Supplementation of Diets with the Black Rice Pigment Fraction Attenuates Atherosclerotic Plaque Formation in Apolipoprotein E Deficient Mice[1]

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ABSTRACT Apolipoprotein (apo)E-deficient mice were used to study the antiatherogenic effect of black rice pigment fraction (BRF) and the possible mechanisms by which it inhibits atherogenesis. The apoE-deficient mice ($n = 45$) were randomly divided into three groups and received AIN-93G diet (positive group), AIN-93G with 5 g of black rice pigment fraction/100 g (BRF group) and AIN-93G with 5 g of white rice outer layer fraction/100 g (WRF group) for 16 wk. C57BL/6J mice ($n = 15$) received AIN-93G and were used as a control group. Blood samples were collected for measurement of lipid concentration, antioxidized LDL antibody and nitric oxide concentration. Livers were extracted for determination of cholesterol concentrations, and aortas were used to determine cholesterol concentrations and inducible nitric oxide synthase protein and mRNA expression. Hearts were used to assess atherosclerotic plaque formation. The apoE-deficient mice fed the black rice pigment fraction diet had 48% ($P < 0.01$) less atherosclerotic lesion area compared with apoE-deficient mice fed only the AIN-93G diet and 46% ($P < 0.01$) less lesion area compared with mice fed the white rice outer layer fraction diet. This observation corresponded with significantly ($P < 0.05$) lower total serum cholesterol, lower liver and aorta cholesterol ($P < 0.01$) and higher HDL cholesterol ($P < 0.05$) concentrations and lower ($P < 0.05$) antioxidized LDL antibody titer in apoE-deficient mice fed the black rice pigment fraction diet compared with positive and WRF groups. Notwithstanding this, mice fed the black rice pigment fraction diet also had lower $CD4^+$ T lymphocyte expression ($P < 0.05$) and weaker inducible nitric oxide synthase expression ($P < 0.05$) compared with mice fed the AIN-93G diet and the white rice outer layer fraction diet, respectively. We concluded that the inhibition of atherosclerotic lesions of the black rice pigment fraction is attributed to the improvement in cholesterol accumulation and reduction in oxidative stress and inflammation. J. Nutr. 133: 744–751, 2003.

KEY WORDS: • *black rice pigment fraction* • *apoE-deficient mice* • *LDL oxidation* • *atherosclerosis* • *inflammatory response*

Atherosclerotic cardiovascular disease is a major cause of death in the United States, Europe and some parts of Asia (1). There are few effective therapeutic options available to treat the disease due to its pathological complexity. Current data support the hypothesis that atherosclerosis involves inflammatory features (2,3). The inflammatory process includes early oxidative modification of LDL, which plays an important role in the initiation and progression of atherosclerosis (4). Oxidized LDL (OxLDL)[3]-induced macrophage adhesion into the subendothelium is an inflammatory response that promotes cholesterol accumulation and foam cell formation, the hallmark of early atherosclerosis. Many in vitro and some in vivo studies have suggested that oxidative modification of LDL involves the onset of atherosclerosis and exacerbates its clinical manifestations (5). Both blood monocytes and plasma lipoproteins at the arterial wall are exposed to atherogenic modifications under oxidative stress status. This indicates that it may be of great importance in regulating the oxidative stress and inhibiting the inflammatory response that accompany the development of atherosclerosis.

Epidemiological and clinical studies with antioxidants have reported contradictory findings; for example, individual antioxidants used in supplements have yielded mostly negative results, whereas a large number of studies both in vitro and from experiments in animals have indicated that oxidative stress is positively associated with the progression of atherosclerosis (6). It is important to note that a large number of observations in different populations have consistently shown that natural foods that are rich in antioxidants are associated with a low incidence of atherosclerotic cardiovascular disease (7–11). China also has a low incidence of atherosclerosis,

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[3] Abbreviations used: BRF, black rice pigment fraction; C, cholesterol; cNOS, constitutive nitric oxide synthase; iNOS, inducible nitric oxide synthase; OxLDL, oxidized LDL; PBS, phosphate-buffered saline; PON, paraoxonase; RT-PCR, reverse transcription polymerase chain reaction; TC, total cholesterol; WRF, white rice outer layer fraction.

