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Anthocyanin Extract from Black Rice Significantly Ameliorates Platelet Hyperactivity and Hypertriglyceridemia in Dyslipidemic Rats Induced by High Fat Diets

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ABSTRACT: Our previous studies have demonstrated that anthocyanin extract from black rice (AEBR) inhibits atherosclerosis. Whether dietary AEBR supplementation can affect platelet function, an important factor in the pathogenesis of cardiovascular diseases, remains unclear. The aim of the present study is to explore the effects and mechanisms of dietary AEBR supplementation on platelet function and lipid profile in dyslipidemic rats. We demonstrated herein that thromboxane A_2, the thrombogenic ratio of thromboxane A_2 and prostacyclin, serum calmodulin, and soluble P-selectin were significantly decreased in rats fed a high fat diet supplemented with AEBR. AEBR supplementation also remarkably lowered serum triglyceride and raised hepatic CPT-1 mRNA expression. These findings suggest that dietary intake of AEBR reduces platelet hyperactivity, hypertriglyceridemia, and body weight gain, and facilitates in the maintenance of optimal platelet function in dyslipidemic rats induced by high fat diets.

KEYWORDS: Anthocyanins, black rice, platelet function, cardiovascular diseases, dyslipidemia

INTRODUCTION

Platelet activation, vessel-wall adhesion, aggregation, and the development of inopportune thrombi play important roles in the pathogenesis of cardiovascular diseases (CVDs), such as atherosclerosis and myocardial and cerebral infarction, the leading causes of morbidity and mortality worldwide.[1,2] Hyperactive platelets, characterized by a high predisposition for activation and increased or impaired platelet function, is considered a risk factor for CVD.[3] Hence, maintenance and achievement of optimal platelet function via the reduction of platelet hyperactivity is a feasible approach for preventing and treating CVD.

Platelet activation is a complex signaling cascade, involving many signaling proteins, such as, thromboxane A_2 (TXA_2, measured by its *in vivo* metabolite, TXB_2), prostacyclin (PGI_2, measured by its *in vivo* metabolite, 6-keto-PGFα), calmodulin (CaM), P-selectin, and so on. TXA_2, synthesized and released by activated platelets, stimulates adjacent platelets and promotes platelet aggregation,[4] whereas PGI_2, synthesized by endothelial cells, is an antagonist for platelet activation and adhesion.[5] In resting platelets, CaM is bound directly to the cytoplasmic domains of platelet receptors, glycoprotein (GP) Ib-IX-V and GPVI, which bind von Willebrand factor and collagen, respectively, both which initiate platelet adhesion. Following activation, CaM is released, free to mediate the activity of the calcium (Ca^{2+})-dependent phospholipase A_2 (PLA_2), which cleaves TXA_2 from membrane phospholipids.[6] Finally, α-granules and Weibel−Palade bodies of platelets and endothelial cells, respectively, fuse to the plasma membrane, releasing additional factors and allowing P-selectin translocation to the cell surface. There, P-selectin is shed from the plasma membrane and released into the blood as soluble P-selectin (sP-selectin),[7] serving as a marker for platelet activation. Consequently, the balance between the production of TXA_2 versus PGI_2, the activity of CaM, and the presence of sP-selectin are all considered important for the maintenance of optimal platelet function.

The levels of TXA_2 versus PGI_2 (TXA_2:PGI_2 thrombogenic ratio),[8] CaM[9] and sP-selectin[10] are significantly higher in dyslipidemia. Hypertriglyceridemia, a common feature of dyslipidemia, is characterized by either increased dietary uptake, increased synthesis or decreased oxidation of fatty acids, and can result in platelet hyperactivity and impaired platelet function.[11] Therefore, modification of dyslipidemia could reduce platelet hyperactivity and help maintain optimal platelet function, decreasing adverse events in CVD.[12]

Diet, along with lifestyle, is recognized as the major modifiable risk factor for CVDs; thus, maintenance of optimal platelet function via dietary means is an interesting target to treat CVD. Large numbers of epidemiological investigations have reported that dietary intake of plant foods rich in polyphenols is inversely associated with CVDs.[13,14] Anthocyanins, the most studied polyphenols, are abundant in various fruits, vegetables and beverages, such as grapes, berries, red cabbage and red wine, and have been shown to impart significant beneficial properties.[15,16] Previous

