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Research Paper

Dose-dependent effects of anthocyanin supplementation on platelet function in subjects with dyslipidemia: A randomized clinical trial

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ABSTRACT

Background: Dyslipidemia induces platelet hyperactivation and hyper-aggregation, which are linked to thrombosis. Anthocyanins could inhibit platelet function *in vitro* and *in mice fed high-fat diets* with their effects on platelet function in subjects with dyslipidemia remained unknown. This study aimed to investigate the effects of different doses of anthocyanins on platelet function in individuals with dyslipidemia.

Methods: A double-blind, randomized, controlled trial was conducted. Ninety-three individuals who were initially diagnosed with dyslipidemia were randomly assigned to placebo or 40, 80, 160 or 320 mg/day anthocyanin groups. The supplementations were anthocyanin capsules (Medox, Norway). Platelet aggregation by light aggregometry of platelet-rich plasma, P-selectin, activated GPⅡbⅢa, reactive oxygen species (ROS), and mitochondrial membrane potential were tested at baseline, 6 weeks and 12 weeks.

Findings: Compared to placebo group, anthocyanins at 80 mg/day for 12 weeks reduced collagen-induced platelet aggregation (-3.39±2.36%) and activated GPⅡbⅢa (-8.25±2.45%) ($P < 0.05$). Moreover, compared to placebo group, anthocyanins at 320 mg/day inhibited collagen-induced platelet aggregation (-7.05±2.38%), ADP-induced platelet aggregation (-7.14±2.00%), platelet ROS levels (-14.55±1.86%), and mitochondrial membrane potential (7.40±1.56%) ($P < 0.05$). There were dose-response relationships between anthocyanins and the attenuation of platelet aggregation, mitochondrial membrane potential and ROS levels (P for trend <0.05). Furthermore, significantly positive correlations were observed between changes in collagen-induced ($r = 0.473$) or ADP-induced ($r = 0.551$) platelet aggregation and ROS levels in subjects with dyslipidemia after the 12-week intervention ($P < 0.05$).

Interpretation: Anthocyanin supplementation dose-dependently attenuates platelet function, and 12-week supplementation with 80 mg/day or more of anthocyanins can reduce platelet function in individuals with dyslipidemia.

Abbreviations: ROS, reactive oxygen species; ASCVD, atherosclerotic cardiovascular disease; IPAQ, International Physical Activity Questionnaire; BW, body weight; BH, body height; NC, neck circumference; WC, waist circumference; WHR, waist-hip ratio; HC, hip circumference; HR, heart rate; BP, blood pressure; EDTA, ethylene diamine tetraacetic acid; PRP, platelet-rich plasma; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; TC, total cholesterol; ApoA-1, apolipoprotein A-1; ApoB, apolipoprotein B; FBG, fasting blood glucose; FINS, fasting insulin; UA, uric acid; HOMA-IR, insulin resistance; PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin clotting time; Fib, fibrinogen; MDA, malondialdehyde; TSOD, total superoxide dismutase; 8-iso-PGF2 , 8-iso-prostaglandin F2 ; ANOVA, analysis of variance; SEM, standard error of the mean; ACN, anthocyanin

Data described in the manuscript, code book, and analytic code will be made available upon reasonable request.

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Research in context

Evidence before this study

We searched PubMed for manuscripts published in English from inception and until April 23, 2021, with "anthocyanins" in combination with "platelet", "dose-dependent effects" and "randomized controlled trial". We found zero randomized controlled trials assessing the dose-dependent effects of anthocyanins on platelet function in subjects with dyslipidemia. Previous studies have shed light on the inhibitory effects of anthocyanins on platelet hyperactivation and hyper-aggregation *in vitro* and in mice fed high-fat diets. One RCT with a single dosage of anthocyanin in healthy sedentary population was identified. This study reported that anthocyanin supplementation had the potential to alleviate platelet activation and aggregation in healthy sedentary population.

Added value of this study

The data show that anthocyanins supplementation for 12 weeks can attenuate platelet function and oxidative stress in a dose-response manner, and 80 mg or more per day of anthocyanins are recommended to ameliorate platelet function and oxidative stress in individuals with dyslipidemia. Besides, anthocyanins at 320 mg per day for 12 weeks can also increase serum HDL-C and ApoA-1 levels.

Implications of all the available evidence

This study, combined with previously reported studies *in vitro* and in mice shows that there is a consistent effect of anthocyanins on platelet function and oxidative stress in individuals with dyslipidemia. In future, more randomized controlled trial should be carried out to determine the benefits of anthocyanins on platelet function in individuals with other diseases.

1. Introduction

Atherosclerotic cardiovascular disease (ASCVD) is a major cause of death in individuals with dyslipidemia [1]. Dyslipidemia, a lipid metabolism disorder, is an independent risk factor for atherosclerotic plaque and thrombotic events [2,3]. Early treatment and prevention of ASCVD in individuals with dyslipidemia are of considerable importance.

Platelet hyperreactivity is a strong and independent predictor of thrombotic events [4–6]. In dyslipidemia, blood platelets exhibit hyperreactivity, increased expression of activated GPIIbIIIa and P-selectin and enhanced aggregation which are involved in the development of atherosclerotic plaques and thrombotic events [7–11]. Some studies mechanistically revealed that reactive oxygen species (ROS) in platelets and elevated oxidation reactions also led to platelet hyperactivation and hyper-aggregation in patients with dyslipidemia [7,10,12]. Hence, attenuating platelet activation, aggregation and oxidative stress is a promising strategy for preventing thrombotic events in patients with dyslipidemia.

Currently, many antiplatelet drugs (such as clopidogrel, aspirin and ticagrelor) are widely used for primary and secondary prevention of thrombotic events [13,14]. However, epidemiologic studies have shown that long-term application of antiplatelet drugs might induce bleeding risk, thus affecting the balance of benefits and risks for preventing thrombotic events [15,16]. Therefore, a safe strategy should be developed to inhibit platelet activation and aggregation in patients with dyslipidemia.

Particularly, the use of plant-derived bioactive flavonoids is considered a promising strategy for ASCVD risk control [17]. Certain natural foods, such as tomato extract, have been shown to exhibit beneficial antiplatelet properties *in vitro* and *in vivo* [18–20]. Anthocyanins, a main subclass of flavonoids, are mainly found in grapes, blueberries, and black rice. In our previous studies, we found that supplementation with anthocyanins for 12 weeks positively improved antioxidative, anti-inflammatory, and cholesterol efflux capacity in a dose-dependent manner in individuals with dyslipidemia [21,22]. Kiara Thompson *et al*. reported that anthocyanin supplementation had the potential to alleviate platelet activation and aggregation in healthy sedentary population [23]. Our previous studies have found that anthocyanins can significantly ameliorate platelet activation and aggregation *in vitro* and in mice fed high-fat diets [24–26]. In addition, anthocyanins also inhibited platelet granule secretions in patients with hypercholesterolemia [27]. However, the effects of anthocyanins on platelet activation, aggregation and oxidative stress in individuals with dyslipidemia have not been investigated, and the efficacy and dose of anthocyanins in this respect are unknown yet. Therefore, this double-blind, randomized, controlled trial aimed to study the effects of anthocyanins on platelet function and explore dose responses in individuals with dyslipidemia.

