Supplementation of the Black Rice Outer Layer Fraction to Rabbits Decreases Atherosclerotic Plaque Formation and Increases Antioxidant Status[1]

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ABSTRACT The influence of the supplementation of black and white rice outer layer fractions on atherosclerotic plaque formation induced by hypercholesterolemia was investigated in rabbits. Male rabbits ($n = 32$) were randomly divided into four groups. They were fed nonpurified diet (normal group), a lard (3.5 g/100 g) with high cholesterol (0.5 g/100 g) diet (HC group); the HC diet with 5 g/100 g white rice outer layer fraction (WRF group); or the HC diet with 5 g/100 g black rice outer layer fraction (BRF) for 2 mo. Blood samples were collected for determination of lipid concentration and oxidative and antioxidative status variables, and aortae were taken for the assessment of atherosclerotic plaques. The atherosclerotic plaque area in rabbits fed the BRF diet was 66% lower than that of the HC or WRF rabbits ($P < 0.001$). Supplementation of the black rice outer layer significantly ($P < 0.05$) lowered aortic 8-hydroxy-2′-deoxyguanosine (8-OHdG) (−52%, −44%) compared with the WRF or HC diets ($P < 0.05$). There were no differences in aortic 8-OHdG levels between rabbits fed the BRF and normal diets. The BRF diet significantly ($P < 0.05$) decreased the malondialdehyde (MDA) level of serum (−37%) and aortic artery (−50%) compared with the WRF diet. There were no differences in the concentrations of serum total cholesterol (TC), LDL cholesterol (LDL-C), HDL-C or the ratio of apoprotein (apo)I/apoB among the HC, WRF and BRF groups. Similarly, there were no differences in the serum vitamin E concentration and erythrocyte and aorta superoxide dismutase (SOD) activities among rabbits fed these diets. The serum concentration of most fatty acids except 18:1 did not differ between the WRF and the BRF groups. We conclude that the inhibition of atherosclerotic plaque formation derived from the black rice outer layer fraction in rabbits might be mediated by antioxidative or anti-inflammatory effects. J. Nutr. 132: 20–26, 2002.

KEY WORDS: • *rice* • *atherosclerosis* • *oxidative status* • *lipids* • *rabbits.*

Atherosclerosis cardiovascular disease is the leading cause of death in the United States, Europe and parts of Asia (1). Few effective measures exist to prevent and treat the disease due to its pathological complexity. Research in the last two decades has shown that human atherosclerosis has many characteristics of an inflammatory disorder and does not result simply from the accumulation of lipids. The pathology of atherosclerosis comprises a complex interaction among lipids, the endothelium, circulating and tissue inflammatory cells, platelets and vascular smooth muscle cells (2). Although hypercholesterolemia is important in ~50% of patients with cardiovascular disease, other factors such as increased oxidative stress and serum homocysteine must be considered (3). Modification of these harmful components of inflammation in the arteries would be beneficial in creating new avenues for management of the disease in the 50% of patients who do not have hypercholesterolemia (4).

Oxidative stress plays an important role in the initiation and progression of atherosclerosis (5). Reactive oxygen species (ROS)[3] are ubiquitous and occur naturally in all aerobic species, arising from both exogenous and endogenous sources. They are quite reactive and readily damage biological molecules, including DNA, protein, carbohydrate and lipids (6). It has been reported that there are many oxidative stress indicators in tissues or blood such as 8-hydroxydeoxyguanosine (8-OHdG), malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and lipid peroxides (LPO) (7). Excessive production of ROS is implicated in the development of atherosclerosis or coronary heart disease (CHD) at different stages, including vascular endothelial cell damage, foam cell formation, vascular smooth muscle cell proliferation, gene expression, impaired vasomotor reactivity and plaque instability (8,9). Many in vitro and some in vivo studies have suggested that oxidative modification of LDL is involved in the onset of atherosclerosis

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[3] Abbreviations used: apo, apoprotein; BRF, black rice outer layer fraction; CHD, coronary heart disease; COX, cyclooxygenase; HC, high cholesterol diet; HDL-C, HDL cholesterol; 4-HNE, hydroxynonenal; IEL, internal elastic lamina; iNOS, inducible nitric oxide synthase; LDL-C, LDL cholesterol; LPO, lipid peroxides; LPS, lipopolysaccharide; MDA, malondialdehyde; MUFA, monounsaturated fatty acids; 8-OHdG, 8-hydroxydeoxyguanosine; PG, prostagladins; PUFA, polyunsaturated fatty acids; RBO, rice bran oil; ROS, reactive oxygen species; SFA, saturated fatty acids; SOD, superoxide dismutase; TC, total cholesterol; TG, triglycerides; WRH, white rice outer layer fraction.

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and exacerbates its clinical manifestations (4). Hence, the hypothesis was formulated that antioxidants may at least in part prevent atherosclerosis and cardiovascular disease. Dietary antioxidants, such as vitamins E and C have received considerable attention in this regard (10).

As research progresses and epidemiologic and clinical trials of antioxidants are being reported, an apparent contradiction emerges, i.e., supplementation studies with individual antioxidants are yielding primarily negative results (11). There are large population studies showing that natural foods rich in antioxidants are associated with a low incidence of cardiovascular diseases. For example, the Mediterranean and Japanese diets have shown these cardioprotective aspects (12–14). There is growing evidence that classic risk factors for CHD do not differ greatly in populations of southern Europe and Japan, compared with other Western countries (15,16). This suggests that other unexplored risk factors are favorably affected by these diets. Food composition studies have shown that both the Mediterranean and Japanese diets are rich in antioxidants derived from fruits, tea, red wine and olive oil and in (n-3) fatty acids, derived from fish, nuts and certain seed oils.

China is a country with a low incidence of atherosclerosis or CHD, which might be largely attributable to the Chinese diet. About 60% of the energy intake of the Chinese people is from carbohydrate, which is derived mainly from rice. The cardioprotective effect of rice diets was suggested several decades ago (17,18). There are different kinds of rice including white rice and colored rice. We recently found that supplementation of black rice, compared with white rice significantly reduced atherosclerotic plaque formation induced by hypercholesterolemia in rabbits, and the mechanism of this action was related to improvement of antioxidant status (19). Most of antioxidants, complex carbohydrates and phytochemicals of rice including proteins, lipids, vitamins, minerals and flavonoids exist in the bran and germ. The endosperm contains primarily large amounts of starch. It is postulated that the beneficial effects of black rice in reducing atherosclerotic plaque formation may be due to the enriched nutrients and phytochemicals in the bran.

