Plant Foods for Human Nutrition **62:** 1–6, 2007.

DOI: 10.1007/s11130-006-0031-7

Effect of Anthocyanin-Rich Extract from Black Rice (*Oryza sativa* L. *indica*) on Hyperlipidemia and Insulin Resistance in Fructose-Fed Rats

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Published online: 23 December 2006
Abstract. This study was designed to evaluate the effect of an anthocyanin-rich extract from black rice on hyperlipidemia and insulin resistance in fructose-fed rats. Rats fed fructose diet for 4 weeks exhibited significantly higher plasma insulin levels and lower insulin sensitivity than the control rats fed AIN-93G diet. Dietary supplementation with the anthocyanin-rich extract (5 g/kg of high-fructose diet) prevented the development of fructose-induced insulin resistance. After fructose-induced insulin resistance had been established, 4-week treatment with the anthocyanin-rich extract (5 g/kg of high-fructose diet) or pioglitazone (270 mg/kg of high-fructose diet) ameliorated the glucose intolerance and hyperlipidemia, but the extract failed to reverse the fructose-induced hyperinsulinemia as pioglitazone did. In addition, rats supplemented by the extract exhibited lower oxidative stress than the fructose-fed controls, as indicated by the lower concentrations of plasma thiobarbituric acid reactive substances and blood oxidized glutathione. Overall, these results suggest that the anthocyanin-rich extract from black rice improves certain metabolic abnormalities associated with diets high in fructose.

Key words: Anthocyanin, Black rice, Hyperlipidemia, Insulin resistance, Metabolic syndrome, Oxidative stress

Abbreviations

AREBR	Anthocyanin-rich extract from black rice
AUC	Area under the curve
BW	Body weight
FFA	Free fatty acid
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HDL-C	High-density lipoprotein cholesterol
HF	High fructose
HOMA-R	Relative-value of homeostasis model
LDL-C	Low-density lipoprotein cholesterol
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol
TG	Triglyceride

Introduction

The metabolic syndrome is associated with increased risk for type 2 diabetes and coronary heart disease, and appears to be widely prevalent in both developed and developing countries. This syndrome consists of a cluster of clinical abnormalities including dyslipidemia, specifically elevated triglycerides and low high-density lipoprotein cholesterol, insulin resistance, and hypertension [1]. Several lines of evidences suggest that insulin resistance plays a key role in the development of metabolic syndrome, and the hyperinsulinemia and hyperlipidemia may be a target for treatment of this disease cluster [2].

Recently, much attention has been focused on plant foods that may be beneficial in preventing metabolic syndrome and possibly reduce the risk of diabetes and cardiovascular disease [3, 4]. Dietary patterns high in fruit, vegetable, and cereals content were generally found to be associated with lower prevalence of metabolic syndrome [3, 5]. Anthocyanins are naturally occurring polyphenolic pigments in the plant kingdom [6]. They are widely distributed in fruits, vegetables, and pigmented cereals, suggesting that we ingest considerable amounts of anthocyanins from our plant-based daily diets. *In vivo* and *in vitro* studies indicate that anthocyanins have several salutary effects, such as improving lipid profile, antiinflammatory and antioxidative activities [7]. Black rice (*Oryza sativa* L. *indica*) is a special cultivar of rice that contains rich anthocyanins in the aleurone layer, has been regarded as a heath-promoting food and widely consumed since ancient times in China and other Eastern Asia countries. Our previous studies showed that the supplementation of black rice pigment fraction markedly reduced oxidative stress and improved lipid profile in addition to modulating atherosclerotic lesions in two different animal models [8, 9]. Furthermore, a standardized anthocyanin-rich extract of black rice (AREBR) with relative high anthocyanins content (43.2%) displayed the concordant results [10, 11]. In two recent studies, anthocyanin was shown to ameliorate the insulin resistance, hyperglycemia, and hyperlipidemia in high-fat-fed mice [12, 13]. We hypothesized that anthocyanin from back rice might exert a protective role against the metabolic syndrome, especially affecting the insulin resistance and hyperlipidemia. At present, information about the effects of anthocyanins on the cluster of symptoms related to metabolic syndrome in the body is scarce.

