Original Article

Supplementation of black rice pigment fraction improves antioxidant and anti-inflammatory status in patients with coronary heart disease

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Black rice and its pigment fraction have shown anti-atherogentic activities in several animal models, but whether their beneficial effects will recur in humans remains unknown. The aim of the present study is to investigate the influence of black rice pigment fraction (BRF) supplementation on selected cardiovascular risk factors in patients with coronary heart disease (CHD). Sixty patients with CHD aged 45 - 75 years were recruited from the Second Affiliated Hospital of Sun Yat-Sen University in Guangzhou, China and randomly divided into two groups. In the test group, the diet was supplemented with 10 grams of BRF derived from black rice for 6 months; While in the placebo group, the diet was supplemented with 10 grams of white rice pigment fraction (WRF) derived from white rice. At baseline, plasma antioxidant status and the levels of inflammatory biomarkers and other measured variables were similar between two groups. After 6 months' intervention, compared to WRF supplementation, BRF supplementation greatly enhanced plasma total antioxidant capacity (TAC) (p=0.003), significantly reduce plasma levels of soluble vascular cell adhesion molecule-1 (sVCAM-1) (p=0.03), soluble CD40 ligand (sCD40L) (p=0.002) and high sensitive C-reactive protein (hs-CRP) (p=0.002) in the test group . No significant changes were observed in plasma total superoxide dismutase (T-SOD) activity, lipids level and carotid artery intima-media thickness (IMT) between two groups. These results may suggest that BRF could exert cardioprotective effects on patients with CHD by improving plasma antioxidant status and inhibiting inflammatory factors.

Key Words: black rice pigment fraction, anthocyanins, coronary heart disease, antioxidant status, inflammation

Introduction

CHD, including angina, myocardial infarction, heart failure, arrhythmias and sudden death, is the major killer of both women and men in the United States, Europe and parts of Asia. Clinical studies have demonstrated that CHD is correlated with atheroma (atherosclerotic plaque) and its complex complications such as thrombosis. Researches have shown that the development of atherosclerosis is caused by a complex interaction among reactive oxygen species, lipids, endothelium, circulation and tissue inflammatory cells, platelets and vascular smooth muscle cells and was not simply due to the accumulation of lipids. [1,2] Therefore, in prevention and treatment of atherosclerotic diseases, it is important to take measures to increase antioxidative status and to inhibit hyperlipidemia and inflammation in patients. At present, a variety of life modifications, such as body weight control and an increase in physical activity, are the recommended treatments to lower risk of CHD. However, maintaining a normal BMI and regular exercise might not be sufficient to prevent CHD. Dietary modifications such as higher intakes of fruits, vegetables and cereals than usual could be another important measures to benefit individuals with the increased cardiovascular disease risk factors.[3]

Black rice (*Oryza sativa L. indica*), a special cultivar of rice which contains a much higher content of anthocyanins in the aleurone layer than white rice, has been regarded as a food and widely consumed as a health-promoting food in China and other Eastern Asia countries for thousands of years. Our previous studies have shown that the supplementation of black rice pigment fraction markedly reduced oxidative stress and inflammation, improved plasma lipid level and alleviate atherosclerotic lesions in two different animal models.[4-6] Furthermore, an anthocyanin-rich extract of black rice with a relatively high anthocyanins content (43.2%), displayed similar effects on animal models.[7] However, whether BRF rich in anthocyanins will exhibit its anti-atherosclerotic properties in human remains uncertain.

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Based on our previous findings, this study was designed to investigate the effect of BRF on plasma antioxidant status, inflammation factors and plasma lipids in patients with CHD. Our hypothesis was that BRF supplementation would have beneficial effects in those patients by reducing plasma lipids, decreasing inflammation and acting as a free radical scavenger.

Materials and methods

Subjects

Sixty subjects aged 45 - 75 years old were recruited from the Second Affiliated Hospital of Sun Yat-Sen University. All of them had previously been diagnosed with established CHD (documented myocardial infarction, significant coronary artery stenosis as assessed by angiography of > 50%, or significantly decreased cardiac perfusion based on cardiac imaging, with and without exercise). Patients with one of the following conditions were excluded: intake of antioxidant supplements within the last 2 months; an acute myocardial infarction within the past 6 months; cardiac surgery within the past 6 months; significant renal failure or liver failure. The study protocol was approved by the medicine ethics committee of SunYat-sen University. The purpose and protocol of our research were clearly explained to the patients before their recruitment in this study, and their written informed consents were obtained.