The present study was designed to investigate the influence of supplementation of the outer layer of black rice on atherosclerotic plaque formation induced by hypercholesterolemia in rabbits, and to explore the possible mechanisms by which this fraction inhibits atherosclerotic plaque formation.

MATERIALS AND METHODS

Animal and diets. Male New Zealand white rabbits ($n = 32$) aged 9 wk and weighing 2.16 kg were obtained from the animal center of Guangzhou, PR-China. Rabbits were housed individually in standard stainless steel cages at 24°C with a 12-h light:dark cycle (lights on, 0630–1830 h). Rabbits had free access to food and tap water. All experiments were performed in accordance with the protocol approved by the standing committee on animals of Sun Yat-sen University of Medical Sciences. Preceding the study, all rabbits consumed the same diet for 1 wk. They were then divided into four groups and fed one of the following for 60 d: nonpurified rabbit diet (normal group, $n = 8$); normal diet with high cholesterol (0.5 g/100 g) and lard 3.5 g/100 g (HC group, $n = 8$); the HC diet with 5 g/100 g of the white rice out layer fraction (WRF group, $n = 8$); the HC diet with 5 g/100 g of the black rice outer layer fraction (BRF group, $n = 8$). The raw material of white and black rice was husked, and the outer layer fractions (bran) of white and black rice (~10% of whole grain) were further ground. The compositions of the rice outer layer fraction and experimental diets were analyzed chemically (**Table 1**). The protein and energy in the different diets were adjusted to the same levels by adding casein and cornstarch (**Table 2**). The rabbits were weighed every week. At the end of experiment, all rabbits were deprived of food overnight and killed under diethyl ether anesthesia. Blood was collected, and whole blood and serum were prepared for laboratory analysis. The major organs and aorta of each rabbit were harvested, washed with ice-cold isotonic saline and weighed. The serum samples were stored at −80°C, and aorta samples were stored in liquid nitrogen until analyzed.

TABLE 1

Composition of black and white rice outer layer fractions

Ingredient	Black rice outer layer 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
Water, *g*	9.80	7.96
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*		
Thiamin	2.30	1.20
Riboflavin	0.40	0.14
Vitamin E	0.60	0.30
Niacin	21.00	13.00
Flavonoids, *g*	6.40	1.17

Analysis of constituents of the diets. Protein was determined by the classic Kjeldahl nitrogen analysis, and fat was assayed by Soxhlet extraction. The carbohydrate, crude fiber, moisture and ash contents were measured according to Osborne and Voogt (20); iron, zinc, and selenium were assayed using an atomic absorption spectrophotometer (SpectrAA40, Varian, Mulgrave, Victoria, Australia). Thiamin was determined on a fluorospectrophotometer (Hitachi F3010, Tokyo, Japan); vitamin E was measured by using a HPLC system (Bio-Rad Model 1706, Hercules, CA); the flavonoids were analyzed by using UV-spectrophotometer (Shimadzu Multi-Purpose 5000 spectrophotometer, Kyoto, Japan) (20).

Processing of aorta fragments. Immediately after the aorta fragment was taken between its origin and bifurcation into the iliac arteries, it was washed with ice-cold sterile physiologic saline to remove debris and blood residues. The proximal ascending aortic arch was dissected from each rabbit and divided into three 3-mm cross sections. The three sections were immersion-fixed in 4% formaldehyde solution for 24 h for morphological analyses. The remaining aorta was opened longitudinally and its adventitial coat was removed, and the tunica media and tunica intima were stored in liquid nitrogen for biochemical analyses.

Assessment of atherosclerotic plaques. The excised samples were embedded in paraffin and sliced 4 μm thick. Hematoxylin, eosin and elastica-van Gomori staining were performed. The cross sections with elastica-van Gomori staining were recorded by a three-chip CCD video camera (JVCky-F 30B, Tokyo, Japan). The area within the internal elastic lamina (IEL, as the indicator of artery size) and lumen area (as the area for blood flow) were measured in the three sections using a digital image analyzer (Kontron IBAS2.5, Eching, Germany). The plaque size was calculated with the following formula: [(IEL − lumen area)/IEL] × 100. The arithmetic mean was calculated and used for further statistical analyses of the plaque size in the proximal ascending aortic arch (21,22).

Serum lipid profile. Serum triglyceride (TG), total cholesterol (TC), LDL cholesterol (LDL-C), HDL-C, apoprotein (apo)AI and

TABLE 2

Composition of the rabbit diets[1,2]

Ingredient	Normal	HC	WRF	BRF
		Units/100 g		
Energy, *kJ*	1377.36	1377.36	1377.36	1377.36
Cornstarch, *g*	58.29	49.56	48.28	48.37
Casein, *g*	18.03	18.03	18.03	18.03
Total fat, *g*	2.58	6.48	7.05	7.01
Corn oil, *g*	2.58	2.48	3.05	3.01
Lard, *g*	0	3.5	3.5	3.5
Cholesterol, *g*	0	0.5	0.5	0.5
Ash, *g*	8.30	7.97	7.94	7.92
Iron, *mg*	12.2	12.2	12.4	11.89
Zinc, *mg*	0.298	0.298	0.529	0.731
Selenium, *mg*	0.9	0.9	0.858	0.863
Thiamin, *mg*	0.5	0.5	0.59	0.535
α-tocopherol, *mg*	7.35	7.35	7.01	6.99
Moisture, *g*	7.45	7.15	7.18	7.27
Fiber, *g*	14.02	13.38	12.93	13.00

[1] Abbreviations used: Normal, rabbits were fed the nonpurified diet; HC, normal diet with 0.5 g/100 g cholesterol and 3.5 g/100 g lard; WRF, HC diet with white rice outer layer fraction (5 g/100 g); BRF, HC diet with black rice outer layer fraction (5 g/100 g).