Fructose has been implicated as a contributor to nearly all of the classic manifestations of the metabolic syndrome [14]. In Sprague-Dawley rats, feeding a high-fructose (HF) diet induces an increase in blood pressure associated with hyperinsulinemia and hyperlipidemia, a pathologic status resembling human type 2 diabetes, and is an excellent laboratory animal model to examine the role of dietary components that could modulate the progression of metabolic

syndrome [14, 15]. The present study was carried out to evaluate the activity of an anthocyanin-rich extract from black rice on the insulin sensitivity and lipid profile as well as oxidative stress in fructose-fed rats, using an insulin-sensitizing drug, pioglitazone, as a positive control [16].

Materials and Methods

Preparation of AREBR

Black rice pigment fraction (about 10% outer layer of whole grain) was kindly provided by the Key Laboratory of Functional Food, Ministry of Agriculture of the P. R. China. Black rice pigment fraction was extracted with 10 volumes 65% ethanol (0.1% HCl) at room temperature for 12 h, with occasional shaking to increase the extraction capacity. Ethanol was removed from the filtered solution by rotary evaporation at ≤ 42 °C. After defatting using petroleum ether, the extraction solution was passed through 0.1% HCl preconditioned Amberlite XAD-7HP resin column (Rohm and Haas, Philadelphia, USA). The adsorbed anthocyanins were eluted by 80% ethanol. The lyophilized powder obtained from the evaporated eluent was called AREBR.

Animals and Experimental diets

The experiment used an animal protocol approved by the Standing Committee on Animal Care at Sun Yat-sen University. Male Sprague-Dawley rats [$n = 60$, body weight (BW) ~ 180 g] were obtained from the Experimental Animal Center of Guangdong Province, China. The rats were housed individually in stainless cages in an air-conditioned room maintained at 22 ± 2 °C with a 12-h light-dark cycle. The rats consumed food and distilled water *ad libitum*. Food intake and body weight were measured weekly during the experimental period.

After five-day acclimatization, the animals were randomly divided into five groups to receive experimental diets. One group received the AIN-93G purified chow diet, while the other four groups were fed with fructose-enriched diet. The compositions of the control and fructose diets are given in Table 1 [15, 17]. The mineral mixture and vitamin mixture were all AIN-93G formulas, obtained from Harlan Teklad (Madison, USA). Diets were freshly mixed in small amounts every 5–7 days and stored at 4 °C. The diets were served daily to prevent deterioration of the AREBR and moisture-absorption of the fructose. The following experimental groups consisting of 12 rats each were maintained for a total experimental period of 8 weeks.

Table 1. Composition of the experimental diets (g/kg diet)

Ingredient	Control diet	High-fructose diet
Casein	200	200
Cornstarch	530	—
Sucrose	100	—
Fructose	—	630
Soybean oil	70	70
Mineral mix	35	35
Vitamin mix	10	10
Fiber	50	50
L-cystine	3	3
Choline bitartrate	2.5	2.5

Experimental Groups

Group 1 (Control), received the AIN-93G purified chow diet for 8 weeks.

Group 2 (High-fructose), received the HF diet for 8 weeks.

Group 3 (AREBR), received the HF diet supplemented with AREBR (5 g/kg diet) for 8 weeks.

Group 4 (AREBR-treated), received the HF diet for 8 weeks; AREBR treatment (5 g/kg of HF diet) was started from week 5 of the experimental period.

Group 5 (Pio-treated), received the HF diet for 8 weeks; pioglitazone treatment (270 mg/kg of HF diet) was started from week 5 of the experimental period.