2. Materials & methods

2.1. Study population

All the subjects were recruited from local communities in Guangzhou, China, through flyers, medical record reviews, or clinicians' recommendations at community health centers. In 2018, we recruited 176 eligible subjects to complete a randomized controlled trial and found that anthocyanins could improve antioxidative and anti-inflammatory effects in a dose-dependent manner in individuals with dyslipidemia [21,22]. Due to limited laboratory conditions at that time, we cannot perform platelet function tests as these tests need to be completed within two hours after blood collection. To determine the effects of anthocyanins on platelet activation, aggregation and oxidative stress in individuals with dyslipidemia, we later recruited additional 104 eligible subjects from the Dadong Street Community Health Service Center and conducted this trial in 2019. A total of 93 subjects completed this trial. The enrollment and intervention were conducted in accordance with the Declaration of Helsinki guidelines. The Ethics Committee of Sun Yat-sen University approved the study protocol. All participants signed written informed consent forms prior to enrollment. The trial was registered at ClinicalTrials.gov (NCT03415503).

The inclusion criteria were as follows: [1] aged 35 to 70 years; [2] dyslipidemia comprising any two or more of the following four criteria: fasting serum triglyceride (TG) $\geq$ 150 mg/dL (1.70 mmol/L), total cholesterol (TC) $\geq$ 200 mg/dL (5.20 mmol/L), low-density lipoprotein cholesterol (LDL-C) $\geq$ 120 mg/dL (3.12 mmol/L), or high-density lipoprotein cholesterol (HDL-C) $\leq$ 35 mg/dL (0.91 mmol/L); [3] less eating out of home; [4] and having stable body weight in the past 3 months [28,29].

The exclusion criteria included: [1] taking any medications known to affect platelet or lipid metabolism, currently or within the past 6 months; [2] taking any anthocyanin supplements or anthocyanin-rich foods currently or within the past 2 months; [3] lactating or pregnant women; [4] or suffering from severe acute or chronic illness.

2.2. Study design

This was a 12-week randomized, double-blind, placebo-controlled trial. To evaluate the dose-response relationship of anthocyanins on platelet function, we set five doses of anthocyanins according to the results of previous clinical trials [21,22,27].

First, a total of 104 eligible subjects were stratified by gender and then randomly assigned to one of the five groups: placebo (n = 20) or anthocyanin at 40 mg/day (n = 20), 80 mg/day (n = 20), 160 mg/day (n = 22), or 320 mg/day (n = 22) by random numbers generated by SPSS v22.0 (SPSS Inc., Chicago, IL, USA) (**Fig. 1**).

Blinding was performed according to our previous research [21,22]. Briefly, a technician who did not participate in the experiments, data collection or analysis was responsible for randomization and the management of the packaged supplements. Participants, investigators, and laboratory technicians were blinded to the treatment assignments.

During the intervention period, three types of oral capsules with the same weight, appearance, taste, and packaging were used: 40 or 80 mg anthocyanin (Medox) and placebo capsules. In detail, subjects in placebo group consumed four placebo capsules, subjects in the 40 mg anthocyanins group consumed one 40 mg anthocyanins and three placebo capsules, subjects in the 80 mg anthocyanins group consumed one 80 mg anthocyanins and three placebo capsules, subjects in the 160 mg anthocyanins group consumed two 80 mg anthocyanins and two placebo capsules and subjects in the 320 mg anthocyanins group consumed four 80 mg anthocyanins capsules per day. Subjects were asked to take two capsules in the morning and two in the evening after meals. Subject compliance was assessed by counting the number of returned capsules when they received their supplements once every 2 weeks.

2.3. Study supplements

Anthocyanin (Medox) and placebo capsules were provided by Medpalett AS (Sandnes, Norway). The Medox capsules contain 80 or 40 mg anthocyanins, both of which comprise 17 different natural anthocyanins purified from bilberry (*Vaccinium myrtillus*) and blackcurrant (*Ribes nigrum*; refer to **Supplemental Tables 1, 2, and 3** for the ingredients of the anthocyanin capsules). The Medox capsules also contained 4% pullulan, maltodextrin and citric acid to maintain stability, whereas the placebo capsules contained only pullulan and maltodextrin.

The subjects were asked to maintain their usual dietary intake and physical activities. The 24 h dietary recall data were recorded on 3 consecutive days, and international physical activity questionnaire (IPAQ) scores were collected by the trained staff via face-to-face interviews at baseline and after 12 weeks, as described in our previous studies [21,22].

2.4. Anthropometric analyses

Anthropometric measurements were performed by a trained examiner as previously described [21,22]. Body weight (BW), body height (BH), neck circumference (NC), waist circumference (WC), waist-hip ratio (WHR), hip circumference (HC), heart rate (HR), and blood pressure (BP) were measured according to standard protocols.

2.5. Biological samples collection and assessment of biochemical biomarkers

At baseline, and after 6 and 12 weeks, overnight fasting venous blood samples were collected in tubes containing sodium citrate anticoagulation, separation gel coagulation, or ethylene diamine tetraacetic acid (EDTA) between 8:00 and 9:00. Samples were separately

