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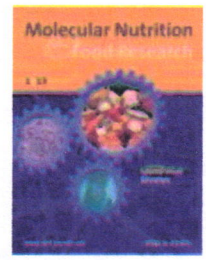

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Foods and Function Article

Black rice extract supplementation attenuates LDL oxidation and regulates the PRMT/ADMA/DDAH pathway in rabbits fed a high-fat diet.

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Abbreviation: PRMT, protein methyltransferase; **ADMA**, asymmetric dimethylarginine; **DDAH**, dimethylarginine dimethylaminohydrolase.

Key Words: asymmetric dimethylarginine, black rice extract, dimethylarginine dimethylaminohydrolase-1, LDL oxidation, protein methyltransferase-1,

Abstract

Scope: Previous investigations have demonstrated that black rice extract reduces or retards endothelial dysfunction in hypercholesterolemic rabbits. However, the effects of such extract on the serum concentrations of oxidized LDL, asymmetric dimethylarginine (ADMA), and mRNA expressions of protein methyltransferase-1 (PRMT-1) and dimethylarginine dimethylaminohydrolase-1 (DDAH-1) remain unclear. This study assessed the effect of black rice extract supplementation on LDL oxidation and the PRMT/ADMA/DDAH pathway in rabbits fed a high-fat diet.

Methods and results: Black rice extract supplementation for 4 weeks significantly decreased the serum concentrations of total cholesterol, F_2-isoprostanes, oxidized LDL, and ADMA; decreased PRMT-1 mRNA expression; and increased DDAH-1 mRNA expression ($p<0.05$).

Conclusion: Black rice extract supplementation can attenuate LDL oxidation and regulate the PRMT/ADMA/DDAH pathway in rabbits fed a high-fat diet. This information is crucial for the prevention of cardiovascular diseases; because an increase in ADMA concentration is an early risk factor for vascular endothelial dysfunction, limiting the excessive synthesis of ADMA can be considered in atherosclerosis treatment.

1 Introduction

Several prospective epidemiological studies have concluded that moderately elevated asymmetric dimethylarginine (ADMA) concentrations are associated with a notably increased risk of coronary events and cardiovascular mortality in the general population. In a meta-analysis of relevant studies on 2015, the risk ratios for the top third versus the bottom third of serum ADMA concentrations were 1.42 (95% CI 1.29–1.56) for cardiovascular disease, 1.39 (1.19–1.62) for coronary heart disease, and 1.60 (1.33–1.91) for stroke.[1]

In the cardiovascular system, ADMA is produced by protein methyltransferase-1 (PRMT-1) and expressed in the heart, smooth muscle cells, and endothelial cells.[2] Dimethylarginine dimethylaminohydrolase (DDAH) catalyzes ADMA to produce dimethylamine and L-citrulline. DDAH-1 is the major enzyme that degrades ADMA. [3] Decreased DDAH activity plays a key role in the accumulation of ADMA.[4] Therefore, the PRMT/ADMA/DDAH pathway may be an alternative target for the treatment of endothelial dysfunction.

Hyperlipidemia is a state characterized by an increased concentration of lipids, including cholesterol and triglycerides, and can predispose to endothelial. Possible mechanisms underlying hyperlipidemia-induced endothelial dysfuntion include the following:[5] (1) upregulation of nicotinamide adenine dinucleotide phosphate oxidase, increased production of O_2^-, and oxidative stress; (2) increased plasma concentrations of ADMA; and (3) oxidation of LDL. ADMA is an endogenous inhibitor of endothelial nitric oxide synthase (eNOS) and competes with L-arginine for the same binding site on eNOS, thus resulting in eNOS uncoupling, increased O_2^- production, and consequently decreased NO production. Plasma concentrations of ADMA have been reported to increase in hypercholesterolemia. Therefore, ADMA considered being both a marker of and a risk factor for endothelial dysfunction.[6]

In addition to scavenging NO, excess O_2^- modifies LDL cholesterol to form oxidized LDL (ox-LDL), which plays a major role in the development of endothelial activation and atherogenesis. Both LDL and ox-LDL have been reported to increase the activity of S-adenosylmethionine-dependent methyltransferases, leading to increased ADMA synthesis. Therefore, LDL and ox-LDL may be attributed to the increased plasma concentrations of ADMA in hypercholesterolemia.[7]

Optimized nutrition through diet supplementation with plant-derived phytochemicals has attracted considerable attention to prevent the onset of several chronic diseases, including cardiovascular impairments, cancer, and metabolic disorders. Studies have extensively demonstrated that rice diets exhibit cardioprotective effects and are effective in the treatment of cardiovascular disease.