[2] Only some minerals and vitamins were measured in the diets, including iron, zinc and selenium, thiamin and Vitamin E.

apoB were measured using a Hitachi Automatic Analyzer (Tokyo, Japan). Serum TG concentrations were assayed by hydrolyzing the triglycerides and measuring the released glycerol (23). Serum TC was determined by using a cholesterol esterase and cholesterol oxidase assay (24). Serum concentrations of HDL-C were assayed by the same method, as was serum TC after the removal of LDL-C and VLDL-C with magnesium dextran sulfate. Serum LDL concentrations were calculated according to the Friedwald formula (25), which assumes that circulating VLDL consist of 80% triglycerides and 20% cholesterol. ApoAI and apoB were determined by the turbidity immunoassay method (26).

Fatty acids level in serum. The levels of serum fatty acids were determined by capillary gas phase chromatography after extraction by methanol and phenol (4:1, v/v) and transesterification directly in a one-step reaction (27). The relative amount of each fatty acid (g/100 g) was quantified. Serum saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) were calculated by summing the four SFA acids, three MUFA and ten PUFA measured, respectively. The PUFA/SFA ratio (P:S) and (n-3) PUFA were also calculated.

8-OHdG level in aorta. Aortic segments were homogenized in a Polytron apparatus (Glas-Col Homogenizer, Jet-Pak, Terre Haute, IN) in 250 mmol/L sucrose and 50 mmol/L Tris HCl (pH7.4, ice-cold). A 50 g/L homogenate was centrifuged at 1500 $\times$ *g* for 15 min; the resulting supernatants were used for measurement of MDA and superoxide dismutase (SOD). The pellet containing nuclei was dispersed in 8 volumes of 10 mmol/L Tris-HCl, 0.1mol/L EDTA, 20 mg/L RNase, 0.5%SDS (pH 8.0), incubated for 1 h at 37°C and then lysed at 37°C for 8 h in the presence of 100 mg/L proteinase K. The suspension was extracted twice with phenol/water/chloroform and twice with chloroform alone. DNA was precipitated from the aqueous phase with 2 volumes of anhydrous alcohol and 0.2 volume sodium caproate. Collected DNA was further washed with ice-cold 70% ethanol.

DNA was suspended in 200 μL of 20 mmol/L sodium acetate buffer (pH4.8) and digested with 20 μg nuclease P1 at 37°C for 30 min. The solution was neutralized with 20 μL of 1.0 mol/L Tris-HCl, pH7.4, before the addition of 1.3 U *Escherichia coli* alkaline phosphatase. The reaction was incubated at 37°C for 1 h, after which the digest was passed through a 30,000 MWCO filter (Millipore, Bedford, PA) (28).

Analysis of DNA hydrolysates was performed with a Bio-Rad (Hercules, CA) model 700 HPLC system. An aliquot was injected

TABLE 3

Serum fatty acid composition in rabbits fed normal or high cholesterol (HC) diets or the HC diet containing a white or a black rice fraction for 60 d[1]

Fatty acid	Normal	HC	WRF	BRF
		g/L		
14:0	0.051 ± 0.020^b	0.085 ± 0.027^b	0.099 ± 0.037^a	0.088 ± 0.046^a
16:0	0.905 ± 0.415^b	3.041 ± 0.803^a	3.838 ± 0.856^a	3.736 ± 1.363^a
16:1	0.066 ± 0.028^b	0.822 ± 0.449^a	0.785 ± 0.412^a	0.599 ± 0.205^a
18:0	0.455 ± 0.243^b	1.212 ± 0.375^a	1.412 ± 0.379^a	1.506 ± 0.687^a
18:1	0.578 ± 0.110^c	4.947 ± 1.327^b	6.740 ± 2.307^a	5.925 ± 2.105^b
18:2	0.445 ± 0.143^a	2.346 ± 0.563^b	3.306 ± 0.868^c	3.378 ± 1.156^c
18:3	0.018 ± 0.005^b	0.112 ± 0.034^a	0.145 ± 0.043^a	0.138 ± 0.054^a
20:0	0.016 ± 0.010^b	0.034 ± 0.014^a	0.044 ± 0.013^a	0.044 ± 0.023^a
20:1	undetectable^b	0.020 ± 0.006^a	0.029 ± 0.009^a	0.026 ± 0.012^a
20:2	undetectable^b	0.020 ± 0.008^a	0.028 ± 0.006^a	0.031 ± 0.012^a
20:3	undetectable^b	0.037 ± 0.027^a	0.032 ± 0.008^a	0.036 ± 0.016^a
20:4	0.032 ± 0.015^c	0.181 ± 0.057^b	0.227 ± 0.042^a	0.253 ± 0.067^a
20:5	0.002 ± 0.003^b	0.033 ± 0.011^a	0.044 ± 0.026^a	0.040 ± 0.014^a
22:0	undetectable^b	0.037 ± 0.014^a	0.041 ± 0.012^a	0.044 ± 0.020^a
22:4	undetectable^b	0.018 ± 0.013^a	0.014 ± 0.007^a	0.018 ± 0.005^a
22:5	undetectable^b	0.027 ± 0.024^a	0.040 ± 0.047^a	0.036 ± 0.025^a
SFA	1.746 ± 0.682^b	4.729 ± 1.191^a	5.754 ± 1.237^a	5.738 ± 2.107^a
MUFA	0.676 ± 0.117^b	5.789 ± 1.711^a	7.553 ± 2.645^a	6.551 ± 2.307^a
PUFA	0.512 ± 0.166^c	2.774 ± 0.657^b	3.838 ± 1.015^a	3.929 ± 1.298^a
P/S	0.315 ± 0.100^b	0.592 ± 0.088^b	0.665 ± 0.072^a	0.694 ± 0.061^a
(n-3)	0.035 ± 0.013^b	0.208 ± 0.066^a	0.262 ± 0.110^a	0.249 ± 0.082^a

[1] Values are means ± SD, $n = 8$. Values in a row without common superscripts differ, $P < 0.05$.

Abbreviations used: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUPA, polyunsaturated fatty acids; (n-3), (n-3) fatty acids. P/S, ratio of PUFA/SFA. See Table 2 for diet abbreviations.