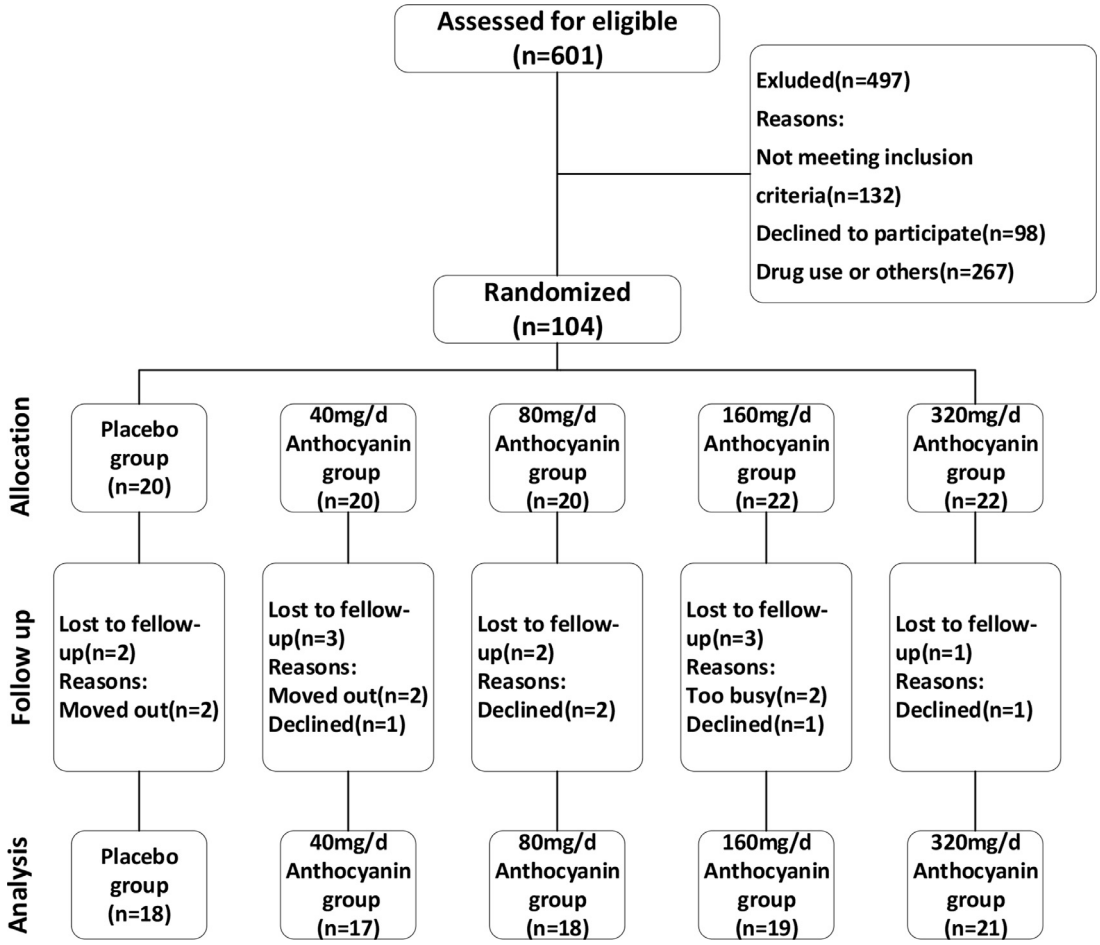

Fig. 1. The participant flowchart of the study.

centrifuged to prepare serum or platelet-rich plasma (PRP) as described in a previous study [24]. PRP was immediately used to assay platelet aggregation and activation or other related parameters. Serum samples and first-morning urine samples were stored at -80°C until subsequent analyses.

Fasting blood samples were subjected to lipid profile analysis, including LDL-C, HDL-C, serum TG, serum TC, apolipoprotein A-1 (ApoA-1), and apolipoprotein B (ApoB). Lipid profile analyses were performed on the Cobas c311 automated assay analyzer (c311, Roche Diagnostics, Switzerland). Enzymatic methods were used to determine the concentrations of HDL-C, LDL-C, TC, and TG. The concentrations of ApoA-1 and ApoB were determined by immunonephelometry. Other biochemical analyses included fasting blood glucose (FBG), insulin (FINS), and uric acid (UA) concentrations that were measured using the Cobas c311 automated assay analyzer. The homoeostasis model assessment of insulin resistance (HOMA-IR) was calculated based on FBG and FINS: HOMA-IR = FINS (mU/L) × FBG (mmol/L)/22.5. Prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin clotting time (TT), and plasma fibrinogen (Fib) were performed on a Sysmex5100 system (Siemens Healthineers, Malvern, USA).

To assess the changes in platelet mitochondrial membrane potential and ROS, freshly isolated PRP was separately stained with 40 nmol/L tetramethylrhodamine methyl ester (TMRM, Abcam, MA, UK) or 20 μmol/L 2',7' –dichlorofluorescin diacetate (DCFDA, Abcam, UK) for 30 min at 37 °C, and was analyzed via a calibrated CytoFLEX flow cytometer (Beckman Coulter, CA, USA) [7]. Serum malondialdehyde (MDA) was determined using a commercial ELISA kit (catalog no. S0131, Beyotime, Shanghai, China). The serum total superoxide dismutase (T-SOD) was measured using a T-SOD assay kit (catalog no. A001-1-2, Jiancheng, Nanjing, China). Urine 8-iso-prostaglandin F2α (8-iso-PGF2α) was determined by a competitive enzyme immunoassay kit (catalog no.516351, Cayman Chemical Company, Ann Arbor, MI, USA) in spot urine samples. Helmersson and Basu have reported that urinary F2-isoprostane isomer concentrations in spot urine showed no significant variation from those measured in 24 h urine samples in the same individuals by radioimmunoassay [30].

2.6. Flow cytometric analysis of platelet activation

Analysis of platelet activation was performed on a flow cytometer as previously described [24,31]. Briefly, freshly isolated PRP was separately incubated with different fluorescent-labeled antibodies activated GPⅡbⅢa (FITC-conjugated mouse anti-human PAC-1, BD Biosciences, San Jose, CA, USA) or P-selectin (FITC-conjugated mouse anti-human CD62p, BD Biosciences, San Jose, CA, USA) at room temperature for 30 min. Platelets were initiated by adding 100 μmol/L adenosine diphosphate (ADP) at room temperature, followed by fixation with 1% paraformaldehyde (pH = 7.2) before analysis. All samples were analyzed via a calibrated CytoFLEX flow cytometer.

2.7. Assessment of platelet aggregation

Platelet aggregation was performed on a Chronolog aggregometer (Chrono-Log Corp., PA, USA) as previously described [31,32]. Briefly, fresh PRP prepared from subjects was stimulated by ADP or collagen at baseline, at 6 weeks, and at 12 weeks. Platelet aggregation was evaluated on a Chronolog aggregometer (Chrono-Log Corp., PA, USA) in PRP (3.0×10^8 platelets/mL) at 37 °C with a sample stir speed of 1000 rpm, and the change in light transmission was monitored and recorded for at least 6 minutes.

2.8. Sample size estimation

The sample size was calculated via PASS software (version 11.0, NCSS Inc.). In our previous randomized controlled trial, supplementation with 320 mg/day anthocyanin resulted in an 8.4% change in blood levels of platelet-derived Regulated on Activation Normal T cell Expressed and Secreted (RANTES) compared to those in the placebo group [27]. Based on the conventional assumption of a two-tailed α level of 0.05 and β level of 0.10, it was determined that 15 subjects should be recruited per group. Taking into account a 10% loss to follow-up rate, at least 17 individuals were needed in each group. We also performed a *post hoc* power analysis to extrapolate the sample size according to the effect of 40 mg/day anthocyanin on P-selectin-positive platelets in our current study. This sample size was approximately equal to the sample size in our current study.

2.9. Statistical analysis

All statistical analyses were two-tailed and performed using SPSS v22.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $P < 0.05$. The data are expressed as the mean ± standard error of the mean (SEM) unless otherwise stated. For categorical variables, analysis was performed using the chi-square test. For continuous variables, variables were checked for normal distribution and were natural log-transformed if they were not normally distributed. The mean substitution method was used to handle missing values. We also performed sensitivity analysis in which the missing values were not imputed. The changes after 6 weeks and 12 weeks were calculated as values after intervention deducted from the values at baseline. The comparability of the five groups at baseline was assessed by one-way analysis of variance (ANOVA) with the *post hoc* Dunnett's test.