Study design and dietary assessment

The clinical efficacy of the BRF was evaluated in CHD patients in a randomised, double-blind, placebo-controlled six-month intervention. During six months the subjects received daily 30 g BRF product (a pack of powder contains 10 g BRF derived from black rice, 12 g starch and 8 g sucrose) daily in the test group whereas 30 g WRF product (a pack of powder contains 10 g WRF derived from white rice, 12 g starch and 8 g sucrose) was consumed in the placebo group. All participants were requested to orally ingest the BRF products or placebo steeped in 300 ml warm water daily after breakfast. During the intervention period, participants were instructed to maintain their ordinary level of physical activity and keep their dietary habits unchanged. Moreover, a 24-h dietary recall was conducted at three different time points (at the baseline, the 3-month follow-up visit and the terminal visit) to ascertain whether their macronutrient, micronutrient and energy intake changed during the study. These dietary questionnaires were administered by trained medical personnel in a face- to-face interview. Food consumption data were converted into nutrients by using the Nutrient Elements Calculator V1.6 (Institute of CDC Nutrition and Food Safety, Peking, China).

Blood specimen collections

For the plasma biomarkers assaying, venous blood was obtained at the baseline and at the end point of the intervention after an overnight fasting. Samples were then placed into tubes with 10% citrate as anticoagulant . After being centrifuged at 3000 rpm for 15 min at 4℃, plasma was collected and stored at -80℃ frozen in portions until analyses. Six volunteers (4 males and 2 females) were selected for assessment of anthocyanin levels in plasma. After overnight fasting (12h - 14h), 4ml plasma samples were collected individually from the volunteers, and then 30 g BRF product (n = 3) or 30 g WRF (n = 3) product were orally ingested. Plasma samples were taken subsequently at 0.5, 1, 1.5, 2 and 4 h after consumption of BRF or WRF product.

Plasma antioxidant status, inflammatory factors and lipids

The plasma TAC was measured using the Ferric Reducing Ability of Plasma (FRAP) method and detected on Shimadu UV-160 spectrophotometer.[8] The plasma T-SOD activity was assayed by employing an indirect inhibition assay in which xanthine and xanthine oxidase serve as a superoxide generator, and nitroblue tetrazolium (NBT) was used as a superoxide indicator.[9] Plasma levels of sVCAM-1 and sCD40L were determined in duplicate by using ELISA kits (Biosource system, Nivelles, Belgium and Bender Medsystems Vienna, Austria). Plasma hs-CRP was assayed by Hitachi 911 automated assay analyzer (Orion D, Espoo, Finland) using the immunoturbidimetric method. Plasma triglyceride was assayed by colorimetric method. Plasma total cholesterol (TC) and HDL cholesterol (HDL-C) were measured using cholesterol esterase and cholesterol oxidase assays.[10] Plasma LDL cholesterol (LDL-C) concentration was determined by the direct method.[11] ApoAI and ApoB were determined using the immunoturbidimetric method.

IMT in carotid arteries

Ultrasound examination of the carotid arteries of all patients was performed by the same investigator using the same high-resolution ultrasound unit with a 7.5 MHz sector scanner and Doppler facility (LOGIQ700, GE Medical. Systems, Milwaukee, WI, USA). Three anatomically predefined sites on both sides of the neck (six sites in total) were examined: the distal 20 mm of the common carotid artery, the carotid bifurcation, and the proximal 10 mm of the internal carotid artery. A mean IMT was defined as the mean values of three sites on each side of the neck of the patient.

Plasma anthocyanins

An ODS solid phase extraction cartridge (Sep-Pak C18, Waters,Milford, MA) was used for extraction of plasma anthocyanins. The cartridge was washed with 5 ml methanol and equilibrated with 5 ml of 1.5 mol/L formic acid aqueous solution before use. Then, 2.0 ml of plasma sample diluted with 0.2 ml of 0.44 mol/L trifluoroacetic acid (TFA) was applied to the cartridge. Water-soluble compounds, polar lipids and neutral lipids were respectively eluted from sample plasma with 5 ml of 1.5 mol/L formic acid aqueous solution, 5 ml of dichloromethane and 5 ml of benzene. Anthocyanins were recovered finally with 0.44 mol/L TFA solution in methanol. The methanol was then evaporated to dryness with a rotary evaporator. The dried extract was redissolved with 200 μL of 0.44 mol/L TFA aqueous solution and the 100 μL portion was subjected to the UV-HPLC analysis to determine anthocyanin concentration. Anthocyanins in plasma were determined with a UV-HPLC system at 520 nm detection. An C18-OSD column (4.6 mm × 250 mm) was