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studies have demonstrated that, through their antioxidative and anti-inflammatory properties, and by improving dyslipidemia, intake of anthocyanin-rich beverages, anthocyanin-rich extract or pure anthocyanins inhibits dyslipidemia, obesity, hyperglycemia and atherosclerosis.[17−20] Several studies have also shown that foods rich in anthocyanins or their *in vivo* metabolites are able to inhibit platelet function.[21] Interestingly, a series of studies from our group also found that anthocyanins can attenuate hyperlipidemia and inhibit the progression of atherosclerosis.[22,23] However, the relationship between anthocyanins and platelet hyperactivity and function and dyslipidemia remains to be elucidated.

Using an *in vivo* rat model of dyslipidemia induced by a high fat diet, in the present study, we show that dietary supplementation with anthocyanin extract from black rice (AEBR) reduces body weight gain, platelet hyperactivity and hypertriglyceridemia, and facilitates in the maintenance of optimal platelet function. Several possible mechanisms behind the beneficial effects of anthocyanins are discussed.

MATERIALS AND METHODS

Animals and Diets. Male SD rats (n = 36), aged 8−9 weeks and weighing 180−220 g, were obtained from the animal center of Guangzhou, P. R. China. Rats were housed at 22 °C with a 12 h light−dark cycle with free access to food and water. Rats were randomly and evenly divided into three groups and fed one of the following for twelve weeks: AIN-93G normal diet (control group), AIN-93G diet containing high fat and cholesterol (HF group), or the high fat diet supplemented with AEBR rich extract (5 g/kg diet, HF+AEBR group). As described previously,[24] the raw material of black rice was husked in the same way (unpolished) so that the rice used underwent the same minimal amount of grinding. Characterization and quantification of anthocyanins present in the extract from black rice were conducted by HPLC (a 250 × 4.6 mm i. d., 5 μm Hypersil GOLD C18 column, Waters) followed by LC−MS analysis. Anthocyanins were identified by both retention time and mass profile in comparison with authentic standards. Other components of the AEBR were analyzed by routine laboratory techniques (Table 1). The compositions of the different diets were analyzed chemically (Table 2). The rats were weighed every week during the experiment. At the end of experiment, all rats were deprived of food overnight[22] and sacrificed under ether anesthesia. Blood was collected by heart puncture, and serum was prepared by centrifugation. Serum samples were stored at −20 °C until used for analysis. The liver of each rat was harvested, washed with ice-cold isotonic saline and weighed. All measurements were done in triplicate, and mean values were obtained. All experiments were approved by the Animal Care and Use Committee of Sun Yat-sen University.

TXB_2 and 6-Keto-PGF1α Assay. The measurement of serum thromboxane B_2 and 6-keto-PGF1α were performed using Correlate-CLIA Thromboxane B_2 and 6-keto-PGF1α Immunoassay kits (Assay Designs Inc., Ann Arbor, MI) according to the manufacturer's instructions.

sP-Selectin and CaM Assay. Serum sP-selectin and CaM levels were measured by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Serum Lipid Profile. Serum total cholesterol (TC) and HDL cholesterol (HDL-C) were measured using cholesterol esterase and cholesterol oxidase assays. Serum LDL cholesterol (LDL-C) concentrations were determined via the direct method. Serum triglyceride (TG) concentrations were assayed by hydrolyzing the TGs and measuring the glycerol released.

Hepatic CPT-1 mRNA by Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assay. Total RNA was extracted from liver by using TriZOL (InVitrogen, US) reagent according to the manufacturer's instructions. RNA was reverse-transcribed using the SUPERSCRIPT First-Strand Synthesis System for RT-PCR (InVitrogen, US). The primers for CPT-1 were 5′-CAGCTCGCACATTACAAGGA-3′ (sense) and 5′-TGCACAAAGTTGCAGGACTC-3′ (antisense). Semi-quantitative estimation was done by comparing mRNA expression of CPT-1 with GADPH represented by the amount of the PCR product formed.