Repeated-measures ANOVA was used to analyze the main effect on groups over time. To compare the effect of anthocyanins at different weeks, Student's t tests for paired data were used to assess changes within the group at baseline and after 6 or 12 weeks, and differences in the change in biomarkers between the intervention groups were analyzed by ANOVA with the *post hoc* Dunnett's test. We also analyzed the dose-related effects of anthocyanins supplementation on metabolic changes via linear trend analysis. Stratified analyses were performed with ANOVA and the *post hoc* Dunnett's test to compare the changes of platelet function and oxidative stress after 12-week treatment stratified by lipids levels. Pearson correlation coefficients (r) were calculated to assess the associations between changes in oxidative stress and changes in platelet aggregation over the 12-week study period.

2.10. Role of funding source

The funders of the present study (namely National Natural Science Foundation of China, Guangzhou Science, Technology, and Innovation Commission, and Shenzhen Science, Technology, and Innovation Commission) only provided financial support and did not participate in the study design, data collection, data analysis, interpretation and writing of the report.

3. Results

3.1. Characteristics, diet monitoring and ***coagulation function***

A total of 104 eligible subjects were randomly assigned to one of the five groups (**Fig. 1**). The characteristics of all 104 eligible subjects with dyslipidemia are shown in **Table 1**. Participants were predominantly females (74.0%) with ages ranging from 36 to 70 years. Participants in the five groups were comparable in age, sex, smoking status, BW, BMI, NC, WC, WHR, HR, BP, lipid profiles, FBG, FINS, and HOMA-IR at baseline (**Table 1**). A total of 93 subjects completed the trial and were included in the analysis (**Fig. 1**). The characteristics of 93 subjects with dyslipidemia are shown in **Supplemental Table 4**. At baseline, 93 subjects in the five groups were comparable in terms of age, sex, smoking status, BW, BMI, NC, WC, WHR, HR, BP, lipid profile,

Table 1
Baseline characteristics of subjects with dyslipidemia.

	Placebo (n=20)	40 mg ACN (n=20)	80 mg ACN (n=20)	160 mg ACN (n=22)	320 mg ACN (n=22)	*P* value[b]
Age, y	56.20±1.56[a]	56.55±1.99	61.05±1.07	58.77±1.59	58.45±1.77	0.247
Gender (M/F)	6/14[c]	6/14	5/15	5/17	5/17	0.964
Weight (kg)	61.14±2.11	62.05±3.19	59.93±2.47	59.86±2.01	62.37±2.63	0.928
Smoking (%)	1(5.0)	2(10.0)	0(0)	1(4.5)	0(0)	0.570
BMI (kg/m^2)	24.76±0.4	23.65±0.78	23.81±0.68	23.53±0.52	24.75±0.8	0.486
NC (cm)	33.91±0.81	33.89±0.81	33.37±0.71	33.00±0.65	33.86±0.81	0.882
WC (cm)	84.29±1.86	83.52±2.38	83.75±1.74	84.06±1.41	85.49±2.03	0.954
WHR	0.89±0.01	0.88±0.01	0.89±0.01	0.89±0.01	0.89±0.01	0.940
SBP (mmHg)	122.81±3.64	117.82±3.3	114.02±3.24	119.81±2.59	118.39±2.13	0.359
DBP (mmHg)	78.99±1.91	73.53±1.83	72.45±2.26	73.73±1.57	73.75±1.74	0.126
HR (beats/min)	79.77±1.59	77.32±2.54	76.74±2.10	75±1.54	74.75±2.34	0.426
TC (mmol /L)	6.24±0.23	6.37±0.18	6.06±0.12	6.13±0.14	6.53±0.17	0.284
HDL-C (mmol /L)	1.39±0.06	1.52±0.09	1.44±0.10	1.53±0.08	1.48±0.06	0.675
LDL-C (mmol /L)	4.41±0.21	4.65±0.18	4.16±0.16	4.21±0.14	4.56±0.17	0.191
TG (mmol/L)	2.06±0.20	1.56±0.16	2.17±0.23	1.81±0.18	1.70±0.17	0.156
ApoA-1 (g/L)	1.45±0.04	1.44±0.06	1.44±0.05	1.49±0.05	1.45±0.03	0.935
ApoB(g/L)	1.36±0.05	1.39±0.05	1.25±0.04	1.26±0.04	1.35±0.05	0.109
FBG (mmol/L)	5.38±0.21	5.23±0.16	5.19±0.12	5.30±0.13	5.46±0.22	0.809
FINS (mU/L)	11.39±1.04	10.32±1.32	9.5±0.95	9.11±0.95	10.30±1.04	0.611
HOMA-IR	2.75±0.27	2.48±0.34	2.21±0.23	2.21±0.26	2.56±0.34	0.662

[a] Data are presented as mean ± SEM or n (%).
[b] P values are for comparison among the five groups (either a one-way analysis of variance or chi-square test for independent data).
[c] For categorical variables, analysis was performed using the chi-square test. SEM, standard error of mean; ACN, anthocyanin; M, male; F, female; BMI, body mass index; NC, neck circumference; WC, waist circumference; WHR, waist-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, total triglyceride; ApoA-1, apolipoprotein A-1; ApoB, apolipoprotein B; FBG, fasting blood glucose; FINS, fasting insulin; HOMA-IR, homoeostasis model assessment of insulin resistance.

FBG, FINS, and HOMA-IR (**Supplemental Table 4**). After 12 weeks of intervention, there was no significant difference among the groups in terms of BW, BMI, NC, WC, WHR, HR, BP, FBG, FINS, or HOMA-IR (**Supplemental Table 4**). However, significant differences in HDL-C levels ($P < 0.05$) and ApoA-1 levels ($P < 0.05$) were observed among the five groups after 12 weeks (**Supplemental Table 5**). Compared with placebo, supplementation with 320 mg/day of anthocyanin significantly increased HDL-C levels and ApoA-1 levels with baseline levels of 1.49±0.07 mmol/L and 1.45±0.04 g/L and post-intervention levels of 1.54±0.07 mmol /L and 1.53±0.04 g/L, respectively ($P < 0.05$, **Supplemental Table 5**).

There was no significant difference in adverse events, blood coagulation function, daily physical activity status or the intake of energy, total protein, carbohydrates, lipids, dietary fiber, anthocyanins and vitamins with antioxidative capacity among the five groups at baseline and after intervention (**Supplemental Tables 6, 7 and 8**). The mean substitution method was used to impute missing values on coagulation function indexes. The analysis in which the missing values were not imputed produced similar result that there was no significant difference among the groups in blood coagulation function (**Supplemental Table 9**).

3.2. Effects of anthocyanins on platelet activation and aggregation

Anthocyanin supplementation at different doses for 6 weeks did not cause significant changes in platelet functions. After 12 weeks, 80 mg/day, 160 mg/day, and 320 mg/day of anthocyanins significantly reduced the percentage of activated GPⅡbⅢa positive platelets, with baseline levels of 54.77±2.23%, 59.57±2.22% and 55.07±1.36% and post-intervention levels of 46.52±2.60%, 45.58±1.42% and 40.29±1.98%, respectively ($P < 0.05$, **Table 2**). Twelve weeks of 320 mg/day anthocyanins significantly decreased the percentage of P-selectin positive platelets, with baseline values of 53.17±1.75% and a post-intervention value of 43.63±1.42% ($P < 0.05$, **Table 2**). Similarly, after 12 weeks, 80 mg/day, 160 mg/day, and 320 mg/day anthocyanins resulted in a moderately significant reduction in platelet aggregation stimulated by collagen, with baseline values of 73.67±3.44%, 79.37±1.97% and 77.67±2.12% and post-intervention values of 70.28±3.73%, 72.84±2.15% and 70.62±3.03%, respectively ($P < 0.05$, **Table 2**). In addition, 12 weeks of anthocyanins at dosages of 160 mg/day and 320 mg/day significantly decreased ADP-induced platelet aggregation, with baseline values of 69.74±2.78% and 69.24±2.81% and post-intervention values of 63.32±3.12% and 62.10±3.13%, respectively ($P < 0.05$, **Table 2**).