TABLE 4

Oxidant and antioxidant status of rabbits fed normal or high cholesterol (HC) diets or the HC diet containing the white rice outer layer fraction (WRF), or the black rice fraction (BRF) for 60 d[1,2]

			SOD		MDA	
	Aorta	Serum α-tocopherol	Erythrocyte	Aorta	Serum	Aorta
	8-OHdG/10^5 dG	*mol/(mmol TC + TG)*	*kU/g Hb*	*U/g protein*	*μmol/L*	*nmol/mg protein*
Normal	9.13 ± 2.94^b (7)[2]	0.033 ± 0.007^a (8)	83.50 ± 1.74^a (8)	77.93 ± 36.98^a (7)	0.96 ± 0.32^c (8)	0.49 ± 0.08^c (7)
HC	20.45 ± 8.41^a (7)	0.018 ± 0.004^b (8)	60.92 ± 1.32^b (8)	35.71 ± 18.12^b (8)	4.30 ± 1.11^a (8)	2.06 ± 0.55^a (8)
WRF	24.12 ± 6.01^a (7)	0.023 ± 0.006^b (8)	47.66 ± 2.15^b (8)	23.03 ± 7.36^b (8)	4.57 ± 0.94^a (8)	1.77 ± 0.32^a (8)
BRF	11.64 ± 4.93^b (8)	0.023 ± 0.010^b (7)	53.89 ± 1.39^b (8)	33.89 ± 14.76^b (8)	2.88 ± 0.78^b (8)	0.88 ± 0.10^b (8)

[1] Values are means ± SD (*n*). Values in a column without common superscripts differ, $P < 0.05$.

Abbreviations used: Hb, hemoglobin; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; dG, 2-deoxyguanosine; TC, total cholesterol; TG, triglyceride; SOD, superoxide dismutase; MDA, malonyldialdehyde. See Table 2 for diet abbreviations.

onto a Supelcosil LC-18-DB deactivated reversed-phase column (4 mm × 30 cm, 10-μm particle size; Millipore) with a 2-cm guard cell. Nucleosides were separated using a mobile phase consisting of 12.5 mmol/L citric acid, 25 mmol/L sodium acetate, 30 mmol/L NaOH and 10 mmol/L acetic acid, pH 5.1, at a flow rate of 1 mL/min. Detection of 8-OHdG was performed using a model 1049A electrochemical detector (Hewlett-Packard, Waldronn, Germany) with the applied potential set to 0.6 V. UV detection of 2-deoxyguanosine was performed using a model 1706 UV detector (Bio-Rad) with UV absorption at 254 nm (29).

Determination of MDA. MDA in the serum and aorta supernatants was measured by the thiobarbituric acid reaction using HPLC with a C18 column and UV-visible detector (30); tetraethoxypropane was used as standard.

Vitamin E level in serum. The vitamin E in the serum was determined by HPLC analysis using benzo(a)pyrene as the standard after extraction with ethanol (100 μL) and *n*-hexane (0.7 mL) as described elsewhere (31). The column used was a Spherisorb C18 column (4.6mm × 10cm, 3-μm particle size; Millipore) protected by a 2-cm guard column. The absorption of α-tocopherol is affected by serum lipid, and carried in serum lipoproteins; thus, α-tocopherol analysis should take account of serum lipid concentrations. Serum level of α-tocopherol was expressed relative to serum total cholesterol and triglyceride contents as its lipid-normalized concentrations (32,33).

SOD activity. Erythrocyte and aorta supernatants were analyzed for SOD activity by using the xanthine oxidase method (34). Briefly, cytochrome C (10 μmol/L) reduction was measured after 30 s of incubation at 25°C with 50 μmol/L xanthine and 2.5 μmol/L xanthine oxidase in 50 mmol/L potassium phosphate buffer (pH 7.8). Absorption at 550 nm was recorded continuously on a Shimazu Multi-Purpose 5000 spectrophotometer (Shimadzu).

Statistical analysis. Results are expressed as means ± SD, and the differences were determined by one-way ANOVA coupled with the Student-Newman-Keuls multiple comparison test. Differences with $P < 0.05$ were considered significant.

RESULTS

Body weight gain. The rabbits fed the different diets did not differ in body weight throughout the experiment (data not shown).

Atherosclerotic plaque formation. There were no visible atherosclerotic plaques in aortae of rabbits fed the normal diet, but atherosclerotic plaques of various degrees were visible in aortae of rabbits fed the HC, WRF and BRF diets. Plaque formation was much more severe in rabbits fed the HC or WRF diets than in those fed the BRF diet. Typical atherosclerotic plaques of aortae of rabbits fed HC, WRF or BRF diets showed injury to endothelial cells, infiltration of inflamed cells, assembling foam cells, cellular necrosis and proliferation of fibroblasts and smooth muscle cells (**Fig. 1**). The plaque area in rabbits fed the BRF diet was lower than in the HC- (66%) or WRF- (75%) fed rabbits ($P < 0.001$) which did not differ from one another (**Fig. 2** and **3**).

Serum lipid profile and fatty acid composition. Serum TG, TC, LDL-C/HDL-C and apoAI/apoB in rabbits fed the normal diet differed from other groups ($P < 0.05$). However, there were no differences in serum lipids among the groups fed the HC, WRF and BRF diets (data not shown). Similarly, serum total SFA, MUFA, PUFA, (n-3) fatty acids and the P/S ratio in rabbits fed the normal diet were lower than those in rabbits fed the WRF and BRF diets ($P < 0.05$), but they did not differ among the HC, WRF and BRF groups (**Table 3**). The serum concentration of most individual fatty acids except 18:1 did not differ between the WRF and BRF dietary groups.

Oxidative and antioxidative levels. Although HC, WRF and BRF diets lowered the SOD activity of erythrocytes and aorta, and the level of serum α-tocophecol compared with the normal diet in rabbits ($P < 0.05$) (**Table 4**), there were no differences in these variables among the groups fed the three diets.

Aortic 8-OHdG was significantly lower (−44%, −52%) in the group fed the BRF diet compared with rabbits fed the HC and WRF diets, respectively ($P < 0.05$) (Table 4). There were no differences in the 8-OHdG levels of aorta between the rabbits fed the HC and WRF diets or between the rabbits fed the normal and BRF diets.