Statistical Analysis. Results are expressed as means ± SD. Data were analyzed by one-way ANOVA coupled with the Student−Newman−Keuls multiple comparison tests. Differences were considered significant if $p < 0.05$. The SPSS 16.0 statistical package was employed.

Table 1. Composition of the AEBR

ingredients	%
protein	4.9
polysaccharide	21.6
cyanidin-3-glucose	38.0
peonidin-3-glucose	5.2
other flavones	16.6
water	5.5
other materials	8.2

Table 2. Composition of the Experimental Rat Diet[a]

	content per kilogram of feed		
ingredient	control	HF	HF+AEBR
energy, kJ	3960	4679.5	4679.5
cornstarch, g	397.5	84.8	84.8
dextrinized cornstarch, g	132.0	116.5	116.5
sucrose, g	100.0	201.3	201.3
casein, g	200.0	233.0	233.0
L-cystine, g	3.0	3.5	3.5
soybean oil, g	70.0	29.1	29.1
cholesterol, g	0	5	5
lard, g	0	206.8	206.8
fiber, g	10.0	58.7	58.7
mineral mix, g	35.0	52.4	52.4
vitamin mix, g	10.0	11.6	11.6
choline bitartrate, g	2.5	2.3	2.3
tert-butylhydroquinone, g	0.014	0.014	0.014

[a] Control group fed normal diet; HF group fed high fat diets; HF+AEBR group fed HF diet containing AEBR.

Table 3. Body Weight of Rats during Experimental Period[a]

groups	N	0 wk	3 wk	6 wk	9 wk	12 wk
HF	12	220.29 ± 2.24	296.38 ± 10.36 a	381.63 ± 13.88 a	426.17 ± 13.35 a	455.33 ± 22.64 a
HF+AEBR	12	219.65 ± 7.88	288.49 ± 13.44 a	363.32 ± 28.30 b	411.90 ± 19.80 b	434.29 ± 32.06 b
control	12	219.35 ± 8.58	276.29 ± 11.77 b	349.70 ± 29.84 c	377.29 ± 8.91 c	406.60 ± 16.67 c

[a] Values are means ± SD. Values within the same column with different letters (a, b, c, etc.) are significantly different ($p < 0.05$).

RESULTS

AEBR Suppresses Body Weight Gain. The body weight gain of the HF group significantly increased when compared to the control group after the feeding ($p < 0.05$), while the body weight gain of the HF+AEBR group was significantly lower than that of the HF group from the sixth week to the end of the experimental period ($p < 0.05$) (Table 3).There was no difference in food intake among the three groups throughout experimental period (Table 4). Therefore, AEBR supplementation suppresses body weight gain of the dyslipidemic rats without altering food intake.

AEBR Significantly Decreases Serum TXA_2 and TXA_2:PGI_2 Thrombogenic Ratio. The serum levels of TXA_2 in the HF group significantly increased when compared to the control diet ($p = 0.0003$), while AEBR supplementation significantly decreased serum TXA_2 levels when compared to HF group ($p = 0.0005$) (Figure 1A). AEBR supplementation resulted in an increase of the concentration of PGI_2 (995.0 ± 140.8 pg/mL) as compared to the HF diet (637.6 ± 191.1 pg/mL), but without significant difference. The TXA_2:PGI_2 thrombogenic ratio of the HF+AEBR group was significantly lower than that of the HF group ($p = 0.0004$), and no different from the control group (Figure 1B).

AEBR Decreases Serum CaM and sP-Selectin. When compared to the control group, a significant increase in the serum CaM levels was observed in the HF group ($p = 0.0004$). AEBR supplementation significantly decreased serum CaM levels in HF+AEBR group ($p = 0.0004$) (Figure 2). Similarly, supplementation of AEBR remarkably lowered the serum levels of sP-selectin induced by HF diet ($p = 0.0023$) (Figure 3).