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TABLE 1

Composition of black rice pigment fraction and white rice outer layer fraction

Ingredient	Black rice pigment fraction	White rice outer layer fraction
	units/100 g	
Protein, *g*	13.90	12.20
Fat, *g*	13.20	14.10
Carbohydrate, *g*	47.36	50.95
Moisture, *g*	9.80	7.96
Crude fiber, *g*	8.32	7.04
Minerals, *mg*	7420	7750
Phosphorus	1694.10	1542.50
Calcium	60.20	45.30
Potassium	673.70	624.60
Magnesium	79.40	80.40
Sodium	2.11	4.35
Iron	16.46	6.30
Zinc	8.96	4.92
Copper	1.49	0.91
Selenium	0.15	0.06
Vitamins, *mg*		
Vitamin B-1	2.30	1.20
Vitamin B-2	0.40	0.14
Vitamin E	0.60	0.03
Nicotinic acid	21.00	13.00
Flavonoids, *g*	6.40	1.17

which could be attributed to the fact that ~60% of the daily energy intake is derived from carbohydrate consumption, mainly in the form of rice. The cardioprotective role of rice diets was suggested several decades ago (12,13). There are many different varieties of rice, including white and pigmented rice. We recently have shown that supplementation of the black rice or black rice outer layer fraction (BRF), compared with white rice outer layer fraction (WRF), significantly reduced atherosclerotic plaque formation induced by high dietary cholesterol feeding in rabbits (14). A primary mechanism of action was associated with improved antioxidant status in rabbits fed the black rice; this effect may be related to higher vitamin E, selenium, iron and zinc concentrations in black rice (15). However, due to the pathogenic complexity of atherosclerosis, we hypothesized that the pigmented layer of black rice may have other possible mechanisms in inhibiting atherosclerotic plaque formation such as hypocholesterolemia and antioxidant and anti-inflammatory properties.

ApoE-deficient mice represent an animal model that is widely used to study the accelerated atherogenic process (16) and is similar to familial dysbetalipoproteinemia, a condition that is defined by increased serum chylomicrons and VLDL remnants. The characteristics of atherosclerotic lesions in apoE-deficient mice are similar to the pathogenesis of atherosclerosis in human lesions in several aspects, including changes in lipid profile and increased oxidative stress and inflammation (17). Moreover, this animal model provides an opportunity to examine the role of dietary components that could modulate the progression of atherosclerosis.

In the present study, we investigated the effect of supplementing the AIN-93G diet with BRF on atherosclerotic plaque formation and metabolic events that may underlie the progression of this disease in the apoE-deficient mice, and we further explored the mechanisms that are attributed to the efficacy of BRF against the atherosclerotic lesions in the apoE-deficient mouse model.

MATERIALS AND METHODS

Animals and diets. The apoE-deficient mice and normal C57BL/6J mice were provided by Jackson Laboratories (Bar Harbor, ME). Forty-five apoE-deficient mice and 15 normal C57BL/6J mice were bred and maintained under conventional housing conditions in our animal facility. All animal procedures were followed in accordance with the approved protocol for use of experimental animals set by the standing committee on animal care at Sun Yat-sen University. All mice were fed a purified diet based on the AIN-93G formulation (18). Normal C57BL/6J mice were used as the control (control group, $n = 15$) and fed the AIN-93G diet. Three groups of apoE-deficient mice were fed one of the following diets: AIN-93G diet (positive group, $n = 15$), AIN-93G diet with 5 g of BRF/100 g (BRF group, $n = 15$) or AIN-93G diet with 5 g of WRF/100 g (WRF group, $n = 15$). The raw material of black and white rice was husked, and the pigment fraction of black rice and the outer layer fraction of white rice (~10% of whole grain) were further ground. The BRF and WRF were chemically analyzed **(Table 1)**. The protein, fat and energy concentrations of different experimental diets were adjusted to the same level by adding casein and soybean oil **(Table 2)**. The duration of feeding mice in different groups was 16 wk. At the end of the experiment, all mice were deprived of food overnight and killed under diethyl ether anesthesia. Whole blood was collected and serum was prepared for laboratory analysis. The major organs and aorta of each mouse were harvested, washed with ice-cold phosphate-buffered saline (PBS) and weighed. The serum samples were stored at −80°C, and aorta samples were stored in liquid nitrogen until used for various analyses. The number of samples used for different measurements was not equal for each variable because the amount of organ tissue harvested was insufficient for all parameter analysis in this experiment.

Assessment of atherosclerosis. Quantification of atherosclerotic fatty streaks was done by calculating the lesion size in the aortic sinus as previously described (19) with a few modifications. Briefly, the heart and upper section of the aorta were removed from the mice and the peripheral fat was cleansed carefully. The upper section was embedded in OCT compound (Sigma Chemical, St. Louis, MO) and frozen at −20°C. Every other section (10 μm thick) throughout the aortic sinus (400 μm) was taken for analysis. The distal portion of the aortic sinus was recognized by the three valve cusps that are the junctions of the aorta and the heart. Cryostat sections were evaluated for fatty streak lesions after staining with Oil red O and counterstain-

TABLE 2

Dietary formulation for apolipoprotein E–deficient mice fed AIN-93G diet (positive group), AIN-93G diet plus 5 g of black rice pigment fraction per 100 g (BRF group) or AIN-93G diet plus 5 g of white rice outer layer fraction per 100 g (WRF group) or for C57BL/6J mice fed AIN-93G diet (control group)