The percentage of activated GPⅡbⅢa positive platelets from baseline to 12 weeks significantly decreased in the 80 mg/day (-8.25±2.45%, $P < 0.01$), 160 mg/day (-13.99±2.56%, $P < 0.01$) and 320 mg/day anthocyanin (-14.78±2.01%, $P < 0.01$) groups (Table 2). Similarly, the percentage of P-selectin-positive platelets from baseline to 12 weeks was reduced in the 320 mg/day anthocyanin group (-9.53±1.54%, $P < 0.05$) [Table 2). In addition, there were significant decreases in collagen-induced platelet aggregation from baseline to 12 weeks in the 80 mg/day anthocyanin (-3.39±2.36%, $P < 0.05$), 160 mg/day anthocyanin (-6.53±1.72%, $P < 0.01$), and 320 mg/day anthocyanin (-7.05±2.38%, $P < 0.01$) groups. The change in ADP-induced platelet aggregation from baseline to 12 weeks in the 160 mg/day anthocyanin (-6.42±1.76, $P < 0.01$) and 320 mg/day anthocyanin groups (-7.14±2.00%, $P < 0.01$) was different from the change in the placebo group (6.38±2.08%) (**Table 2**). Stratified analyses (**Supplementary Table 10**) showed that among subjects with high TC, platelet activation, and aggregation have robust dose–response relationship among five groups.

Further analysis showed that the reductions in P-selectin positive platelets (P for trend < 0.001), activated GPⅡbⅢa positive platelets (P for trend < 0.001), ADP-induced platelet aggregation (P for trend < 0.001), and collagen-induced platelet aggregation (P for trend < 0.001) were significantly dependent on the dose of anthocyanins (**Table 2**).

3.3. Effects of anthocyanins on oxidative stress

After 12 weeks, 160 mg/day and 320 mg/day anthocyanins significantly reduced urinary 8-iso-PGF2α from baseline values of 1.25±0.15 pg/mg creatinine and 1.40±0.14 pg/mg creatinine to post-

Table 2
Effects of anthocyanins on platelet activation and aggregation in subjects with dyslipidemia.

	Placebo (n = 18)	40 mg ACN (n = 17)	80 mg ACN (n = 18)	160 mg ACN (n = 19)	320 mg ACN (n = 21)	P value	P for trend [c]
P-selectin positive platelets (%)[b]							
Baseline	52.23±1.92[a]	47.92±3.40	51.84±1.45	53.73±1.70	53.17±1.75	0.348	0.262
6 weeks	51.80±1.79	50.36±2.11	48.37±1.23	55.78±2.08	51.39±1.38	0.050	0.482
12 weeks	51.58±1.84	50.24±2.13	47.14±1.22	46.55±2.26	43.63±1.42##*	0.018	0.001
6-week change	-0.43±2.02	2.44±3.70	-3.47±1.44	2.05±2.44	-1.78±1.68	0.341	0.619
12-week change	-0.65±2.02	2.32±3.74	-4.70±1.42	-7.18±3.33	-9.53±1.54#	0.009	0.001
Activated GPIIbIIIa positive platelets (%)[b]							
Baseline	52.26±3.35	54.52±2.05	54.77±2.23	59.57±2.22	55.07±1.36	0.255	0.356
6 weeks	58.80±1.73	58.21±2.13	54.73±2.59	60.18±2.25	56.71±1.84	0.432	0.755
12 weeks	58.92±1.75	57.35±1.79	46.52±2.60##*	45.58±1.42##*	40.29±1.98##*	<0.001	<0.001
6-week change	6.55±3.23	3.69±2.74	-0.04±1.86	0.62±1.23	1.64±1.88	0.250	0.219
12-week change	6.66±3.24	2.83±2.48	-8.25±2.45##	-13.99±2.56##	-14.78±2.01##	<0.001	<0.001
ADP-induced platelet aggregation (%)							
Baseline	66.83±2.11	69.71±2.82	62.11±2.34	69.74±2.78	69.24±2.81	0.181	0.364
6 weeks	69.50±2.92	72.00±3.28	64.67±3.86	69.26±2.53	65.52±2.55	0.421	0.285
12 weeks	73.22±2.76	74.29±2.48	63.61±2.91	63.32±3.12*	62.10±3.13#*	0.005	0.002
6-week change	2.67±2.49	2.29±3.29	2.56±3.43	-0.47±2.08	-3.71±1.80	0.324	0.036
12-week change	6.38±2.08	4.59±2.17	1.50±2.87	-6.42±1.76##	-7.14±2.00##	<0.001	<0.001
collagen-induced platelet aggregation (%)							
Baseline	77.94±2.85	74.24±2.15	73.67±3.44	79.37±1.97	77.67±2.12	0.445	0.469
6 weeks	80.33±2.18	80.24±2.47	76.00±2.93	79.11±2.51	75.10±1.95	0.384	0.126
12 weeks	82.61±2.28	78.29±2.33	70.28±3.73#*	72.84±2.15*	70.62±3.03##*	0.009	0.007
6-week change	2.39±4.25	6.00±3.17	2.33±2.27	-0.26±2.83	-2.57±1.74	0.303	0.059
12-week change	4.67±1.64	4.06±2.35	-3.39±2.36#	-6.53±1.72##	-7.05±2.38##	<0.001	<0.001

[a] Mean ± SEM (all such values).
[b] $P < 0.05$, the main effect of intervention was analysis by repeated measures ANOVA. There was an interaction effect between intervention and time in P-selectin, activated GPIIbIIIa, ADP-induced and collagen-induced platelet aggregation.
[c] The dose-related effects of anthocyanins supplementation on biomarkers were investigated via linear trend analysis.
* $P < 0.05$, the differences between baseline and 6-week or 12-week data were compared by Student's t test for paired data.
# $P < 0.05$, the differences between placebo group and anthocyanin groups were compared by *post hoc* Dunnett's test for multiple comparisons.
$P < 0.01$, the differences between placebo group and anthocyanin groups were compared by *post hoc* Dunnett's test for multiple comparisons. SEM, standard error of mean; ACN, anthocyanin.

intervention values of 0.76±0.14 pg/mg creatinine and 0.74±0.16 pg/mg creatinine ($P < 0.05$, **Table 3**). Twelve weeks of anthocyanins at 160 mg/day and 320 mg/day significantly decreased serum MDA, with baseline values of 4.24±0.31 μmol/L and 3.53±0.21 μmol/L and post-intervention values of 3.52±0.20 μmol/L and 2.78±0.16 μmol/L, respectively ($P < 0.05$, **Table 3**). In addition, 12 weeks of anthocyanins at 320 mg/day yielded a further reduction in the percentage of ROS-positive platelets and TMRM-positive platelets compared with those observed in the 160 mg/day and 80 mg/day groups, with baseline values of 30.60±2.28% and 57.40±2.53% and post-treatment values of 16.05±1.22% and 64.80±1.65%, respectively ($P < 0.05$, **Table 3**).