AEBR Decreases Serum TG. The HF diet significantly increased serum TG as compared to the control diet ($p = 0.004$). The serum levels of TG in the HF+AEBR group were significantly lower than those of the HF group ($p = 0.039$) and no different when compared to the control group (Table 5). However, there are no differences in the serum levels of TC, LDL-C and HDL-C between HF and the control group.

Table 4. Food Intake of Rats during Experimental Period[a]

groups	*n*	food intake (g/day)
HF	12	16.88 ± 0.36 a
HF+AEBR	12	16.52 ± 0.22 a
control	12	16.84 ± 1.01 a

[a] Values are means ± SD. Values within the same column with different letters (a, b, c, etc.) are significantly different ($p < 0.05$).

AEBR Increases Hepatic CPT-1 mRNA Expression. The CPT-1 mRNA expression of the HF+AEBR group was significantly higher than that of the HF ($p = 0.046$) and control groups ($p = 0.04$), while there was no significant difference in the hepatic CPT-1 mRNA expression between the HF and control groups (Figure 4).

DISCUSSION

The present study demonstrates that, in rats fed a high fat diet, AEBR supplementation attenuates platelet function. To our knowledge, this is the first study showing the positive effects of the AEBR on *in vivo* platelet function. Compared to rats fed the control diet, rats fed a high fat diet exhibited increased body weight gain, increased serum TG levels, and downregulated

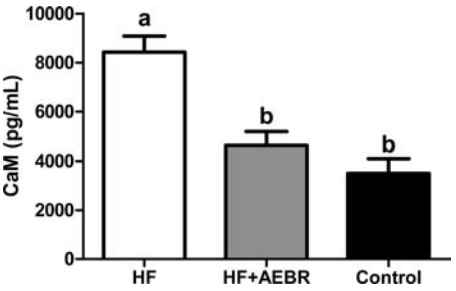

Figure 2. Effect of AEBR on serum CaM level. Serum CaM of rats fed normal (control) or high fat (HF) diets or the HF diet supplemented with anthocyanin extract from black rice (HF+AEBR) were measured at the twelfth week. Values are means ± SD, $n = 10-12$ per group. Values with different letters (a, b, c, etc.) are significantly different ($p < 0.05$).

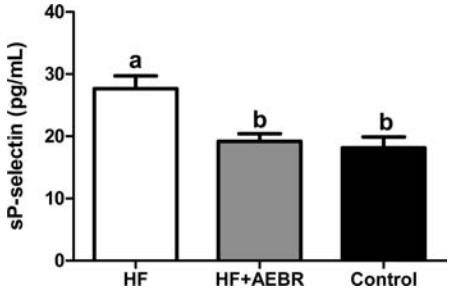

Figure 3. Effect of AEBR on serum sP-selectin level. Serum sP-selectin of rats fed normal (control) or high fat (HF) diets or the HF diet supplemented with anthocyanin extract from black rice (HF+AEBR) were investigated at the end of the experiment. Values are means ± SD, $n = 10-12$ per group. Values with different letters (a, b, c, etc.) are significantly different ($p < 0.05$).

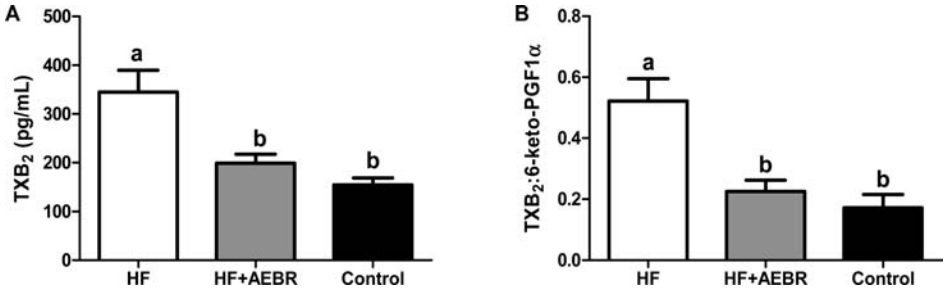

Figure 1. Effect of AEBR on serum TXB_2 and TXB_2:6-keto-PGF1α ratio. Serum TXB_2 synthesis (A) and TXB_2:6-keto-PGF1α ratio (B) of rats fed normal (control) or high fat (HF) diets or the HF diet supplemented with anthocyanin extract from black rice (HF+AEBR) were assessed at the twelfth week. Values are means ± SD, $n = 10-12$ per group. Values with different letters (a, b, c, etc.) are significantly different ($p < 0.05$).