A previous study showed that black rice contains anthocyanin pigments with notable antioxidant and anti-inflammatory properties, thus revealing its potential for use in nutraceutical or functional food formulations.[8] Black rice consumption was reported to reduce or retard the progression of atherosclerotic plaque induced by dietary cholesterol; the mechanisms underlying the antiatherogenic effects of black rice may be increased antioxidant activity and decreased oxidative status.[9] A previous investigation suggested that the antioxidant characteristics of plant extracts can improve endothelial function by regulating the PRMT/ADMA/DDAH pathway.[10]

As mentioned, the PRMT/ADMA/DDAH pathway may be an alternative target related to ED. Because black rice extract (BRE) has antioxidant properties, we postulated that BRE supplementation could regulate the PRMT/ADMA/DDAH pathway to improve endothelial function by inhibiting oxidative stress. Therefore, in this study, we examined whether BRE supplementation could inhibit LDL oxidation and regulate the PRMT/ADMA/DDAH pathway in rabbits fed a high-fat diet.

2 Experimental Section

2.1 Preparation of black rice samples

We selected an "Enrekang" pigmented black rice variety (a local variety from South Celebes, Indonesia) under a botanical expert's supervision. The rice seeds were dehulled, milled in a laboratory mill, and passed through a 60-mesh sieve. The fresh powdered black rice samples were collected immediately in polyethylene bags. The powdered black rice was stabilized according to the method reported by Malekian et al.[11] with some modifications. The samples were heated in an autoclave for 3 min at 120°C, followed by cooling down to room temperature overnight. They were then stored at 4°C in a refrigerator for further analysis.

2.2 Extraction of black rice

The powdered black rice was extracted according to the method developed by Lai et al [12]. Samples of 5 kg were macerated and extracted with 70% ethanol, three extractions each performed overnight at room temperature. The extract was filtered through No. 6 filter paper (Advantech, Dublin, CA, USA). The filtrates were concentrated into dry solids through the sequential use of a rotary evaporator (EYELA, Tokyo, Japan) and lyophilizer (Ilshin Lab. Co., Seoul, Korea). The final dried extract was stored at -20°C for further analysis. The BRE yield represented 3.1% of the powdered black rice weight.

2.3 Animals, diets and sample collection

Twenty-seven male New Zealand white rabbits with an average body weight of 2.0 ± 0.2 kg were obtained from a local supplier and used for this experiment. The rabbits were housed individually in standard stainless-steel cages at 24°C with a 12-h light–dark cycle and were fed standard rabbit pellets for 1 week for acclimatization. The rabbits were randomly divided into three groups ($n = 9$): Group 1, comprising rabbits fed with a normal diet (ND group); Group 2,

comprising rabbits fed with a high-fat diet (normal diet + 5% lard and 12% egg yolk; FD group); and Group 3, comprising rabbits fed with the high-fat diet supplemented with 0.6 g/100 g BRE (FBRED group). The amount of BRE in this dose equals 18.7 g of black rice because the BRE yield was 3.1% of the black rice weight. This BRE supplementation dose was converted from the 200 g/day dose of black rice in humans with a 70 kg body weight; the conversion factor was 0.07.[13] Composition of standard pellet for normal diets are presented in Supplementary Table 1.The preparations were administered for 4 weeks in the treatment protocol, and the dose for each diet was set at 100 g per rabbit per day, with water being available *ad libitum*. Fresh food was provided every day, and the remaining food from the previous day was removed. The body weights of the animals were recorded weekly.

After the rabbits were subjected to overnight fasting, blood samples were collected from the central ear artery before (0 weeks) and at the end of the experiment (after 4 weeks) to measure the serum concentrations of lipids, F_2-isoprostanes, ox-LDL, and ADMA. After centrifugation at 1000 × *g* at 4°C for 15 min, serum was collected and stored at 80°C until assayed. For aortic assessment, rabbits were euthanized by an overdose of sodium pentobarbital and exsanguinated at the end of the experiment. The arcus aorta was removed for measurement of PRMT-1 and DDAH-1 mRNA expression. The study was approved by the Health Research and Ethics Committee of the Medicine Faculty, Hasanuddin University, Makassar, Indonesia (reference number: 256/H4.8.4.5.31/PP36-KOMETIK/2016).