The BRF diet significantly ($P < 0.05$) decreased the MDA levels of serum (−37%) and aorta (−50%) compared with the WRF diet (Table 4). MDA levels in serum and aorta in the groups fed the BRF diet were still higher than those of rabbits fed the normal diet. The levels of 8-OHdG and MDA in aorta were significantly correlated with the atherosclerotic plaque area ($r = 0.719$ and 0.605, $P < 0.01$), respectively.

DISCUSSION

The major finding of the present study was that rabbits consuming the diet containing the black rice outer layer (5 g/100 g) with high cholesterol (0.5 g/100 g) had dramatically reduced aortic atherosclerotic plaque areas compared with the rabbits consuming the WRF diet (5 g/100 g) or the high cholesterol diet. The diet consumed by rabbits was almost the same in the WRF and BRF groups except for the different types of rice outer layer fraction supplemented. Supplementa-

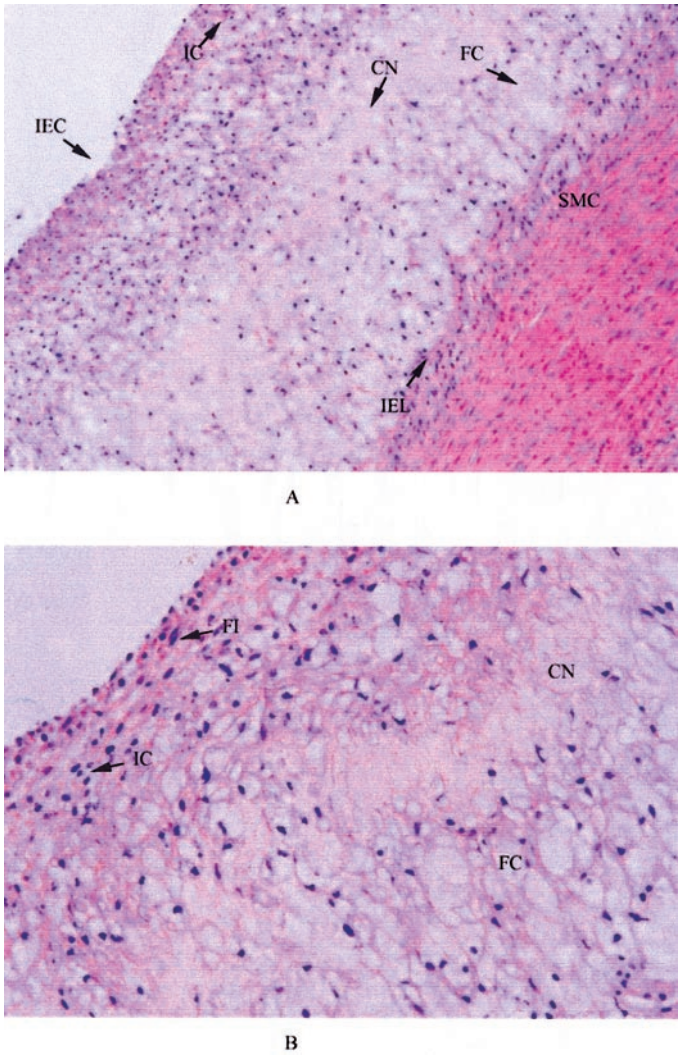

FIGURE 1 Typical atherosclerotic plaque in histological cross section aorta with hematoxylin and eosin staining in a rabbit fed the high cholesterol (HC) diet for 60 d. The typical atherosclerotic plaque structure and composition were characterized by the injury to endothelial cells (IEC), infiltration of inflamed cells (IC), assembling foam cells (FC), cellular necrosis (CN), proliferation of fibroblasts (FI) and smooth muscle cells (SMC). (*A*) Magnification ×200; (*B*) magnification X400. IEL, internal elastic lamina.

tion of the black rice outer layer fraction significantly reduced the atherosclerotic plaque area, suggesting that BRF can prevent atherosclerotic plaque formation and progression. The pathology of atherosclerosis is complex, involving impaired oxidative and antioxidative homeostasis, lipid metabolism and interaction among the endothelium, circulating and tissue inflammatory cells, platelets and vascular smooth muscle cells (2).We speculated that inhibition of atherosclerotic plaque formation by BRF might be mediated by its improvement of antioxidation status, lipid metabolism and the anti-inflammation response.

Currently, MDA and 8-OHdG are among the most valuable makers of lipid peroxidation and DNA oxidative products, respectively (7). In the present study, the BRF diet decreased aortic 8-OHdG and serum and aortic MDA significantly (Table 4). This indicated that supplementation of the black rice outer layer fraction decreased oxidative stress. Because oxidative stress plays a large role in the development of

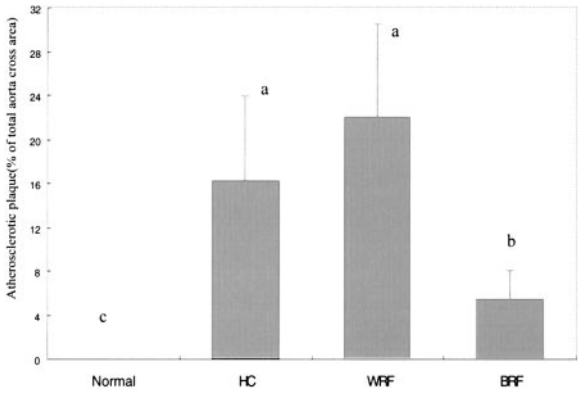

FIGURE 2 Atherosclerotic plaque in aorta of rabbits fed normal or high cholesterol (HC) diets or the HC diet containing the white rice outer layer fraction (WRF), or the black rice outer layer fraction (BRF) for 60 d. Values are means ± SD, $n = 7–8$. Columns without a common letter differ, $P < 0.001$.

atherosclerosis, the decreased peroxidation processes and DNA oxidative damage by supplementation of the BRF reduced the damage of oxidative stress to artery endothelial or smooth muscle cells, contributing to the inhibition of atherosclerotic plaque formation in aorta.