Oral Glucose Tolerance Test

One day before the termination of the experiment, six rats of each group were subjected to an oral glucose tolerance test. Briefly, after overnight fasting, a 0 min blood sample (0.2 mL) was taken by cutting the tail tip, then a glucose solution was immediately administered by gavage (3 g glucose/kg BW), and three more tail vein blood samples were taken at 30, 60 and 120 min after glucose administration for measurement of plasma glucose concentrations. The area under the curve (AUC) for glucose was calculated using the trapezoidal rule.

Biochemical Analysis and Markers of Oxidative Stress

Fasting blood samples (0.2 mL) were taken by cutting the rats tail tips after 4-week feeding to evaluate the insulin sensitivity. After 8 weeks on their diets, all rats were fasted overnight and killed by withdraw blood from femoral artery. Plasma was divided into aliquots and stored at -40 °C until assayed for lipid profile, markers of oxidative stress, and insulin levels.

Glucose was determined immediately by a portable glucometer (LifeScan, Milpitas, USA) using whole blood from tail tips of rats. Plasma triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) levels were

Table 2. Effect of diets on body weight, food intake, epididymal fat pad and liver weight

Parameters	Control	High-fructose	AREBR	AREBR-treated	Pio-treated
Body weight (g)	345.0 ± 7.4	334.9 ± 10.2	323.9 ± 12.5	324.8 ± 5.5	335.5 ± 10.2
Food intake (g/d)	17.1 ± 0.5	16.4 ± 0.8	16.6 ± 0.3	16.2 ± 0.4	16.8 ± 0.8
EFP[a] weight (g)	3.2 ± 0.2*a*	3.1 ± 0.2*a*	3.2 ± 0.2*a*	3.4 ± 0.2*a*	4.1 ± 0.1*b*
liver weight (g)	9.7 ± 0.5	11.9 ± 0.6	11.3 ± 0.5	11.7 ± 0.4	10.8 ± 0.5

Note. Values are means ± SEM, $n = 12$. Values in the same row with different letters are significantly different at $P < 0.01$.
[a]epididymal fat pad.

assayed enzymatically using a Biosystem automatic biochemistry analyzer (Madrid, Spain). Free fatty acid (FFA) was determined by Cu^{2+} method using a spectrophotometric assay kit (Jiancheng Bio., Nanjing, China). Insulin was assayed in plasma samples using a standard ELISA kit (Linco Research, St. Charles, USA). The relative-value of homeostasis model (HOMA-R) was expressed as an index of insulin resistance [18]. HOMA-R was calculated by the formula HOMA-R = fasting glucose (mmol/L) × fasting insulin (μIU/mL)/22.5. Insulin values were expressed as SI units (1 μIU/mL = 6.945 pmol/L).

Plasma concentrations of thiobarbituric acid reactive substances (TBARS), as an index of lipid peroxidation, were measured by the method of Saadani [19]. Blood reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were measured as previously described [20]. Total glutathione (GSH + GSSG) was determined enzymatically at an absorbance of 412 nm. The assay of GSSG was performed after having masked GSH by adding 2-vinylpyridine to the deproteinized extract. The difference between the two values gives the GSH levels in the plasma.

Statistical Analysis

Statistical analyses were performed using the SPSS 11.0 package (SPSS Inc., Chicago, USA). The results are presented as means ± SEM for the number of rats (n) per experimental condition. Data were analyzed by one-way ANOVA and a *post hoc* least significant difference (LSD)-t multiple comparisons test. The level of significance was set at $P < 0.05$.

Results

Animal Characteristics

During the treatment period no obvious toxic effects were noted in the animals. There were no differences in food-intake and weight gain among different groups throughout 8-wk feeding period (Table 2). The pio-treated group had a larger epididymal fat pad than the other four groups ($P < 0.01$) at sacrifice.

Plasma Lipid Profile and Markers of Oxidative Stress

Fructose feeding induced a significant increase in the concentrations of FFA by 136%, TG by 117%, and TC by 14%, and a significant decrease of HDL-C by 18% respectively compared with the control group (Table 3). The treatment of AREBR and pioglitazone significantly ($P < 0.05$) improved plasma FFA, TG and HDL-C concentrations, also resulted in decrease of plasma TC levels, but without significance.