Table 1. Baseline characteristics of the 60 participants in this study

	BRF group (n=30)	WRF group (n=30)	*p*-value
Age ,y	63.7±8.69	64.0±10.7	0.60
Male	63.3%	56.7%	0.79
BMI, kg/m^2	24.0±3.62	23.2±2.85	0.33
Systolic blood pressure, mm Hg	143±23.0	148±26.3	0.38
Diastolic blood pressure, mm Hg	83.3±13.5	86.4±11.2	0.34
Diabetes (%)	13.3%	16.7%	0.72
Hyperlipidemia (%)	53%	60%	0.60
Hypertension (%)	50%	46.7%	0.80
Current cigarette smoking (%)	0%	2.8%	0.15

Data are expressed as mean±SD or proportion (%)

Table 2. The daily dietary intake of nutrients according to intervention assignment

Nutrients	BRF group (n=30)	WRF group (n=30)	*p*-value
Energy (kcal)	1758±423	1820±448	0.67
Protein (g)	80.3±21.2	75.4±21.0	0.49
Fat (g)	47.4±18.4	50.5±26.5	0.69
Cholesterol (mg)	271±134	242±93	0.46
Carbohydrate (g)	261±68.7	275±78.2	0.59
Total fiber (g)	0.6±4.7	8.9±3.6	0.24
Vitamin A (μgRE)	686±316	457±445	0.08
Vitamin C (mg)	121±45.8	91.9±58.0	0.10
Vitamin E (mg)	10.3±4.32	8.16±4.12	0.14
Iron (mg)	19.1±4.53	18.1±4.98	0.53
Zinc (mg)	13.0±3.48	12.2±3.89	0.53
Selenium(μg)	57.6±14.8	56.9±23.0	0.91

used with a mixture of 4% aqueous phosphoric acid and actonitrile (88:12, v/v) as column eluant at a flow rate of 0.8 mL/min. Cynidin 3-o-β-glucoside (Cy-3-g) and Peonidin 3-o-β-glucoside (Pn-3-g) (Polyphenol AS, Norway) were used as the standards. The sample preparation and HPLC analysis procedures followed the methods of Cao G et al and Kay et al with modification.[12,13]

Statistical analyses

Baseline characteristics were compared between the test group and the placebo group using Student's t-test for continuous variables and X^2 tests for categorical variables. The dietary nutrient contents were compared between two groups using one-way ANOVA test. BRF and WRF group parameters were compared at baseline as well as the changes in parameters after 6 months' intervention. The Student's t-test was used for data with normal distribution and the nonparametric Mann-Whiney U-test was used for data not normally distributed. The values are reported as mean ± SD in all the results tables unless otherwise specified. *p*values were two tailed and $p<0.05$ was considered as significant.

Results

All enrolled participants completed the study and no side effects were observed in either group. The characteristics at baseline are presented in Table 1. The medicine which the patients utilized before the trial (such as statin, aspirin, β blockers, angiotensin converting enzyme inhibitors and calcium channel antagonists) were similar between the two groups and there were also no significant differences in daily energy, nutrients or antioxidant observed between the two groups based on the data from the dietary questionnaires mentioned before (Table 2). Patients were generally compliant as assessed by counting returned products, scheduled phone interviews and personal visits. According to the returned products, the average usage of the products was 91.4% in the test group and 90.9% in the placebo group, respectively. There was no significant difference between two groups.

The results for antioxidant status, plamal lipids, inflammatory biomarkers and IMT are presented in Table 3. Overall, the values of measured variables at baseline were similar in the two groups. Concerning the plasma TAC, an increasing change from baseline of 1.29±2.96 U/ml was seen in the test group but a decreasing change from baseline of 0.61±1.69 U/ml for TAC was observed in the placebo group.The difference of change between the two groups was significant ($p=0.003$). There was no significant difference in plasma SOD activity between the two groups after 6 months' intervention. As for the inflammatory biomarkers, our results showed that BRF treatment over the 6-month intervention led to decreasing changes in plasma sVCAM-1 (1367±756 mg/L to 993±532 mg/L), sCD40L (8.36±4.09 mg/L to 5.73±2.37 mg/L) and hs-CRP (3.82±1.82 mg/L to 2.55±1.66 mg/L) in the BRF group, while the levels of these inflammatory biomarkers didn't change significantly after 6 months in the WRF group. The difference in changes of these three biomarkers between two groups was significant, ($p=0.03$,