Consumption of fruits and vegetables, olive oil, red wine and tea is inversely correlated with the rates of heart disease (8). These foods are particularly rich in natural antioxidants, including ascorbate (vitamin C), the tocopherols (vitamin E) and carotenoids. A sufficient supply of dietary antioxidants might help prevent or delay the occurrence of pathological changes associated with oxidative stress. To explore the mechanism by which supplementation of BRF decreased the aortic oxidative stress, vitamin E concentration of serum was measured because it is considered to be a potent antioxidant compound in vivo and in vitro and may improve oxidative

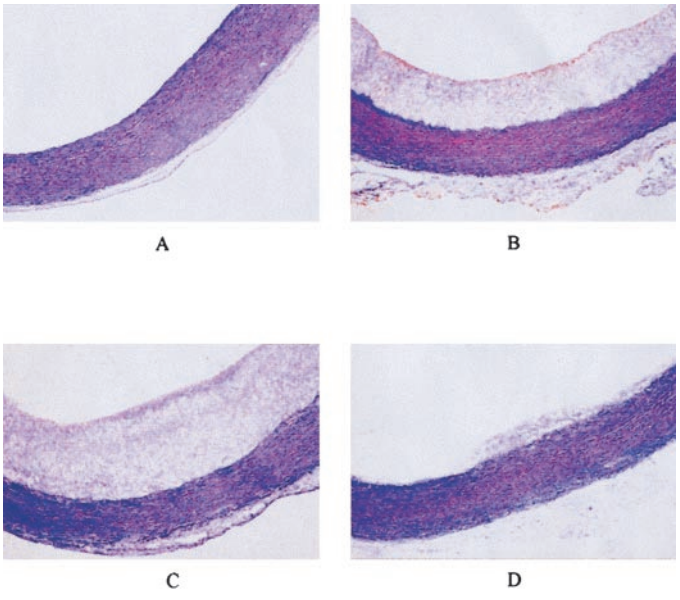

FIGURE 3 Cross sections of aortic arches in rabbits fed normal (*A*) or high cholesterol (HC) (*B*) diets or the HC diet containing the white rice outer layer fraction (WRF) (*C*), or the black rice outer layer fraction (BRF) (*D*) for 60 d. Magnification X40.

stability (35). In this study, the serum vitamin E concentration did not differ between the BRF and WRF groups, indicating that it might not be the primary antioxidant contributing to the lowering of oxidative status in aortic tissue by the BRF diet.

In the present study, antioxidant status in blood was not improved as efficiently as aortic tissue in rabbits fed BRF diet because blood and erythrocyte SOD activities did not differ among the HC, WRF and BRF groups. A number of studies have shown that oxidative stress is increased by high blood lipid levels (36). The elevated oxidative capacity induced by high blood lipids might be too strong to be reduced, and antioxidant status in the circulatory system is improved little by supplementation of BRF. This may explain in part why serum SOD was not significantly improved in rabbits fed the BRF compared with rabbits fed the WRF diets. There is increasing evidence indicating that oxidized LDL play an important role in the development of atherosclerosis. The oxidation of LDL is thought to take place in the arterial intima when the particles have become isolated from circulating water-soluble antioxidants (37). The facts that the BRF diet lowered atherosclerotic plaque and decreased aortic MDA levels suggest that the antioxidants in this diet act efficiently in the artery intima, thereby inhibiting atherosclerotic formation.

Epidemiologic studies have shown dietary flavonoid intake to be inversely associated with mortality from CHD (38). Flavonoids are potent antioxidants and can protect against the oxidation of LDL, a process implicated in atherogenesis. Flavonoids also show antilipoperoxidant and anti-inflammation properties (39). Because the amount of flavonoids in the black rice outer layer fraction was 5.4 times as much as that of white rice outer layer fraction (Table 1), the antiatherogenic effect of the BRF diet in rabbits might be related to the antioxidative properties of flavonoid-like compounds.

Several studies have suggested that some elements such as zinc and selenium may in part be antiatherogenic by inhibiting oxidative stress-responsive events in endothelial cell dysfunction (40–42). The BRF diet contained higher levels of zinc than did the WRF diet, which might also contribute to inhibition of atherosclerosis formation in rabbits fed BRF. In addition, other levels of constituents such as riboflavin and minerals are higher in the BRF than in the WRF diet, which may contribute in part to oxidative improvement.

Epidemiologic studies have shown that the level of dietary fat intake is positively correlated with the average serum cholesterol value and mortality from CHD (43). A number of different investigators have demonstrated that in addition to total fat, the fatty acid composition of diets influences serum TC in humans. In general, SFA elevate serum cholesterol concentration, and unsaturated fatty acids decrease it. Rice bran oil (RBO) is composed mainly of unsaturated fatty acids, including oleic, linoleic and linolenic acids. In addition, RBO contains unsaponifiable materials including tocopherols, γ-oryzanol, phytosterols, tocotrienols and squalene (34). The beneficial effects of rice consumption were reported to derive from the RBO or bran fiber. In the present study, the beneficial influence of the rice outer layer fraction appeared not to be due to the fiber because the fiber content of the two rice diets was virtually the same. A number of studies in humans and animals have revealed that RBO consumption lowered serum total TG, TC and LDL-C, and increased plasma HDL-C concentrations (44–46). In the present study, serum lipids and apo B concentrations did not differ among the HC, WRF and BRF groups. Also the serum concentration of most fatty acids except 18:1 did not differ between the WRF and BRF groups. Therefore, RBO did not appear to be responsible for lowering atherosclerotic plaque formation in rabbits fed the BRF diet.

The atherogenic process is largely a fibroproliferative inflammatory response involving macrophages and T lymphocytes, which are present in the arterial intima at all stages in the development of atherosclerotic lesions. It has been reported that flavonoids and anthocyanidin or anthocyanin have anti-inflammatory properties. A series of studies (47–49) found that flavonoid derivatives possess anti-inflammatory activity in vitro and in vivo. Flavonoid derivatives including prenylated compounds such as morusin, kuwanon C and sanggenon D and biflavonoids such as bilobetin and ginkgetin inhibited nitric oxide (NO) production from lipopolysaccharide (LPS). Inhibition of NO production was mediated by suppression of inducible nitric oxide synthase (iNOS), but not by direct inhibition of iNOS enzyme activity. Because NO produced by iNOS plays an important role in inflammatory disorders, inhibition of NO production by these flavonoids may contribute at least in part to their anti-inflammatory and immunoregulating potentials in vivo. In addition to antioxidation, flavonoid- and anthocyanidin-like compounds in the BRF diet may be antiatherogenic through their anti-inflammatory action, and thereby inhibit the development of atherosclerotic plaques.