Ingredient	Control group	Positive group	BRF group	WRF group
	units/kg of diet			
Energy, *kJ*	15,756.944	15,756.944	15,756.944	15,756.944
Cornstarch, *g*	397.486	397.486	397.486	397.486
Casein, *g*	200	200	200	200
Dextrinized cornstarch, *g*	132	132	132	132
Sucrose, *g*	100	100	100	100
Soybean oil, *g*	70	70	70	70
Fiber, *g*	50	50	50	50
Mineral mix, *g*	35	35	35	35
Vitamin mix, *g*	10	10	10	10
L-Cystine, *g*	3	3	3	3
Choline bitartrate, *g*	2.5	2.5	2.5	2.5
tert-Butylhydroquinone, *g*	0.014	0.014	0.014	0.014

ing with hematoxylin. Each section was evaluated for Oil red O staining area by capturing images directly from an RGB camera (JVCky-F 30B; Tokyo, Japan) attached to an Olympus BX-50 light microscope and displaying them on a Trinitron RGB monitor (Kontron IBAS2.5; Eching, Germany). Image analysis was determined using Optimas 4.1 software. Results were expressed as the percentage of the total cross-sectional vessel wall area (normal plus diseased area/section, excluding the lumen) stained with Oil red O.

Serum lipid profile. Blood samples were obtained from mice via retro-orbital bleeding under anesthesia, and serum was prepared by centrifugation. Serum total cholesterol (TC) and HDL cholesterol (HDL-C) were measured using cholesterol esterase and cholesterol oxidase assays (20). Serum LDL cholesterol (LDL-C) concentrations were determined via the direct method (21).

Assay for cholesterol accumulation in liver. Immediately after the mice were killed, the livers were removed and heat processed at 110°C for 11 h. Weighed dried livers (300 mg) were extracted with Folch reagent [chloroform/methanol (2:1 vol/vol)] and stored at −20°C until analysis (22,23).

Assay for cholesterol accumulation in aorta. Immediately after the mice were killed, the aortic arch was dissected from the aortic root to the right renal artery and adventitial fat was removed. After being washed with cold PBS, the tissue was minced and weighed before removal of the lipids according to the method of Folch et al. (23,24). Fluorescence was measured with a spectrofluorophotometer (R-540; Shimadzu, Tokyo, Japan) (excitation, 325 nm; emission, 415 nm). Samples for each aorta were run in duplicate. All values are expressed as nanomoles per gram of aorta (wet weight) for each mouse. Means and SD were determined for each group.

Detection of anti-OxLDL antibodies by ELISA. Serum anti-OxLDL antibody concentrations were determined with a Protein Detector kit. (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The measurement was made according to the manual provided with the kit with a few modifications (25). In brief, polystyrene plates with 96 wells were coated with either copper-OxLDL or native LDL overnight at 4°C. After washing, the plates were blocked with blocking solution for 2 h at room temperature. Diluted (1:50) serum fractions were added in blocking solution. After additional overnight incubation at 4°C, the serum samples were washed three times, and secondary antibody solution (1:10,000 alkaline phosphatase conjugated goat anti-mouse IgG in blocking solution) was added to each well for 1 h at room temperature. After extensive washing, 100 μL of substrate solution (1 g of *p*-nitrophenyl phosphate/L in 50 mmol of carbonate buffer/L containing 1 mmol of $MgCl_2$/L) was dispensed into each well. The reaction was stopped after 30 min by the addition of 100 μL of stop solution (1 mol of NaOH/L) to each well. Absorbance was detected at 405 nm in a Titertek ELISA reader (Bio-Tek Instruments, Highland Park Winooski, GA), and results were expressed as absorbance at 405 nm. Anti-OxLDL concentrations were calculated as follows: OD value of binding to native LDL subtracted from OxLDL binding.

Serum nitrite measurement. Serum nitrite concentration from mice was measured as an indicator of NO production using the Griess reaction. Serum (100 mL) was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). The absorbance at 550 nm was determined with a curve calibrated on sodium nitrite standards (26).

Assay for inducible nitric oxide synthase (iNOS) protein by Western blotting. iNOS protein was analyzed by immunoblotting with the anti-iNOS antibody (27). Protein extracts of the aorta were made by homogenization in 5 volumes of ice-cold Tris-buffered saline (0.15 mol of sodium chloride and 20 mmol of Tris-HCl/L, pH 7) containing 1% Triton X-100, 1 mmol of phenylmethylsulfonylfluoride/L and 1 mg of aprotinin/L. Extracts containing 60 μg of total protein were loaded onto 8% SDS-PAGE using a protein assay (Bio-Rad Laboratories, Hercules, CA), and the separated proteins were electrophoretically transferred to nitrocellulose membranes. The membrane was blocked in Tris-buffered saline with 0.1% Tween-20/5% nonfat milk and probed with the polyclonal antibody to iNOS (1:1,000 dilution) (Rt anti-Mo iNOS; Santa Cruz Biotechnology, Santa Cruz, CA) overnight, followed by a horseradish peroxidase linked secondary antibody (1:1,000 dilution). Specific protein bands were revealed by enhanced chemiluminescence and visualized by immediate exposure to autoradiographic film.