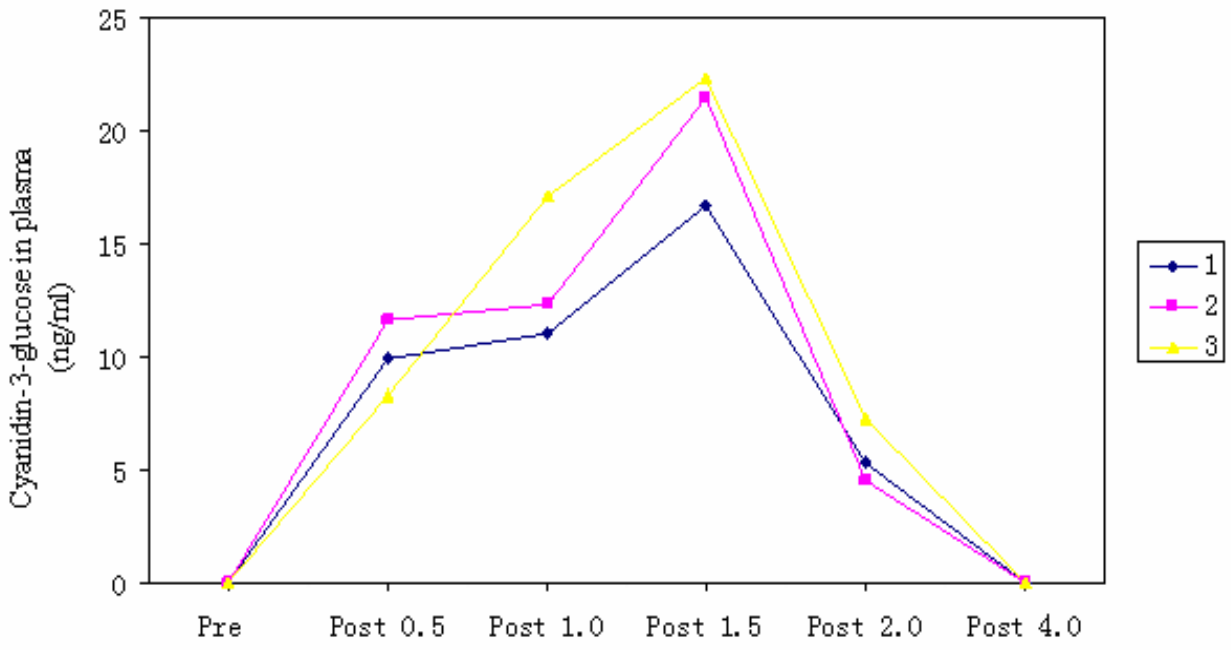

Figure 1. Pharmacokinetic parameters of cyanidin-3-glucose in human plasma after 3 subjects consumed a BRF product. Pre = pre consumption, Post 0.5 = post-consumption 0.5 hour, Post 1.0 = post-consumption 1.0 hour, Post 1.5 = post-consumption 1.5 hours, Post 2.0 = post-consumption 2.0 hours, Post 4.0 = post-consumption 4.0 hours.

Table 3. The plasma levels of antioxidant status, inflammatory biomarkers, lipids and IMT of carotid artery