2.4 Biochemical assays

The serum concentration of total cholesterol was determined using an enzymatic method. A reagent set from Roche Diagnostics Ltd. (Basel, Switzerland) was used for the determination, which was conducted according to the manufacturer's instruction for the Cobas Integra 400 Plus system.

The serum concentration of F_2-isoprostanes was determined using an immunoassay kit (8-Isoprostane ELISA Kit, item No. 516351, Cayman Chemical, MI, USA). The analysis was conducted according to the manufacturer's instruction.

The serum concentration of ox-LDL was determined through the quantitative sandwich enzyme immunoassay technique using a Rabbit ox-LDL ELISA Kit (Cat. Number CSB-E06991Rb, Cusabio, Houston, TX, USA). The analysis was conducted according to the manufacturer's instruction.

The serum concentration of ADMA was determined using an immunoassay kit (Rabbit ADMA ELISA Kit, Cat. Number E1301Rb, Wuhan EIAab Science Co, Ltd. Wuhan, China). The assay was conducted according to the manufacturer's instruction.

PRMT-1 and DDAH-1 mRNA expression levels were analyzed through real-time quantitative PCR (RT-qPCR). Briefly, DNA was extracted from freshly collected arcus aorta tissue samples according to the diatom guanidinium thiocyanate (GuSCN) method described by Hatta et al.[14] The extraction of DNA from the aorta tissue samples was conducted as follows: a 100 μg freshly collected tissue sample was mixed with 900 μL of lysis buffer (50 mM Tris-HCl, 5.25 M GuSCN, 20 mM EDTA, 0.1% Triton X-100) and centrifuged at 12,000 × *g* for 10 min. To obtain the DNA, 20 μL of diatom suspension was added. The diatom containing the bound DNA was sedimented by centrifugation at 12,000 × *g* for 15 s. The diatom pellet was washed with washing buffer (5.25 M GuSCN in 0.1 M Tris-HCl, pH 6.4), rinsed with 70% ethanol and acetone, and dried by incubation at 56°C for 10 min. The pellet was mixed with 60 μL of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA buffer, and the DNA was eluted through incubation at 56°C for 10 min. After the sedimentation of the diatom, the supernatant was collected for RT-PCR examination. [14,15]

The prepared PCR mix included the following: 14 μL MQ Baker, 5 μL master mix, 0.5 μL forward primer (PRMT-1 mRNA), 0.5 μL reverse primer (PRMT-1 mRNA), and 5 μL DNA

template in a total reaction volume of 25 μL (Bio-Rad, CA, USA). RT-PCR was performed in an optical 96-well plate through a CFX-2000 RT-PCR Sequence Detection System (Bio-Rad, CA, USA) using EvaGreen detection chemistry (Biotium Inc, Hayward, CA, USA). The run protocol was as follows: initial denaturation at 95°C for 10 min and 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min. After the PCR process, a dissociation curve was constructed by increasing the temperature from 65°C to 95°C to detect PCR product specificity. In addition, a no-template control (H_2O control, Baker) was analyzed for possible contamination in the master mix. A cycle threshold (Ct) value was recorded for each sample. PCRs were set up in triplicate, and the mean of the three Ct values was calculated. A comparative Ct method was applied to the raw Ct values to determine relative gene expression. The same procedure was run for DDAH-1 mRNA expression. Specific forward and reverse primers were designed using Primer Express software, version 2.0.0 (Applied Biosystems Inc, Foster City, CA, USA), as follows:

PRMT-1, 5'-TTGACTCCTATGCCCACT-3' (sense) and
5'-CCACATCCAGCACCACC-3' (antisense);
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH),
5'CGGCAAGTTCAACGGCACAG-3' (sense) and
5'-GAAGACGCCAGTAGACTCCACGAC-3' (antisense);
DDAH-1, 5'-GCCTGATGACATAGCAGCAA-3' (sense) and
5'-CCATCCACCTTTTCCAGTTC-3' (antisense); and GAPDH,
5'-CTGCACCACCAACTGCTTAG-3' (sense) and
5'-AGGTCCACCACTGACACGTT-3' (antisense).[16,17]