Assay for iNOS mRNA by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from aorta by using TriZOL reagent according to the manufacturer's instructions (InVitrogen, Carlsbad, CA). RNA was reverse-transcribed from 5 μg of total RNA in a final volume of 20 μL using the SUPERSCRIPT First-Strand Synthesis System for RT-PCR (InVitrogen). The reaction was stopped by the addition of 0.1 units of *Escherichia coli* RNase H/μL. and heating of the samples for 20 min. cDNA was subjected to DNA amplification by PCR (28) using 1 U of *Tag* DNA polymerase and both primer pairs complementary to murine iNOS cDNA and actin cDNA at a final concentration of 20 pmol of each primer/L. The reaction was predenatured at 95°C for 5 min. The amplification cycle (denaturing at 94°C for 45 s, annealing at 60°C for 1 min and extension at 72°C for 1 min) was repeated 35 times and followed by a final extension for 10 min at 72°C. The primers for iNOS were 5′-AGACATGGCTTGCCCCTGG-3′ (sense) and 5′-GATCAGGAGGGATTTCAAAGACCT-3′ (antisense). The expected product length was 674 bp. The primers for the "housekeeping gene" β-actin were 5′-GGACTCCTATGTGGG TGACGAGG-3′ (sense) and 5′-GGGAGAGCATAGCCCTCGTAGAT-3′ (antisense). The expected product length was 366 bp. Final PCR products were separated on 2% agarose gels and detected by ethidium bromide staining. Semiquantitative estimation was done by comparing mRNA expression of iNOS with β-actin represented by the amount of the PCR product formed.

Immunohistochemistry. Immunohistochemical staining for CD4 and CD8 was performed on 10-μm-thick cryostat sections of the aortic sinus. The sections were fixed in cold acetone at 4°C and pretreated with 3% H_2O_2 to exhaust endogenous peroxidase activities. Unless specified, the sections were blocked with nonimmune goat serum, followed by treatment with primary antibody using the following antibodies: rabbit anti-mouse CD4 monoclonal antibody (Santa Cruz Biotechnology) and rabbit anti-mouse CD8 polyclonal antibody (Dako, Glostrup, Denmark). Sections were then incubated with horseradish peroxidase conjugated secondary antibody and peroxidase-conjugated streptavidin. After washing in PBS, color was developed with DAB solution and counterstained with hematoxylin. Staining in the absence of first or second antibody was used as a negative control.

Statistical analysis. Results are expressed as means ± SD. Data were analyzed by one-way ANOVA coupled with the Student-Newman-Keuls multiple comparison test. Differences were considered significant if $P < 0.05$. SPSS Version 10.0 software was used for all statistical analysis.

RESULTS

Body weights. The means ± SD body weight of mice before the start of the experiment was 17 ± 1 g compared with 25–27 g at the end of the experiment. There were no significant differences in final body weights among mice fed the various dietary treatments.

Atherosclerotic plaque formation in the aortic sinus. There was no visible atherosclerotic plaque in the aortic sinus of the C57BL/6J mice fed the AIN-93G diet (control group). Atherosclerotic plaques of varying degrees were visible in the aortic sinus of apoE-deficient mice fed AIN-93G (positive group), the BRF group and the WRF group (**Fig. 1**A). The data show less deposition of plaque in the BRF group mice compared with the positive and WRF groups. The plaque area in the BRF group mice was 48% and 46% lower than that of the positive and WRF groups, respectively ($P < 0.01$), which did not differ from each other (Fig. 1B).

Serum lipid profile. Serum TC, LDL-C and HDL-C concentrations in C57BL/6J mice were significantly different from those of the other groups ($P < 0.05$) (e.g., positive, BRF and