	BRF group (n=30)			WRF group (n=30)			*p*-value
	Baseline	6-month	Change	Baseline	6-month	Change	
TAC,10^3 u/L	11.1±3.53	12.4±4.24	1.29±2.96 [a]	10.3±2.73	9.70±2.30	-0.61±1.69[b]	<0.01
SOD, 10^3 u/L	51.1±11.2	51.5±23.5	0.38±23.3	49.4±8.89	50.5±13.0	1.11±19.0	0.90
sVCAM-1, mg/L	1367±756	993±532	-373±492[a]	1236±548	1208±690	-28.2±691[b]	0.03
sCD40L, mg/L	8.36±4.09	5.73±2.37	-2.63±3.27 [a]	8.25±4.53	8.85±4.73	0.60±4.43[b]	<0.01
hs-CRP, mg/L	3.82±1.82	2.55±1.66	-1.27±1.66 [a]	3.58±1.71	3.82±1.96	0.24±1.99[b]	<0.01
TG, mmol/L	1.56±0.80	1.30±0.92	-0.26±1.03	1.68±0.99	1.28±0.58	-0.39±1.24	0.78
TC, mmol/L	4.16±1.02	3.89±0.77	-0.27±1.08	4.11±0.78	4.10±0.71	-0.01±0.89	0.22
LDL-C, mmol/L	2.39±0.71	2.17±0.50	-0.22±0.91	2.34±0.60	2.35±0.64	0.02±0.87	0.24
HDL-C, mmol/L	1.03±0.33	1.17±0.42	0.14±0.31	1.03±0.22	1.06±0.28	0.03±0.33	0.22
ApoAI, g/L	0.96±0.21	1.05±0.23	0.09±0.18	0.95±0.14	0.94±0.21	-0.02±0.25	0.16
ApoB, g/L	0.83±0.20	0.76±0.15	-0.06±0.22	0.77±0.12	0.73±0.21	-0.04±0.24	0.70
IMT (left), mm	1.03±0.25	1.03±0.28	0.00±0.24	1.14±0.34	1.15±0.33	0.01±0.12	0.87
IMT (right), mm	1.02±0.31	1.02±0.35	0.00±0.25	1.18±0.36	1.20±0.38	0.02±0.17	0.80

Significant difference between the change value of each groups is indicated by different superscript letter (paired t-test, $p<0.05$)

p=0.002, p=0.002) respectively.

The mean changes of TG, TC, LDL-C, HDL-C, apoAI and apoB between the test group and the placebo group, however, were not significant different after 6-months' intervention. Furthermore, there was no significant change in carotid IMT in both groups throughout the study.

After the intake of the BRF products, only the Cy-3-g (the dominant anthocyanin in BRF) appeared in plasma and arrived at a maximum level (21.5±4.48 ng/ml) at 1.5 h, but disappeared quickly after 4 hours (Fig 1, 2). Our data indicated that the human plasma contained no detectable anthocyanins before the intake of BRF (baseline, t=0) and we could not detect anthocyanins in the participants' plasma in WRF group at any time.

Discussion

The present study shows that treatment with a subfraction of black rice can benefit patients with CHD by increasing the plasma antioxidant status and by reducing inflammation. The effectiveness of the BRF in this clinical trial was comparable with that of WRF, which served as a placebo in this study. BRF is composed of different kinds of nutrients and phytochemicals which mainly include anthocyanins.[14] On the basis of previous studies in our group, phytochemicals rather than fiber or vitamins contained in the black rice are responsible for the atheroprotective effect.[7] Anthocyanins have recently been considered as important phytochemicals with potential health-promoting activities, such as anti-oxidation, anti-inflammation, etc.[15] Similarly, other studies have also documented that plant anthocyanins are beneficial to cardiovascular health.[16,17]

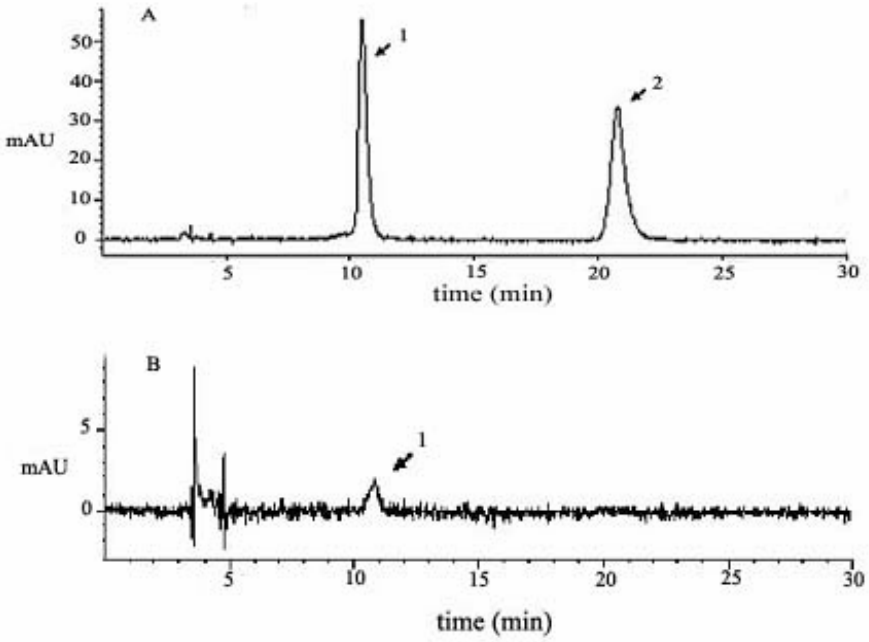

Figure 2. Representative HPLC chromatograms of two anthocyanin standards (A) and anthocyanins in human plasma sample 1.5 h after 30 g BRF intake (B). Peaks 1 and 2 were confirmed as Cyanidin-3-glucose and Peonidin-3-glucose respectively according to the standards. mAU = milliabsorbance units.