The high-fructose group exhibited significantly higher concentrations of plasma TBARS than the other four groups ($P < 0.05$), which all had equivalent values (Table 4). AREBR supplementation significantly reduced the blood GSSG and GSSG/GSH ratio when compared to the fructose-fed controls ($P < 0.01$).

Plasma Glucose and Insulin Concentrations

The fasting plasma glucose levels of all groups were similar at week 4 (Table 5). However, plasma insulin levels and insulin sensitivity index of HOMA-R among the three

Table 3. Effect of diets on fasting plasma lipid profile

Plasma	Control	High-fructose	AREBR	AREBR-treated	Pio-treated
FFA (μmol/L)	290.9 ± 37.4*a*	687.9 ± 61.4*b*	345.8 ± 41.0*a*	361.4 ± 34.4*a*	301.5 ± 44.7*a*
TG (mmol/L)	1.08 ± 0.09*a*	2.34 ± 0.35*b*	1.67 ± 0.23*a*	1.67 ± 0.20*a*	0.98 ± 0.35*a*
TC (mmol/L)	1.63 ± 0.06*a*	1.86 ± 0.07*b*	1.72 ± 0.09*ab*	1.76 ± 0.06*ab*	1.73 ± 0.06*ab*
LDL-C (mmol/L)	0.26 ± 0.05	0.32 ± 0.05	0.27 ± 0.03	0.29 ± 0.02	0.32 ± 0.05
HDL-C (mmol/L)	0.87 ± 0.03*a*	0.71 ± 0.04*b*	0.83 ± 0.04*a*	0.87 ± 0.04*a*	0.85 ± 0.03*a*

Note. Values are means ± SEM, $n = 12$. Values in the same row with different letters are significantly different at $P < 0.05$.

Table 4. Effect of diets on blood markers of oxidative stress

Parameters	Control	High-fructose	AREBR	AREBR-treated	Pio-treated
TBARS (μmol/L)	$4.2 \pm 0.4a$	$7.4 \pm 0.4b$	$4.7 \pm 0.5a$	$4.7 \pm 0.4a$	$4.9 \pm 0.4a$
GSSG (μmol/L)	$10.3 \pm 1.1a$	$17.0 \pm 1.6b$	$9.6 \pm 1.3a$	$11.2 \pm 1.4a$	$12.8 \pm 1.2a$
GSH (μmol/L)	414.8 ± 50.0	365.8 ± 17.5	432.5 ± 54.5	407.6 ± 27.0	395.5 ± 23.2
GSSG/GSH $\times 10^2$	$2.6 \pm 0.2a$	$4.7 \pm 0.5b$	$2.2 \pm 0.2a$	$2.8 \pm 0.4a$	$3.5 \pm 0.5c$

Note. Values are means $\pm$ SEM, $n = 12$. Values in the same row with different letters are significantly different at $P < 0.05$.

fructose feeding groups were significantly higher than the control group ($P < 0.05$). Rats preventively fed AREBR exhibited comparable insulin sensitivity with control rats, as indicated by the HOMA-R at week 4 (2.4 ± 0.3 *vs* 2.2 ± 0.2) and week 8 (2.4 ± 0.3 *vs* 2.3 ± 0.3). After 8 weeks of diets feeding, a moderate hyperglycemia was observed in the high-fructose rats compared with the controls (6.4 ± 0.20 *vs* 5.4 ± 0.17 mmol/L). Pioglitazone administration reversed the insulin resistant status in the pio-treated rats. While the HOMA-R obtained from the AREBR-treated rats was intermediate between, and had significant differences ($P < 0.05$) with that of the control and the high-fructose groups (Table 5).

Oral Glucose Tolerance Test

During the two hours following glucose ingestion, plasma glucose levels in the high-fructose group were always significantly higher than those in the control group (Figure 1A), and were also higher than that of the other three groups at 30 min ($P < 0.05$). Compared to the fructose-fed controls, AREBR and pioglitazone supplementations significantly ($P < 0.01$) reduced the AUC for glucose (Figure 1B).