The urinary 8-iso-PGF2α from baseline to 12 weeks significantly decreased in the 80 mg/day anthocyanin (-0.36±0.20 pg/mg creatinine, $P < 0.05$), 160 mg/day anthocyanin (-0.50±0.16 pg/mg creatinine, $P < 0.01$) and 320 mg/day anthocyanin (-0.65±0.12 pg/mg creatinine, $P < 0.01$) groups (**Table 3**). The decrease in serum MDA from baseline to 12 weeks in the 160 mg/day anthocyanin group (-0.73±0.34 μmol/L, $P < 0.01$) and 320 mg/day anthocyanin group (-0.75±0.25 μmol/L, $P < 0.01$) was different from the change in the placebo group (0.49±0.29 μmol/L) (Table 3). In addition, the percentage of ROS-positive platelets from baseline to 12 weeks was significantly reduced in the 160 mg/day anthocyanin group (-14.34±2.88%, $P < 0.01$) and 320 mg/day anthocyanin group (-14.55±1.86%, $P < 0.01$) (**Table 3**). Stratified analyses (**Supplementary Table 10**) showed that among subjects with high TC, T-SOD, urinary 8-iso-PGF2α, MDA, percentage of TMRM-positive platelets, percentage of ROS-positive platelets have robust dose-response relationship among five groups.

The decrease in urinary 8-iso-PGF2α (P for trend < 0.001), serum MDA (P for trend < 0.05) and percentage of ROS-positive platelets (P for trend < 0.01) was significantly dependent on the dose of anthocyanin supplementation (**Table 3**). The improvement in the percentage of TMRM-positive platelets (P for trend < 0.001) and T-SOD (P for trend < 0.05) was significantly dependent on the anthocyanin dose (**Table 3**).

3.4. Association between changes in platelet oxidative stress and platelet aggregation

After the 12-week intervention, the decrease in urinary 8-iso-PGF2α (r = 0.341, $P < 0.01$), serum MDA level (r = 0.251, P = 0.015) and platelet ROS level (r = 0.473, $P < 0.01$) exhibited positive associations with the alteration in collagen-induced platelet aggregation (**Fig. 2**). Positive correlations were also found between the change in urinary 8-iso-PGF2α (r = 0.386, $P < 0.01$), serum MDA level (r = 0.320, $P < 0.01$) or platelet ROS level (r = 0.551, $P < 0.01$) and the change in ADP-induced platelet aggregation (**Fig. 2**).

Table 3
Effects of anthocyanins on oxidative stress in subjects with dyslipidemia

	Placebo (n = 18)	40 mg ACN (n = 17)	80 mg ACN (n = 18)	160 mg ACN (n = 19)	320 mg ACN (n = 21)	P value	P for trend[c]
Urine 8-iso-PGF2α (pg/mg creatinine)							
Baseline	1.32±0.12 [a]	1.24±0.12	1.25±0.12	1.25±0.15	1.40±0.14	0.898	0.484
6 weeks	1.41±0.16	1.17±0.20	1.48±0.17	1.13±0.13	1.30±0.16	0.526	0.667
12 weeks	1.54±0.19	1.22±0.19	0.89±0.21#	0.76±0.14#*	0.74±0.16##*	0.008	0.003
6-week change	0.09±0.14	-0.06±0.23	0.23±0.23	-0.12±0.18	-0.10±0.15	0.635	0.385
12-week change	0.22±0.20	-0.02±0.14	-0.36±0.20#	-0.50±0.16##	-0.65±0.12##	0.001	<0.001
MDA (μmol/L) [b]							
Baseline	3.51±0.19	3.91±0.25	3.77±0.27	4.24±0.31	3.53±0.21	0.204	0.808
6 weeks	3.53±0.37	3.89±0.38	3.51±0.32	3.22±0.30	3.38±0.28	0.692	0.401
12 weeks	3.99±0.18	3.78±0.28	3.44±0.23	3.52±0.20*	2.78±0.16##*	0.001	<0.001
6-week change	0.02±0.43	-0.02±0.38	-0.27±0.39	-1.03±0.45	-0.15±0.37	0.351	0.598
12-week change	0.49±0.29	-0.13±0.41	-0.33±0.42	-0.73±0.34#	-0.75±0.25#	0.072	0.015
T-SOD (U/ml)							
Baseline	147.53±2.84	152.38±3.82	149.24±3.49	148.99±3.08	147.75±2.64	0.838	0.653
6 weeks	153.90±3.84	155.35±5.31	159.92±5.34	156.68±3.20	153.97±2.96	0.838	0.791
12 weeks	148.11±3.34	156.75±3.91	157.18±3.49	157.78±3.04*	163.54±4.73#*	0.081	0.012
6-week change	6.37±2.98	2.98±5.08	10.68±5.58	7.69±2.34	6.22±1.74	0.710	0.930
12-week change	0.58±2.12	4.38±2.30	7.94±3.42	8.79±3.07	15.79±4.96##	0.033	0.012
ROS positive platelets (%)[b]							
Baseline	29.28±2.00	33.17±2.51	32.21±1.96	37.09±2.66	30.60±2.28	0.158	0.871
6 weeks	28.51±1.29	29.04±1.38	33.08±2.03	30.01±1.14	29.91±0.92	0.181	0.780
12 weeks	30.78±1.87	25.56±1.65	25.79±1.72	22.75±1.59##*	16.05±1.22##*	<0.001	<0.001
6-week change	-0.77±2.27	-4.13±3.01	0.87±2.51	-7.08±3.07	-0.69±2.39	0.222	0.997
12-week change	1.50±1.64	-7.61±3.47	-6.42±2.36	-14.34±2.88##	-14.55±1.86##	<0.001	<0.001
TMRM positive platelets (%)[b]							
Baseline	60.19±2.88	59.11±2.68	61.87±2.40	54.99±2.04	57.40±2.53	0.360	0.245
6 weeks	59.75±2.01	57.13±2.41	60.47±2.82	55.96±2.18	58.69±1.93	0.624	0.765
12 weeks	52.93±4.17	55.49±2.32	56.52±1.95	59.15±2.64	64.80±1.65#*	0.020	0.001
6-week change	-0.44±2.54	-1.99±1.67	-1.41±1.28	0.97±2.39	1.29±1.77	0.721	0.259
12-week change	-7.25±3.54	-3.62±2.68	-5.35±2.70	4.16±2.05##	7.40±1.56##	<0.001	<0.001

[a] Mean ± SEM (all such values).
[b] $P < 0.05$, the main effect of intervention was analysis by repeated measures ANOVA. There was an interaction effect between intervention and time in ROS and TMRM.
[c] The dose-related effects of anthocyanins supplementation on biomarkers were investigated via linear trend analysis.
* $P < 0.05$, the differences between baseline and 6-week or 12-week data were compared by Student's t test for paired data.
# $P < 0.05$, the differences between placebo group and anthocyanin groups were compared by *post hoc* Dunnett's test for multiple comparisons.
$P < 0.01$, the differences between placebo group and anthocyanin groups were compared by *post hoc* Dunnett's test for multiple comparisons. SEM, standard error of mean; ACN, anthocyanin; 8-iso-PGF2α, 8-iso-prostaglandin F2α; MDA, malonaldehyde; T-SOD, total superoxide dismutase; ROS, reactive oxygen species.