The higher amount of anthocyanins contained in BRF compared to WRF might be the major component responsible for the BRF-induced cardioprotective effects which were not shown in the WRF group. Although BRF, compared with WRF, also contains higher amounts of iron, selenium and zinc ,all of which were reported to possess certain cardioprotective effects in vivo,[18,19] non-selectively supplementing these minerals, even in an amount higher than that consumed by the patients in the present study, did not show any favorable effect on atherosclerosis.[20] These results implies that the anthocyanins contained in the BRF may be the main cause for the beneficial effects observed in the BRF group, although it can not be ruled out that some minerals in BRF may contribute partially to those beneficial effects.

Anthocyanins have been shown to display certain antioxidant activities in vitro and in vivo.[21,22] Consistent with those studies, our study shows that supplementation of BRF rich in anthocyanins could increase the total plasma antioxdant capacity in patients with CHD, which may be due in part to the intact absorption of anthocyanin(Figure 1). It's well known that the total antioxidant system in plasma includes anti-oxidative enzymatic and non-enzymatic systems mostly from the diet. In this study, plasma SOD activity is not significantly different between the two groups, which may suggest that the anthocyanin in BRF is probably unrelated to the antioxidative enzymatic systems but act as a direct reactive oxygen species scavenger by itself in vivo. Another possibility is that the anthocyanins in BRF may enhance SOD activity in tissue but not in plasma.

Emerging evidences have strongly suggested that atherosclerosis is a multifactorial and multistep disease that involves chronic inflammation at every stage, from initiation to progression, and eventually, plaque rupture.[23-25] Therefore, inflammatory factors are considered to play an important role in the development and progression of atherosclerotic lesions.[26] VCAM-1,CD40L and hs-CRP, all of which play important roles in the inflammatory process, have recently been shown to circulate in increased amounts in patients with CHD.[27-29] The soluble forms of these molecules may thus be regarded as markers of atherosclerotic plaque's activity. Similar to our previous reports, this study demonstrates that compared with WRF, chronic intake of BRF rich in anthocyanins can exert significant inhibitory effects on sVCAM-1,sCD40L and hs-CRP in patients with CHD. Since a previous study reported that VCAM-1, CD40L and hs-CRP are stimulated by ROS,[30] the decrease in these inflammatory factors maybe due largely to the improvement in plasma antioxidant status by anthocaynins contained in BRF.

Concerning the plasma lipids, the results in this reserach are not in accordance with our previously published studies.[4-7] However, it should be emphasized that our study was performed as a clinical trial in patients with atherosclerotic disease and not in animal models or isolated cells. As far as we know, most hyperlipidemia participants had been taking statins and keeping a low-lipid diet before being recruited in this study, which led to a relatively normal plasma lipids level before the BRF treatment. Therefore, we assume that the lipid-lowering effects may be masked by the long-term use of statins and consumption of low-lipid diets in patients since the lipid-lowering effects of different treatments are always almost significantly related to pretreatment cholesterol levels.[31,32] Another possible explanation could be that the in vivo beneficial effects of BRF rich in anthocaynins are probably not caused by its lipid-lowering capacity in this study. This hypothesis is derived from one similar study in which consumption of black or green tea had no effect on plasma lipids for 4 weeks in smokers.[33] Carotid IMT is commonly used as a surrogate marker of atherosclerosis. Supplementation of BRF could not reduce carotid IMT in our study. No changes in the carotid IMT may be explained by the unchanged of lipids level because the progression of carotid atherosclerosis is tightly related to the

the increased lipids level in plasma.[34]

In conclusion, the present study provides the first clinical evidence that black rice pigment fraction can reduce some cardiovascular risk factors in patients with CHD. Those positive effects of BRF may be attributed to the antioxidant and anti-inflammatory activities of anthocyanins contained in BRF. These observations suggest that BRF may serve as an important diet supplement for those individuals with CHD.

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