Prostaglandins (PG) are present in a wide variety of tissues and play a central role in inflammation. It has been reported that PGE_2 contributes to the pathogenesis of cardiovascular disease through proinflammatory activities. The inflammatory PG is converted from arachidonic acid in membrane phospholipids through the function of the enzyme cyclooxygenase (COX). At least two forms of the enzyme COX exist, a constitutive (COX-1) form and an inducible (COX-2) form. COX-1 is mainly responsible for the biosynthesis of PG involved in homeostatic regulation, whereas the second isoform, COX-2, is primarily involved in producing PG in response to a wide spectrum of environmental insults and internal stimuli. COX-2 may participate in the initiation and pathogenesis of atherosclerosis via a rapid and excessive production of prostanoids, which has a proatherosclerotic effect (50). Certain flavonoids are potent inhibitors of PG production and COX-2 expression in a concentration-dependent manner (48,51). Inhibition of COX-2 expression by flavonoids may be one of the mechanisms responsible for their anti-inflammatory effect (48). In the present study, inhibition of COX-2 expression and PG production or release by the higher flavonoid contents in the BRF diet could be another mechanism by which it inhibited atherosclerotic plaque formation in rabbits. However, the anti-inflammatory action and antiatherogenic properties of the BRF diet warrant further elucidation.

LITERATURE CITED

1. Breslow, J. L. (1997) Cardiovascular disease burden increases, NIH funding decreases. Nat. Med. 3: 600–601.
2. Orford, J. L., Selwyn, A. P., Ganz, P., Popma, J. J. & Rogers, C. (2000) The comparative pathobiology of atherosclerosis and restenosis. Am. J. Cardiol. 86: 6H–11H.
3. Braunwald, E. (1997) Shattuck Lecture—cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. N. Engl. J. Med. 337: 1360–1369.
4. Ross, R. (1999) Atherosclerosis—an inflammatory disease. N. Engl. J. Med. 340: 115–126.
5. Liao, D. F., Jin, Z. G., Baas, A. S., Daum, G., Gygi, S. P., Aebersold, R. & Berk, B. C. (2000) Purification and identification of secreted oxidative stress-induced factors from vascular smooth muscle cells. J. Biol. Chem. 275: 189–196.
6. Shackelford, R. E., Kaufmann, W. K. & Paules, R. S. (2000) Oxidative stress and cell cycle checkpoint function. Free Radic. Biol. Med. 28: 1387–1404.
7. Chao., W. H., Askew, E. W., Roberts, D. E., Wood, S. M. & Perkins, J. B. (1999) Oxidative stress in humans during work at moderate altitude. J. Nutr. 129: 2009–2012.