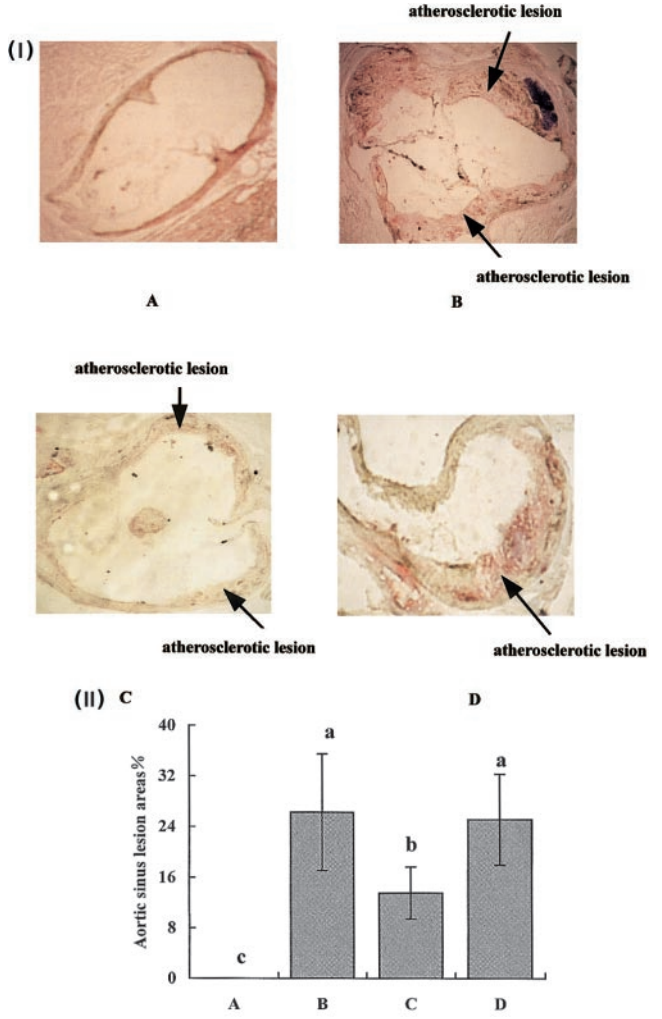

FIGURE 1 *(I)* Crystat sections of aortic sinus stained by Oil red O in mice fed AIN-93G diet [control group *(A)* and positive group *(B)*] or AIN-93G diet supplemented with the black rice pigment fraction [BRF group *(C)*] or with the white rice outer layer fraction [WRF group *(D)*] for 16 wk. The fatty-streak lesions stained red *(arrows)* (original magnification, ×40). *(II)* Atherosclerotic plaque area in aortic sinus of mice fed AIN-93G diet [control group *(A)* and positive group *(B)*] or AIN-93G supplemented with black rice pigment fraction [BRF group *(C)*] or with white rice outer layer fraction [WRF group *(D)*] for 16 wk. Values are means ± SD, $n = 15$. Bars without a common letter are significantly different, $P < 0.01$.

WRF groups). Concentrations of TC and LDL-C in the BRF group mice were lower than those in the positive and WRF groups, ($P < 0.05$). There were no differences in these variables between the positive and WRF groups. Both BRF and WRF groups had higher HDL-C concentrations than the positive group ($P < 0.05$); furthermore, the BRF group had a lower LDL/HDL ratio than the WRF group ($P < 0.05$) (**Table 3**).

Antibodies to OxLDL. There was a significant reduction of the serum anti-OxLDL antibody titer in BRF group mice compared with WRF and positive group mice ($P < 0.05$). However, there were no significant differences in the titers of OxLDL antibody between the positive and WRF groups (**Table 4**).

Cholesterol accumulation in aortas and livers. There was less accumulation of TC in aorta and liver tissue from C57BL/6J mice compared with all groups of apoE-deficient

TABLE 3

Serum lipid concentrations in apolipoprotein E–deficient mice fed AIN-93G diet (positive group) or AIN-93G diet supplemented with the black rice pigment fraction (BRF group) or white rice outer layer fraction (WRF group) or C57BL/6J mice fed AIN-93G diet (control group) for 16 wk[1,2]

Group	TC	LDL-C	HDL-C	LDL/HDL
	mmol/L			
Control	2.88 ± 0.76[c]	0.28 ± 0.08[c]	1.78 ± 0.44[c]	0.16 ± 0.04[c]
Positive	15.68 ± 2.64[a]	1.43 ± 0.42[a]	2.55 ± 0.44[b]	0.57 ± 0.12[a]
BRF	12.12 ± 1.83[b]	0.75 ± 0.25[b]	2.66 ± 0.40[a]	0.34 ± 0.17[b]
WRF	17.24 ± 3.65[a]	1.32 ± 0.56[a]	2.65 ± 0.66[a]	0.45 ± 0.18[a]

[1] TC, total cholesterol; C, cholesterol.
[2] Values are means ± SD, $n = 10$. Values in a column without a common superscript letter are significantly different, $P < 0.05$.

mice ($P < 0.01$) (**Figs. 2** and **3**). Accumulation of cholesterol in aortas and livers in the BRF group was reduced significantly compared with that in the positive and WRF groups, ($P < 0.01$). There were no significant differences between the positive and WRF groups.

Immunohistochemistry. The T lymphocyte expression of antibodies to CD4 and CD8 in aortic sinus is shown in **Figure 4**. $CD4^+$ T lymphocyte expression in C57BL/6J mice was less than that in all groups of apoE-deficient mice ($P < 0.05$). The BRF group mice had a lower CD4 expression than the positive and WRF groups ($P < 0.05$). There were no significant differences in positive $CD4^+$ T lymphocyte concentrations between the positive and WRF groups, and there were no significant differences in $CD8^+$ T lymphocyte expression among the different groups.

Western blot analysis for iNOS protein expression. The C57BL/6J mice had lower iNOS protein expression than all groups of apoE-deficient mice ($P < 0.05$). The BRF group mice had lower iNOS protein expression than the positive and WRF group mice ($P < 0.05$), suggesting that BRF inhibited iNOS protein expression in apoE-deficient mice. No significant difference was observed between the positive and WRF groups (**Fig. 5**).