Discussion

In the present study, we show for the first time that the anthocyanin-rich extract from black rice has the property of preventing metabolic syndrome by improving lipid profile and increasing insulin sensitivity in fructose-fed rats. Based on our previous results [8–11], the high amount of anthocyanin contained in the extract might be the major component responsible for the extract-induced anti-metabolic syndrome property.

There are considerable evidences supporting that high fructose diet upregulate the lipogenesis, resulting in high production of FFA and TG [14]. Elevated plasma FFA levels promote fat oxidation and decrease carbohydrate oxidation. Consequently, extensive oxidations of fatty acids produce an abundant of highly reactive molecular species, and increase oxidative stress [21]. It is well known that oxidative stress plays critical roles in the pathogenesis of various diseases, including diabetes, hypertension and atherosclerosis [22]. Most recently, Houstis N et al. demonstrated that reactive oxygen species have a causal role of insulin resistance in two cellular models [23]. Considering the role of free radical activity in insulin resistance, Faure P et al. observed an impairment of the antioxidant defense systems in rats fed a HF diet, and supplementation with the antioxidant, vitamin E, could improve the oxidative stress and have a beneficial effect on insulin sensitivity of these rats [24]. This study clearly showed that HF diet led to oxidative stress, as indicated by the higher levels of plasma TBARS and ratio of blood GSSG/GSH in the high-fructose group (Table 4). Anthocyanin significantly inhibited the oxidative stress, prevented the insulin resistance occurrence in the AREBR group, suggesting that the decreased oxidative stress by AREBR may contribute to the increased insulin sensitivity in this fructose animal model.

The glitazones, peroxisome proliferators activated receptor-γ (PPARγ) agonists, including pioglitazone and rosiglitazone, are extensively used to improve insulin

Table 5. Effect of diets on fasting plasma glucose and insulin levels

Groups	Plasma glucose (mmol/L)		Plasma insulin (pmol/L)		Insulin sensitivity (HOMA-R)[a]	
	4 wk	8 wk	4 wk	8 wk	4 wk	8 wk
Control	5.6 ± 0.17	5.4 ± 0.17a	61.2 ± 4.7a	67.0 ± 13.7a	2.2 ± 0.2a	2.3 ± 0.3a
High-fructose	5.7 ± 0.21	6.4 ± 0.20b	137.3 ± 10.9b	108.4 ± 18.3b	5.0 ± 0.3b	4.4 ± 0.4b
AREBR	5.4 ± 0.15	5.3 ± 0.28a	69.9 ± 14.7a	70.5 ± 17.4a	2.4 ± 0.3a	2.4 ± 0.3a
AREBR-treated	5.4 ± 0.18	5.6 ± 0.21a	135.3 ± 18.3b	93.9 ± 19.4b	4.7 ± 0.4b	3.4 ± 0.3c
Pio-treated	5.6 ± 0.20	5.7 ± 0.11a	140.4 ± 9.6b	72.9 ± 14.9a	5.0 ± 0.4b	2.6 ± 0.3a

Note. Values are means $\pm$ SEM, $n = 12$. Values in the same column with different letters are significantly different at $P < 0.05$.
[a]HOMA-R = fasting glucose (mmol/L) $\times$ fasting insulin (μIU/mL, 1 μIU/mL = 6.945 pmol/L)/22.5.