8. Giugliano, D. (2000) Dietary antioxidants for cardiovascular prevention. Nutr. Metab. Cardiovasc. Dis. 10: 38–44.
9. Kunsch, C. & Medford, R. M. (1999) Oxidative stress as a regulator of gene expression in the vasculature. Circ. Res. 85: 753–766.
10. Porkkala, E., Sarataho, E., Salonen, J. T., Nyyssonen, K., Kaikkonen, J., Salonen, R., Ristonmaa, U., Diczfalusy, U., Brigelius, Flohe, R., Loft, S. & Poulsen, H. E. (2000) Long-term effects of vitamin E, vitamin C, and combined supplementation on urinary 7-hydro-8-oxo-2′-deoxyguanosine, serum cholesterol oxidation products, and oxidation resistance of lipids in nondepleted men. Arterioscler. Thromb. Vasc. Biol. 20: 2087–2093.
11. Rimm, E. B. & Stampfer, M. J. (1997) The role of antioxidants in preventive cardiology. Curr. Opin. Cardiol. 12: 188–94.
12. Massaro, M., Carluccio, M. A. & De Caterina, R. (1999) Direct vascular antiatherogenic effects of oleic acid: a clue to the cardioprotective effects of the Mediterranean diet. Cardiologia 44: 507–513.
13. Caruso, D., Berra, B., Giavarini, F., Cortesi, N., Fedeli, E. & Galli, G. (1999) Effect of virgin olive oil phenolic compounds on in vitro oxidation of human low density lipoproteins. Nutr. Metab. Cardiovasc. Dis. 9: 102–107.
14. Sugano, M. (1996) Characteristics of fats in Japanese diets and current recommendations. Lipids 31 (suppl.): S283–S286.
15. Van den Hoogen, P. C., Feskens, E. J., Nagelkerke, N. J., Menotti, A., Nissinen, A. & Wiseman, H. (1999) The bioavailability of non-nutrient plant factors: dietary flavonoids and phyto-oestrogens. Proc. Nutr. Soc. 58: 139–146.
16. Akerblom, H. K., Viikari, J., Raitakari, O. T. & Uhari, M. (1999) Cardiovascular Risk in Young Finns Study: general outline and recent developments. Ann. Med. 31 (suppl. 1): 45–54.
17. Genest, J. (1986) Nutritional management of hypertension: past, present, and future. Can. J. Physiol. Pharmacol. 64: 836–839.
18. Kempner, W. (1946) Some effects of the rice diet treatment of kidney disease and hypertension. Bull. N.Y. Acad. Med. 22: 350–370.
19. Ling, W. H., Cheng, Q. X., Ma, J. & Wang, T. (2001) Red and black rice decrease atherosclerotic plaque formation and increase antioxidant status in rabbits. J. Nutr. 131: 1421–1426.
20. Osborne, D. R. & Voogt, P. (1978) The Analysis of Nutrients in Foods, pp. 46–55. Academic Press, London, UK.
21. Clarkson, T. B, Anthony, M. S. & Jerome, C. P. (1998) Lack of effect of raloxifene on coronary artery atherosclerosis of postmenopausal monkeys. J. Clin. Endocrinol. Metab. 83: 721–6.
22. Bruck, B., Brehme, U., Gugel, N., Hanke, S., Finking, G., Lutz, C., Benda, N., Schmahl, F. W., Haasis, R. & Hanke, H. (1997) Gender-specific differences in the effects of testosterone and estrogen on the development of atherosclerosis in rabbits. Arterioscler. Thromb. Vasc. Biol. 17: 2192–2199.
23. Bucolo, G. & David, H. (1973) Quantitative determination of serum triglycerides by the use of enzymes. Clin. Chem. 19: 467–486.
24. Allain, C. C., Poon, L. S., Chan, C.S.G., Richmond, W. & Fu, P. C. (1974) Enzymatic determination of total serum cholesterol. Clin. Chem. 20: 740–745.
25. Friedewald, W. T., Levy, R. I. & Frederickson, D. S. (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. Clin. Chem. 18: 499–502.
26. Ikeda, T., Shibuya, Y., Senba, U., Sugiuchi, H., Araki, S., Uji, Y. & Okabe, H. (1991) Automated immunoturbidimetric analysis of six serum apoliproteins: correlation with radial immunodiffusion assays. J. Clin. Lab. Anal. 5: 90–95.
27. Lepage, G. & Roy, C. C. (1986) Direct transesterification of all classes of lipids in a one-step reaction. J. Lipid Res. 27: 114–120.
28. Cahill, A., Wang, X. & Hoek, J. B. (1997) Increased oxidative damage to mitochondrial DNA following chronic ethanol consumption. Biochem. Biophys. Res. Commun. 235: 286–290.
29. Fiala, E. S., Conaway. C. C. & Mathis, J. E. (1989) Oxidative DNA and RNA damage in the livers of Sprague-Dawley rats treated with the hepatocarcinogen 2-nitropropane. Cancer Res. 49: 5518–5522.
30. Chen, C. Y., Huang, Y. L. & Lin, T. H. (1998) Association between oxidative stress and cytokine production in nickel-treated rats. Arch. Biochem. Biophys. 356: 127–132.
31. Driskell, W. J., Neese, J. W., Bryant, C. C. & Bashor, M. M. (1982) Measurement of vitamin A and vitamin E in human serum by high-performance liquid chromatography. J. Chromatogr. 231: 439–444.
32. Kontush, A., Reich, A., Baum, K., Spranger, T., Finckh, B., Kohlschutter, A. & Beisiegel, U. (1997) Plasma ubiquinol-10 is decreased in patients with hyperlipidaemia. Atherosclerosis 129: 119–126.
33. Araujo, F. B., Barbosa, D. S., Hsin, C. Y., Maranhao, R. C. & Abdalla, D. S. (1995) Evaluation of oxidative stress in patients with hyperlipidemia. Atherosclerosis 117: 61–71.
34. Oyanagui, Y. (1984) Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. Anal. Biochem. 142: 290–296.
35. Rukmini, C. & Raghuram, T. C. (1991) Nutritional and biochemical aspects of the hypolipidemic action of rice bran oil: a review. J. Am. Coll. Nutr. 10: 593–601.
36. Ibrahim, W., Lee, U. S., Yeh, C. C., Szabo, J., Bruckner, G. & Chow, C. K. (1997) Oxidative stress and antioxidant status in mouse liver: effects of dietary lipid, vitamin E and iron. J. Nutr. 127: 1401–1406.
37. Wiseman, H., Tikkanen, M. J., Wahala, K., Ojala, S., Vihma, V. & Adlercreutz, H. (1998) Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance Proc. Natl. Acad. Sci. U.S.A. 95: 3106–3110.
38. Wiseman, H. (1999) The bioavailability of non-nutrient plant factors: dietary flavonoids and phyto-oestrogens. Proc. Nutr. Soc. 58: 139–46.
39. La Casa, C., Villegas, I., de la Alarcon, L. C., Motilva, V. & Martin, C.M.J. (2000) Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. J. Ethnopharmacol. 71: 45–53.
40. Kang, B. P., Bansal, M. P. & Mehta, U. (1998) Selenium supplementation and diet induced hypercholesterolemia in the rat: changes in lipid levels, malonyldialdehyde production and the nitric oxide synthase activity. Gen. Physiol. Biophys. 17: 71–78.
41. Oteiza, P. I., Olin, K. L., Fraga, C. G. & Keen, C. L. (1995) Zinc deficiency causes oxidative damage to proteins, lipids and DNA in rat testes. J. Nutr. 125: 823–829.
42. Hennig, B., Meerarani, P., Ramadass, P., Toborek, M., Malecki, A., Slim, R. & McClain, C. J. (1999) Zinc nutrition and apoptosis of vascular endothelial cells: implications in atherosclerosis. Nutrition 15: 744–748.
43. Dietschy, J. M. (1998) Dietary fatty acids and the regulation of plasma low density lipoprotein cholesterol concentrations. J. Nutr. 128 (suppl. 2): 444S–448S.
44. Nicolosi, R. J., Ausman, L. M. & Hegsted, D. M. (1991) Rice bran oil lowers serum total and low density lipoprotein cholesterol and apo B levels in nonhuman primates. Atherosclerosis 88: 133–142.
45. Seetharamaiah, G. S. & Chandrasekhara, N. (1989) Studies on hypocholesterolemic activity of rice bran oil. Atherosclerosis 78: 219–223.
46. Purushothama, S., Raina, P. L. & Hariharan, K. (1995) Effect of long term feeding of rice bran oil upon lipids and lipoproteins in rats. Mol. Cell. Biochem. 146: 63–69.
47. Cheon, B. S., Kim, Y. H., Son, K. S., Chang, H. W., Kang, S. S. & Kim, H. P. (2000) Effects of prenylated flavonoids and biflavonoids on lipopolysaccharide-induced nitric oxide production from the mouse macrophage cell line RAW 264.7. Planta Med. 66: 596–600.
48. Kim, H. K., Cheon, B. S., Kim, Y. H., Kim, S. Y. & Kim, H. P. (1999) Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. Biochem. Pharmacol. 58: 759–765.
49. Raso, G. M., Meli, R., Di-Carlo, G., Pacilio, M. & Di Carlo, R. (2001) Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A.1. Life Sci. 68: 921–31.
50. Wu, D., Hayek M. G. & Meydani, S. N. (2001) Vitamin E and macrophage cyclooxygenase regulation in the aged. J. Nutr. 131: 382S–388S.
51. Manthey, J. A. (2000) Biological properties of flavonoids pertaining to inflammation. Microcirculation 7: S29–S34.