RT-PCR analysis for iNOS mRNA expression. To investigate whether the alteration of iNOS activity by different diets was due to altered iNOS mRNA, a RT-PCR analysis for

TABLE 4

Serum antioxidized LDL antibody titer in apolipoprotein E-deficient mice fed AIN-93G diet (positive group) or AIN-93G diet supplemented with the black rice pigment fraction (BRF group) or white rice outer layer fraction (WRF group) or C57BL/6J mice fed AIN-93G diet (control group) for 16 wk[1]

Group	Optical density
Control	0.012 ± 0.001[c]
Positive	0.079 ± 0.028[a]
BRF	0.036 ± 0.013[b]
WRF	0.061 ± 0.020[a]

[1] Values shown are means ± SD, $n = 15$. Values in the column without a common superscript letter are significantly different, $P < 0.05$.

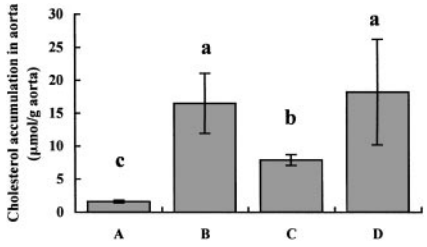

FIGURE 2 Cholesterol accumulation in aortas of mice fed AIN-93G diet [control group *(A)* and positive group *(B)*] or AIN-93G supplemented with the black rice pigment fraction [BRF group *(C)*] or with white rice outer layer fraction [WRF group *(D)*] for 16 wk. Values are means ± SD, $n = 6$. Bars without a common letter are significantly different, $P < 0.01$.

total mRNA samples extracted from the aorta was performed and β-actin was used as the housekeeping gene. Significantly lower concentrations of iNOS mRNA were expressed in C57BL/6J mice than in apoE-deficient mice ($P < 0.05$). The BRF group mice showed a weaker expression of iNOS mRNA than the positive and WRF group mice ($P < 0.05$). No significant difference was observed between the positive and WRF groups (**Fig. 6**).

DISCUSSION

The present study showed for the first time that supplementation of experimental diets with the BRF reduced atherosclerotic plaque areas in aortic sinus compared with the WRF in apoE-deficient mice. The BRF thus has the capacity to prevent atherosclerotic plaque formation and progression in apoE-deficient mice.

ApoE is a circulating 34-kDa glycoprotein component of several lipoproteins, including LDL, chylomicron, VLDL remnants, β-migrating VLDL and HDL. The protein has a role in the uptake and degradation of chylomicron and VLDL remnants by the LDL receptor and the LDL receptor related protein (29). Genetic deficiency of apoE in humans has been shown to result in the accumulation of plasma remnant lipoproteins and the development of atherosclerosis. ApoE-deficient mice also develop severe hypercholesterolemia and atherosclerosis when they are fed a regular low fat/low cholesterol diet (30). Consistent with former findings, the present study showed that after 16 wk of dietary intervention, apoE-deficient mice fed AIN-93G diet (positive group) exhibited

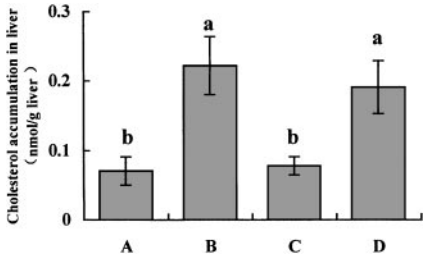

FIGURE 3 Cholesterol accumulation in livers of mice fed AIN-93G diet [control group *(A)* and positive group *(B)*] or AIN-93G supplemented with the black rice pigment fraction [BRF group *(C)*] or with the white rice outer layer fraction [WRF group *(D)*] for 16 wk. Values are means ± SD, $n = 8$. Bars without a common letter are significantly different, $P < 0.01$.

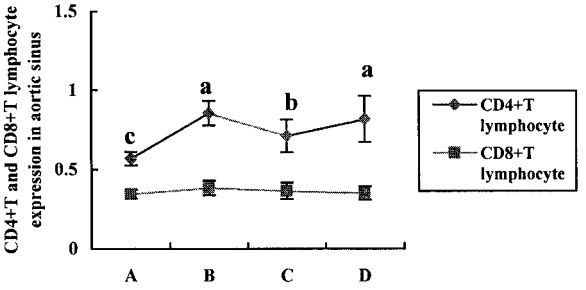

FIGURE 4 T-lymphocyte expression in aortic sinus of mice after being fed AIN-93G diet [control group *(A)* and positive group *(B)*] or AIN-93G diet supplemented with the black rice pigment fraction [BRF group *(C)*] or with the white rice outer layer fraction [WRF group *(D)*] for 16 wk. Values are as means ± SD, $n = 15$. Columns without a common letter are significantly different, $P < 0.05$.