Table 5. Serum Lipid Concentrations at the End of the Experimental Period[a]

groups	*n*	TG (mmol/L)	TC (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	HDL-C:LDL-C
HF	11	1.71 ± 0.45 a	1.98 ± 0.30 a	0.60 ± 0.13 a	0.37 ± 0.05 a	1.66 ± 0.45 a
HF+AEBR	12	1.40 ± 0.26 bc	1.95 ± 0.32 a	0.65 ± 0.09 a	0.39 ± 0.06 a	1.68 ± 0.21 a
control	10	1.27 ± 0.28 b	1.81 ± 0.27 a	0.67 ± 0.16 b	0.26 ± 0.06 b	2.75 ± 0.87 b

[a] Values are means ± SD. Values within the same column with different letters (a, b, c, etc.) are significantly different ($p < 0.05$).

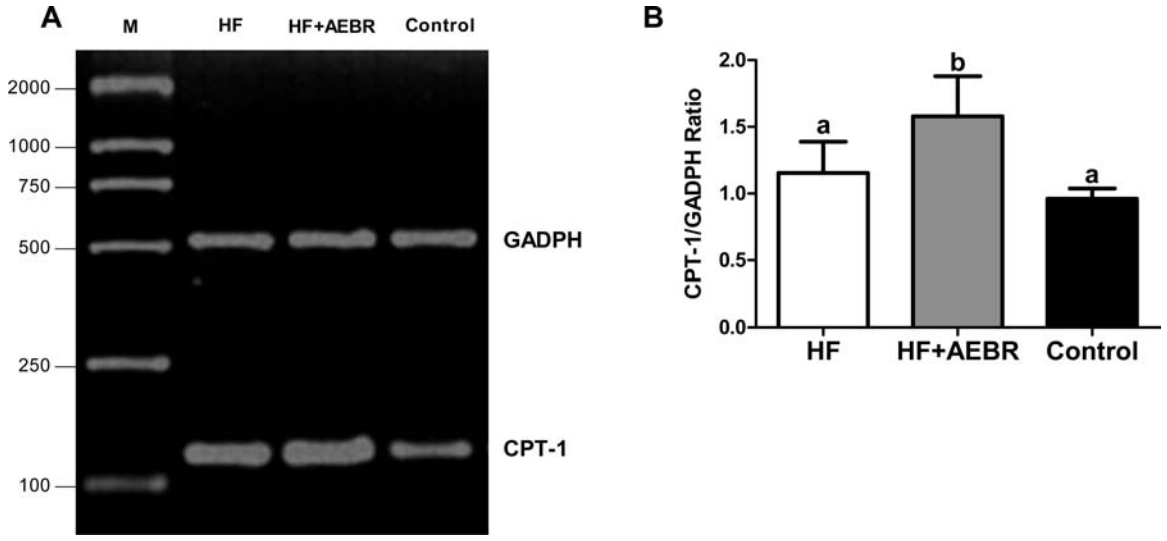

Figure 4. Effect of AEBR on hepatic CPT-1 mRNA expression. (A) RT-PCR analysis of AIN-93G diet (control), high fat diet (HF), and high fat diet supplemented with anthocyanin extract from black rice (HF+AEBR) groups for twelve weeks. (B) The hepatic CPT-1 mRNA expression concentrations were calculated as the ratio of CPT-1 versus GADPH mRNA expression. Values are means ± SD. Values with different letters (a, b, c, etc.) are significantly different ($p < 0.05$).

platelet function, indicated by increased CaM and sP-selectin levels and increased TXA_2 production combined with increased TXA_2:PGI_2 ratio. AEBR supplementation was able to significantly reduce body weight gain, and restore serum TG levels and platelet function by decreasing CaM and sP-selectin levels, TXA_2 production and TXA_2:PGI_2 ratio.