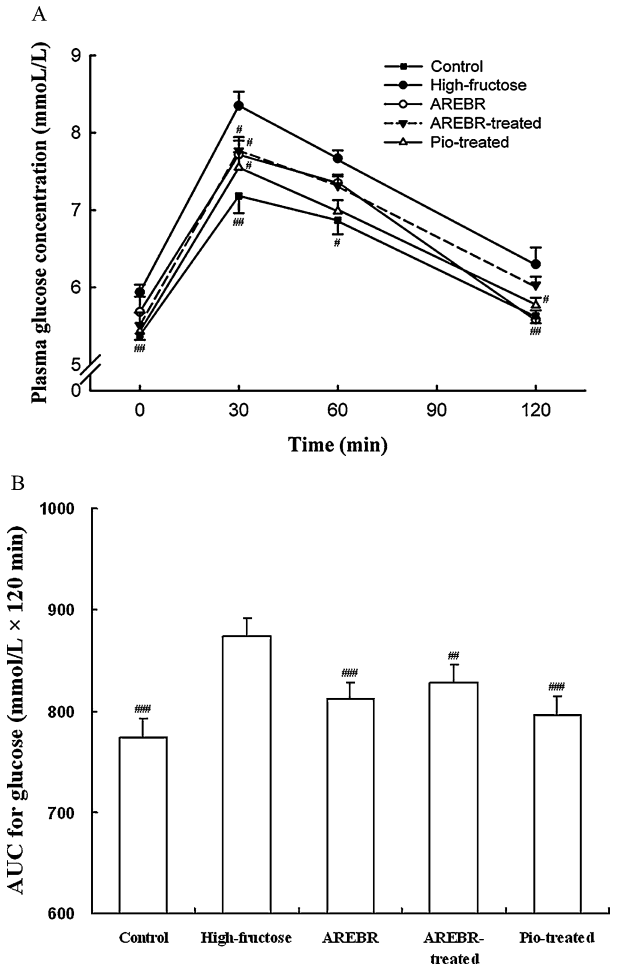

Figure 1. Changes of plasma glucose (A) and total area under curve (AUC) for glucose (B) in an oral glucose test (3 g glucose/kg BW). Values are means ± SEM, $n = 6$. Statistically significant differences between treatments are indicated, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to the high-fructose group.

sensitivity in type 2 diabetic and obese non-diabetic subjects with impaired glucose tolerance [25]. We recently demonstrated that treatment of peritoneal macrophages and macrophage-derived foam cells with cyanidin 3-glucoside, the predominant anthocyanin in black rice pigment fraction [10], causes PPARγ activation in a concentration-dependent manner [26]. The present results showed that chronic intake of AREBR, similar to pioglitazone, induced ameliorating effects on lipid homeostasis and insulin sensitivity. However, AREBR couldn't efficiently reverse the established hyperinsulinemia as pioglitazone did in this study. Further studies are required for elucidating the detailed mechanisms underlying these effects.

Elevated plasma FFA, and resultant lipid intermediate compounds, can directly interrupt insulin signaling by impairing the process of insulin-dependent protein phosphorylation and glucose transport, which develop to insulin resistance [21, 27]. The lipid-lowering properties of anthocyanins have been demonstrated by a number of investigators [12, 13, 28]. Tsuda T et al. showed, that anthocyanin-rich purple corn color supplementation, could suppress the mRNA levels of enzymes involved in FFA and TG synthesis in high-fat fed mice [12]. Grape polyphenols were shown to alter lipoprotein metabolism by decreasing plasma TG and apolipoprotein B concentrations [28]. In the present study, AREBR treatment improved the parameters of FFA and HDL-C to levels comparable with that of the pio-treated and control rats (Table 3). Meanwhile, pio-treated animals exhibited larger epididymal fat pad may attribute to the side effect of pioglitazone [25].

In conclusion, this study has shown that dietary anthocyanins-rich extract from black rice is capable of preventing and ameliorating the hyperlipidemia and insulin resistance induced by a high-fructose diet. The underlying mechanism may be related mainly to inhibiting oxidative stress and improving the plasma lipid profile. The results indicate that AREBR is a promising nutraceutical ingredient, and may possess clinical importance in preventing diabetes and metabolic syndrome.

Acknowledgments

We particularly thank Prof. Mingwei Zhang in the Key Laboratory of Functional Food, Ministry of Agriculture of the P.R. China, for providing the black rice pigment fraction. This work was supported by the research grant from National Natural Science Foundation of China (#30025-037) and China Medical Board of New York Inc. (CMB, # 98-677).

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