higher serum cholesterol concentrations, which in turn corresponded to greater cholesterol accumulation in both liver and aortic tissue. ApoE-deficient mice fed a diet supplemented with BRF had relatively lower serum TC and higher HDL-C concentrations compared with both the positive and WRF groups, respectively. This alteration is one explanation for the reduced atherosclerotic plaque noted in the mice. Elevated serum HDL has been correlated with reduced risk of atherosclerosis (31), on the basis that HDL has an important role in transporting cholesterol or cholesterol ester from peripheral tissues to the liver where cholesterol is metabolized and transformed into bile acids. This pathway represents one critically important mechanism for reducing cholesterol concentrations in both blood and peripheral tissues, thus protecting against atherosclerotic plaque formation. In addition, although the mice fed BRF or WRF had relatively higher HDL-C concentrations than the positive group, it was the apoE-deficient mice fed the BRF-supplemented diet that exhibited the lowest LDL/HDL ratio. This ratio is often used to characterize the athero-

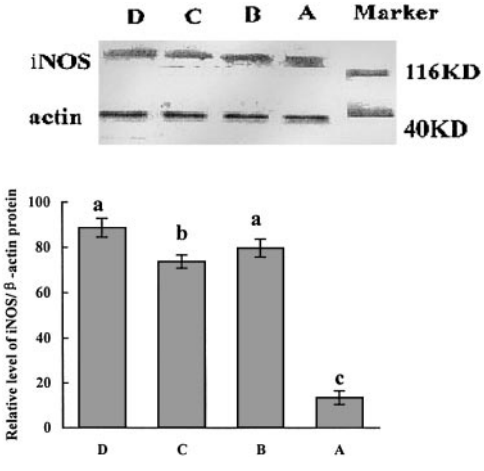

FIGURE 5 *(Top)* Immunoblotting analysis of inducible nitric oxide synthase (iNOS) protein expression in aortas of mice fed AIN-93G diet [control group *(A)* and positive group *(B)*] or AIN-93G supplemented with the black rice pigment fraction [BRF group *(C)*] or with the white rice outer layer fraction [WRF group *(D)*] for 16 wk. *(Bottom)* The iNOS protein expression concentrations were calculated as the ratio of iNOS to β-actin protein expression. Values are means ± SD, $n = 4$. Bars without a common letter are significantly different, $P < 0.05$.

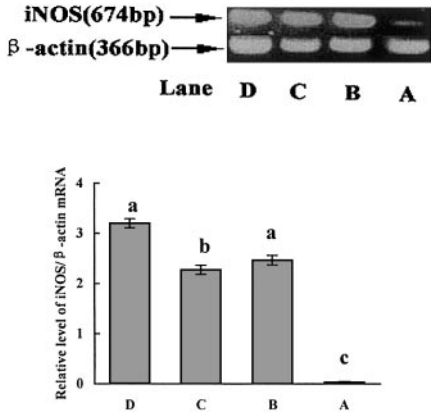

FIGURE 6 *(Top)* Reverse transcription polymerase chain reaction analysis of inducible nitric oxide synthase (iNOS) mRNA expression in aortas of mice fed AIN-93G diet [control group *(A)* and positive group *(B)*] or AIN-93G supplemented with black rice pigment fraction [BRF group *(C)*] or with white rice outer layer fraction [WRF group *(D)*] for 16 wk. *(Bottom)* The iNOS mRNA expression concentrations were calculated as the ratio of iNOS to β-actin mRNA expression. Results are means $\pm$ SD, $n = 4$. Bars without a common letter are significantly different, $P < 0.05$.

genic potential of different dietary constituents, including cholesterol and saturated fatty acids, and has been associated with atherosclerotic plaque deposition in aortic tissue (32). Because a large part of TC was accounted for by the non-LDL/non-HDL fraction in apoE-deficient mice, which is similar to a previous report (30), the influence of the BRF diet on VLDL or IDL fractions remains unclear in this study.

The BRF contains a variety of phytochemicals, such as polyphenols, isoflavones and anthcyanidins, that have several beneficial functions. It has been reported that isoflavones are efficient in reducing blood lipids (33,34). The improvement in the lipid profile induced by the BRF-supplemented diet may be attributed to compounds such as flavones or isoflavones, which are plentiful in the BRF. However, whether these components affect lipid absorption, lipoprotein assembly or turnover and other metabolic pathway needs further elucidation.