There are several possible mechanisms as to how AEBR supplementation is able to restore platelet function. It is possible that AEBR or its metabolites can alter Ca^{2+} and CaM-related signaling pathways and directly affect platelet function. Some polyphenols have been shown to directly affect cell signaling pathways. It has been reported that apigenin, genistein, luteolin and quercetin impaired U46619-induced Ca^{2+} mobilization, platelet tyrosine phosphorylation and ERK 1/2 activation.[25] In addition, resveratrol was found to inhibit elevated Ca^{2+}/CaM-dependent protein kinase II activity.[26] As CaM mediates the stimulation of PLA_2 by Ca^{2+},[27] and then activated PLA_2 cleaves TXA_2 from membrane phospholipids,[6] our data suggests that AEBR affects platelet function and the TXA_2:PGI_2 ratio by decreasing the concentration of serum CaM or Ca^{2+} and inhibiting CaM-related signaling pathways.

Several studies have evidenced that higher serum TG is linked with decreased platelet function in obesity.[28,29] While the mechanism of this link remains unclear, the following pathways may contribute to how hypertriglyceridemia decreases platelet function: changes in lipid content of platelet membranes may facilitate their activation by physiological stimuli;[30] and superoxide anions have been shown to activate platelets and anthocyanins may decrease the activity superoxide anions released by polymorphonuclear leukocytes[31] or produced by the hypercholesterolaemic vascular wall.[32,33] The present study found that AEBR diet significantly reduced serum TG and body weight gain induced by high fat diet in rats, which is consistent with previous findings.[34] Furthermore, Jayaprakasam et al. reported that dietary anthocyanins had an antiobesity effect through regulating TG synthesis or lipid hydrolysis.[35] Therefore, it is possible that AEBR induce the reduction of body weight gain is contributed to sustained lower serum TG.

While TGs may directly affect platelet function, their levels in serum depend on endogenous fatty acid synthesis and catabolism via β-oxidation.[36] Fatty acids are transported across the outer mitochondrial membrane by CPT-1, and then the inner mitochondrial membrane by carnitine.[37] Once inside the mitochondrial matrix, fatty acids undergo β-oxidation, and are broken down to generate acetyl-CoA, the entry molecule for the citric acid cycle.[38] The activity of CPT-1 is the rate-liming step in β-oxidation, and our data suggests that AEBR enhances the expression of CPT-1 mRNA, accelerating fatty acid oxidation and lowering serum TG. This reduction in serum TG may decrease the direct effect of TGs on platelet function.

It is important to note that atherosclerosis is also considered a chronic inflammatory disease.[1] Platelets can induce potent inflammatory responses in adjacent cells, such as leukocytes and endothelial cells,[39] and platelets themselves may also respond to inflammatory mediators produced by these neighboring cells.[40] P-Selectin, an adhesion molecule expressed on and

subsequently released by activated platelets and endothelial cells,[41,42] mediates inflammatory interactions between platelets and leukocytes, and leukocyte rolling on the endothelium.[43] Previous studies by us and others have shown that AEBR acts as an anti-inflammatory by decreasing adhesion molecules CD40L and interleukin (IL)-1β,[44,45] and grape seed-derived proanthocyanidin extract significantly attenuated the increased expression of P-selectin and other adhesion molecules.[46] In line with these studies, our data suggests that AEBR can reduce sP-selectin levels, affecting platelet- and endothelial-mediated inflammatory pathways via P-selectin.

The present study shows that, in rats fed a high fat diet, AEBR supplementation was able to significantly reduce body weight gain, and rescue serum TG levels and platelet function by restoring CaM and sP-selectin levels, TXA_2 production and TXA_2:PGI_2 ratio back to that of controls. This is also the first study that shows the beneficial effects of AEBR on *in vivo* platelet function. While several mechanisms are proposed, the exact mechanism by which AEBR improves platelet function, hypertriglyceridemia and weight gain seen in high fat diets, and how these results are implicated in the development of CVD, requires further investigation.

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