4. Discussion

In the present study, we found a linear dose-response association for the attenuation of platelet activation, aggregation and oxidative stress after anthocyanin supplementation in subjects with dyslipidemia. After 12-week anthocyanin intervention, anthocyanin supplementation at 80 mg/day partially reduced platelet activation, aggregation and oxidative stress biomarkers. Individuals who received 160 or 320 mg/day anthocyanins for 12 weeks showed further improvement in platelet activation, aggregation, and oxidative stress biomarkers. Anthocyanins at 320 mg/day for 12 weeks also increased serum HDL-C and ApoA-1 levels, and this was consistent with our previous studies [21]. In addition, 6 weeks of anthocyanin supplementation did not significantly improve platelet activation, aggregation, oxidative stress or lipid levels. These findings suggested that anthocyanins could inhibit platelet activation, aggregation and oxidative stress in a dose-response manner and that receiving 80 mg or more of anthocyanins per day could help attenuate platelet function in individuals with dyslipidemia.

Anthocyanin-rich foods and anthocyanin extracts could result in beneficial outcomes in the context of metabolic diseases [21,22,33,34]. Many studies have investigated their impact on thrombosis risk, such as platelet oxidative stress, activation, and aggregation. However, most studies were conducted only *in vitro* or in animals to observe the effect of anthocyanins on platelet activation and aggregation. For example, *in vitro* experiments have shown that polyphenol-rich extracts from black chokeberry could reduce platelet adhesion, aggregation and generation of ROS in blood platelets [35]. Besides, anthocyanin-rich extracts from black chokeberry were found to be more effective in inhibiting platelet function than other polyphenols in an animal model of hyperhomocysteinemia [36]. Similarly, our previous study revealed that anthocyanin could inhibit human platelet activation, aggregation and secretion *in vitro* [24,25]. Kiara Thompson et al. found that anthocyanin supplementation had the potential to alleviate platelet activation and aggregation in healthy sedentary population [23]. In addition, our previous human studies have also shown its inhibitory effects on platelet granule release and chemokines in patients with hypercholesterolemia [27]. However, there is no clinical evidence about the effects of anthocyanin

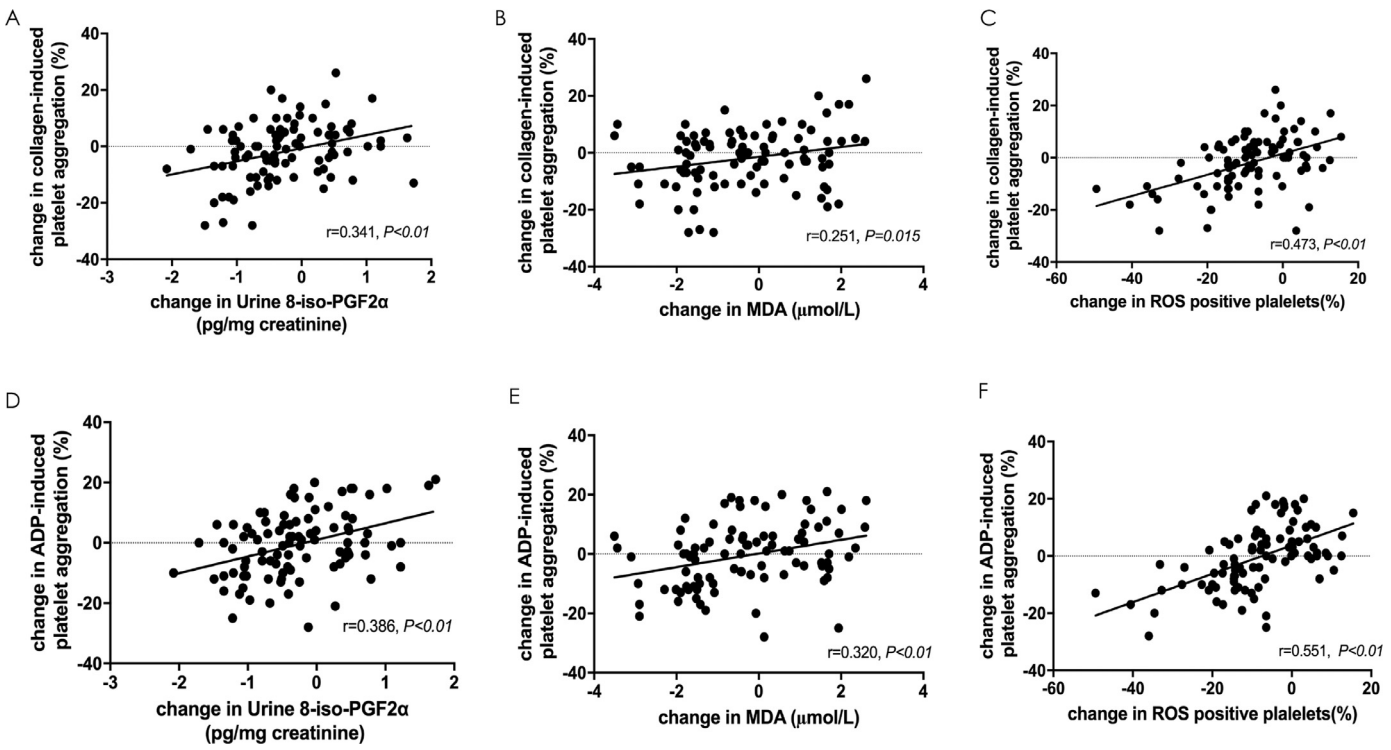

Fig. 2. *Correlation between changes in platelet aggregation and oxidative stress in subjects with dyslipidemia.* The correlation between the change in urine 8-iso PGF 2α and the alteration in collagen-induced platelet aggregation or ADP-induced platelet aggregation after the 12-week intervention are shown in A and D (N = 93); the correlation between the change in serum MDA levels and the alteration in collagen-induced platelet aggregation or ADP-induced platelet aggregation after the 12-week intervention are shown in B and E (N = 93); and the correlation between the change in ROS positive platelets and the alteration in collagen-induced platelet aggregation or ADP-induced platelet aggregation after the 12-week intervention are shown in C and F (N = 93); Pearson's correlation coefficients are noted for each plot. 8-iso-PGF2α, 8-iso-prostaglandin F2α; MDA, malonaldehyde; ROS, reactive oxygen species.

supplementation on platelet activation, aggregation and oxidative stress in individuals with dyslipidemia. The present study first demonstrated that anthocyanins could attenuate platelet function in subjects with dyslipidemia.

