. We suspected that chemokine receptors might be among the targets of anthocyanins. Based on this hypothesis, we conducted an in vitro experiment to explore the effects of anthocyanins on platelet chemokine receptors. Interestingly, our primary data showed that anthocyanins significantly inhibited the expression of CXCR2, CXCR4 and CCR5 on leukocytes and platelets (unpublished data). These results indicate that inhibition of the corresponding chemokine receptors

may be one of the mechanisms of anthocyanins in reducing inflammatory cell recruitment. In addition, some studies have shown that platelet chemokines promote the recruitment of inflammatory cells to injured tissues by forming heterocomplexes with molecules released from neutrophils and other cells, such as HNP1-CCL5 [77], HMGB1-CXCL12 [78], and CXCL4-CCL5 [79]. Hence, selective disruption of the formation of these heterocomplexes may represent a novel mechanism of action of anthocyanins in weakening the interactions between platelets and other inflammatory cells. Further studies are required to investigate the mechanisms underlying the effects of anthocyanins.

Conclusions

In conclusion, the results of this study suggest that platelet chemokines may be important targets of anthocyanins in prevention of the development of early atherosclerosis. Anthocyanins exert their cardiovascular protective effects by influencing platelet chemokines, blood lipids and the inflammatory response.

Abbreviations

ENA-78: Epithelial neutrophil-activating peptide; GRO- α : Growth-regulated oncogene- α ; HDL-C: High-density lipoprotein-cholesterol; HsCRP: High-sensitivity C-reactive protein; HUVECs: Activated human umbilical vein endothelial cells; IL-1 β : Interleukin-1 β ; IL-8: Interleukin-8; LDL-C: Low-density lipoprotein-cholesterol; MCP-1: Monocyte chemoattractant protein-1; MIF: Macrophage migration inhibitory factor; NAP-2: Neutrophil-activating peptide-2; ox-LDL: Oxidized low density lipoprotein; PAI-1: Human plasminogen activator inhibitor1; SDF-1 α : Stromal cell-derived factor 1 α ; sP-selectin: Soluble P-selectin; TNF- α : Tumor necrosis factor- α

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Availability of data and material

The datasets obtained and/or analyzed in the current study are available on reasonable request from the corresponding author.

Authors' contributions

XZ, YZ, WL and YY participated in the study design; XZ, YZ, FS, YY, FY and DL were involved in the collection and analysis of data; XZ wrote the manuscript; and YY and WL provided insightful comments and suggestions during preparation of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

All authors provide consent for publication of this paper.

Ethical approval and consent to participate

The study was approved by the ethics committee of Sun Yat-sen University, and written informed consent was obtained from all participants.

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