Several lines of evidence have indicated that immune complexes formed from OxLDL are relevant to the development of atherosclerosis (35,36). In fact, a positive correlation between the autoantibody titer to OxLDL and the rate of progression of atherosclerosis has been reported (37). Previously reported data also show that titers of oxidatively modified LDL antibodies may have value as a diagnostic or prognostic tool for atherosclerosis (38). Oxidation of LDL results in structural modification and the formation of a large number of neoepitopes (39). For example, covalently bound products result from the reaction between reactive aldehydes, which are generated during lipid peroxidation, and lysine and histidine residues of apoB or similar proteins that compose the LDL molecule (40). Examples of highly immunogenic products of oxidatively modified LDL include malondialdehyde lysines and 4-hydroxynonenal lysine (41). The titers of autoantibodies to OxLDL are dependent on the presence and thus occurrence of OxLDL. Because OxLDL present in atherosclerotic lesions trigger an in vivo humoral immune response (42), it follows that the titer of OxLDL is related to the response of the immune system. Moreover, autoantibodies to OxLDL in atherosclerotic plaques are related to $CD4^+$ T lymphocytes, which promote B cell activation and production of anti-OxLDL antibodies (43).

The milieu of underlying mechanisms defining the progression of atherosclerosis is made more complex by the fact that T cells are also involved in accelerating atherosclerosis. Zhou et al. (44) reported the presence of many $CD4^+$ T lymphocytes in aortic lesions of apoE-deficient mice fed both a standard rodent and Western-type diets. Immunohistochemical and PCR analyses have also reported the presence of T cell cytokines interleukin-2 and interferon-γ expression within human plaques (45,46), which indicates that local T cell activation has occurred. It is also known that interferon-γ will promote atherogenesis by enhancing T cell mediated immune inflammation and upregulating endothelial leukocyte adhesion (47–49).

In the present study, supplementation of the diet fed to apoE-deficient mice with BRF decreased the titer of anti-OxLDL antibodies and the level of $CD4^+$ T lymphocytes compared with the positive and WRF groups. This finding strongly suggests that the pigments present in BRF possess sufficient bioavailable antioxidant activity to suppress the formation of OxLDL, while also providing protection against an immune response to OxLDL by lowering $CD4^+$ T lymphocyte expression. This combined effect of BRF resulted in a lower production of anti-OxLDL antibodies.

A variety of in vitro studies (50–52) originally suggested that paraoxonase (PON), which is a calcium-dependent, HDL-associated organophosphate hydrolase, may protect against LDL oxidation and atherosclerosis in apoE-deficient mice. These data demonstrated that several antioxidants, including pomegranate juice, red wine derived polyphenols and such flavonoids as quercetin or catechin preserved serum PON activity in apoE-deficient mice because they decrease the concentration of lipid peroxides, which inactivates PON. The BRF used in our study has a large amount of flavonoids compared with WRF; whether its antioxidation effects are related to higher concentrations of flavonoids and improved PON activity requires further study.

NOS are present in both constitutive (cNOS) and inducible (iNOS) forms, with iNOS producing NO at both a higher rate and for a longer duration than cNOS (53). NO has antiadhesive properties that influence the interaction between leukocytes and endothelial cells, perhaps by interfering with the migration of monocytes and lymphocytes at the atherosclerotic site. In addition, NO has antithrombotic and antiproliferative properties, which may also provide a local protective effect against the development of atherosclerosis (54). NO contributes to the pathogenesis of inflammatory disorders (55) and is induced in response to a variety of inflammatory cytokines (55), many of which are produced in atherosclerotic conditions that involve further inflammatory cellular damage and apoptosis (56). Mediation of these reactions may occur from the highly reactive oxidant peroxynitrite that is generated from the reaction between NO and the superoxide anion (57). NO produced in large quantities therefore has the potential to cause tissue injury due to the generation of peroxynitrite radicals, which have oxidizing capacity toward cellular protein and lipid macromolecules (56). Our findings show that apoE-deficient mice fed the BRF-supplemented diet had a relatively lower expression of iNOS protein and mRNA. This observation corresponds to the antioxidant and anti-inflammatory properties attributed to black rice reported in an earlier study in rabbits (14,15), which was also related to antiatherogenic effects.

The specific constituents present in the BRF that contribute to the anti-inflammatory and antioxidant properties and

observed reduction in atherosclerosis in experimental animals in this and other studies remain to be determined. BRF contains a mixture of anthocyanins and flavonoids (58) that have well-documented antioxidant and anti-inflammatory properties (59,60). Flavonoid derivatives, including prenylated compounds (e.g., morusin, kuwanon C and sanggenon D) and bioflavonoids such as bilobetin and ginkegtin, are effective inhibitors of lipopolysaccharide-induced NO production (61,62).

In conclusion, this study has demonstrated that the presence of BRF in a diet fed to apoE-deficient mice was effective in decreasing atherosclerotic plaque development in the aortic sinus. This cardioprotective effect was related to several mechanisms that corresponded to lowering serum TC concentration and LDL/HDL ratios, decreasing cholesterol accumulation in aortic arterial tissue and reducing LDL oxidation and $CD4^+$ T lymphocytes in aortic tissue. The present study indicates that black rice or BRF has strong preventive effects against atherosclerotic disease or coronary heart disease. However, the naturally occurring components present in BRF that contribute to the combined inhibition of immune and inflammatory responses that lead to a beneficial antiatherogenic properties require further elucidation and are currently under investigation.

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