Most studies used only a single dose to investigate the effects of anthocyanins, and the dosage at which anthocyanin produces protective functions has not been well investigated. Our recent studies found that supplementation of anthocyanins for 12 weeks led to antioxidative and anti-inflammatory effects in a dose-response manner in a way that anthocyanin supplementation at a dosage over 80 mg/day could produce beneficial effects in individuals with dyslipidemia [21,22]. As discussed before, most studies were conducted only *in vitro*, in animals or in healthy individuals to explore the effects of anthocyanins on platelet function [23,36], and few studies have investigated the effect of anthocyanins on platelet function in individuals with dyslipidemia. The present study first indicated that anthocyanins at a dosage of >80 mg/day could effectively inhibit platelet activation, aggregation and oxidative stress in subjects with dyslipidemia, whereas anthocyanins at a dosage of more than 80 mg/day would result in more beneficial effects on platelet activation, aggregation and oxidative stress. In addition, no significant differences were found in coagulation function and adverse events among the five groups after intervention. This evidence indicated that daily intake of anthocyanins at 80 mg or higher is safe and could have the potential to reduce platelet activation, aggregation and oxidative stress in individuals with dyslipidemia.

The protective function of anthocyanins on platelet activation, aggregation and oxidative stress was significantly obvious after 12 weeks of supplementation but not after 6 weeks of supplementation. Therefore, the duration of anthocyanin supplementation may have an essential impact on the improvement in platelet function. Similarly, in our previous randomized clinical trials in hypercholesterolemic individuals and other randomized clinical trials in patients with hyperlipidemia and patients after myocardial infarction, it was indicated that anthocyanin supplementation for over 12 weeks could exert protective effects, such as improving lipid profiles and inhibiting platelet granule release in plasma [21,22,27,37–39]. One underlying mechanism of anthocyanin may be related to its improvement on the composition of intestinal flora, which needs time to be gradually changed [40–43]. In addition, animal experiments have shown that after long-term administration of anthocyanins to animals, total anthocyanin concentrations were detected in animal tissues, then these may be released from tissues to make the effects time-dependent [44]. In summary, an increased duration of anthocyanin supplementation may result in greater potential clinical benefits on platelet function and oxidative stress, especially in light of the modest efficacy of anthocyanins.

Platelets from patients with dyslipidemia show hyperreactivity, increased surface expression of activated GPIIbIIIa and P-selectin, higher responsiveness of platelet aggregation to stimulus [9,10,45]. Dyslipidemia is associated with elevated levels of oxidative stress [46–48]. Moreover, it has been reported that oxidative stress is one of the major causes of platelet hyperreactivity in individuals with dyslipidemia [7,12]. Our previous study showed that supplementation with anthocyanins for 12 weeks improved antioxidative capacity in a dose-response manner in individuals with dyslipidemia [22]. In the present study, we found that anthocyanins inhibited platelet ROS and improved platelet mitochondrial membrane potential after 12 weeks of treatment. Anthocyanins could reduce platelet ROS levels and urinary 8-iso-PGF2α and MDA levels, which were positively correlated with the change in platelet aggregation after the 12-week treatment. In addition, our findings indicate that the attenuation of platelet hyperreactivity after anthocyanin

intervention was linked to the improvement in oxidative stress among individuals with dyslipidemia.

Some cross-sectional studies conducted in various countries indicated that anthocyanin intake in daily diets was relatively low, ranging from 11.48 to 47.0 mg/day [49]. The daily intake of anthocyanins in participants from our current study was less than 6 mg/day. Overall, anthocyanin intake from the daily diet might not reach 80 mg/day. Thus, the intake of food with adequate anthocyanins is necessary. A previous study has reported that the total anthocyanin contents in fresh bilberry and blackcurrant were approximately 516.3 mg/100 g and 201.0 mg/100 g, respectively [50]. In addition, 100 g of blackberries, black elderberries and black chokeberries contain 85.21−190.62 mg, 295.48−1266.00 mg, and 125.63−989.70 mg of anthocyanins, respectively [51−53]. Therefore, anthocyanin intake of over 80 mg/day can be obtained through daily food intake of anthocyanin-rich foods or nutritional supplements.

There are some strengths and limitations in our present study. The double-blind, randomized, placebo-controlled, dose-response trial design is the major strength of this study. We also maintained the 24 h dietary recall data and IPAQ during the trial to monitor the dietary habits and physical activity of all participants, thus minimizing the chance that our results are attributed to confounding bias. Besides, we tested platelet function assays at baseline, 6 weeks and 12 weeks, respectively, which is difficult to be completed in the intervention trial, due to that platelet function are required to be detected within two hours right after blood collection. Meanwhile, some limitations also existed in the study. The sample size of this study was relatively small as in most other randomized controlled trials which focused on platelet function [19,20,23], because of the difficulty of platelet function assays. But the sample size of this study was calculated via a previous trial [27] and a *post hoc* power analysis, and is adequate to obtain sufficient statistical power. In addition, another limitation of this work was that we did not detect anthocyanins or their metabolites in serum samples as a measure of compliance as in most other randomized controlled trials using anthocyanins as a supplement, mainly because of the short half-life of anthocyanins [54]. The findings of our study which focused on individuals with dyslipidemia can just indicate a potential benefit for individuals with other metabolic diseases, and the improvement effect of anthocyanins on individuals at advanced stages of other metabolic diseases is worthy of further clinical study.

5. Conclusion

Supplementation with anthocyanins could effectively inhibit platelet activation, aggregation and oxidative stress in a dose-dependent manner in subjects with dyslipidemia. Taking 80 mg or more of anthocyanins per day is recommended to reduce platelet activation, aggregation and oxidative stress based on our studies. Our results also add to the evidence that there are relationships between changes in platelet aggregation and changes in oxidative stress among individuals with dyslipidemia after anthocyanin supplementation.

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Z.T. and Y.Y. designed the research. Z.T. performed the data analysis and wrote the initial draft of the manuscript. Z.T., W.L. and Y.Y. critically revised the manuscript. Z.T., K.L., D.F., Y.Z., X.G., X.M., L.X., Y. S., F.Y., J.Z., P.W. and Y.M. conducted the research. W.L. and Y.Y. have primary responsibility for final content, and along with all other authors contributed to critically reviewing the manuscript.

Data sharing

Due to the principles of ethics and data privacy security, we have uploaded all deidentified participant data, study protocol, statistical analysis plan and informed consent form to OneDrive, https://onedrive.live.com/. After publication, deidentified participant data, described in the manuscript, study protocol, statistical analysis plan and informed consent form will be made available upon reasonable request via email to corresponding author.

Clinical trial registration

The trial was registered at ClinicalTrials.gov (NCT03415503).

Declaration of Competing Interest

The authors report no conflicts of interest in this work.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103533.

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