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Full Paper

Rice bran water extract prevents cardiac lipid accumulation and oxidative damage in rats fed a high-fat diet

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Abstract: Rice bran water extract (RBE) has been reported to have cardiovascular protective effects including lipid-lowering and antioxidant effects. The present study aims to investigate the effects of RBE and its possible protective mechanisms against lipid accumulation and oxidative damage to the heart of male Sprague-Dawley rats induced by a high-fat diet (HFD). After four weeks of exclusive HFD feeding, elevated triglyceride, total cholesterol and malondialdehyde contents in the cardiac tissue were examined. The HFD group showed elevated serum levels of free fatty acid, triglyceride and total cholesterol. By contrast, these elevations decreased after four weeks of oral RBE treatment (2,205 mg/kg/day). RBE treatment also increased cardiac free radical-scavenging activity. Mechanistically, expression levels of peroxisome proliferator-activated receptor α, AMP-activated protein kinase α, glucose transporter 4 and heme oxygenase-1 were up-regulated in the heart of the RBE-treated rats. However, down-regulated expression levels of cluster of differentiation 36, sterol regulatory element binding protein-1 and nuclear factor-kappa B p65 were detected in the RBE-treated group as compared with the HFD group. However, cardiomyocyte size and cardiac marker levels were unchanged between the groups. RBE intake may prevent HFD-induced lipid overaccumulation and oxidative damage in the cardiac tissue, resulting in cardioprotective effects.

Keywords: rice bran, high-fat diet, metabolic syndrome, cardiac lipotoxicity, oxidative stress

Maejo Int. J. Sci. Technol. **2019**, *13*(02), 96-109

INTRODUCTION

Metabolic syndrome is commonly associated with the development of heart diseases [1]. Among the various pathological mechanisms, cardiac lipid overaccumulation is one of the most important causes of heart diseases. Recent human and animal studies have suggested that ectopic lipid accumulation in the cardiac tissue is associated with cardiac dysfunctions [2-4]. Although the precise mechanisms of cardiac lipid overaccumulation are not well understood, they are linked to the dysregulation of myocardial lipid metabolism. The major pathogenesis of cardiac lipid accumulation involves: (1) increased delivery of triglyceride (TG)-derived fatty acids (FA) and free fatty acids (FFA) to the heart; (2) increased FA uptake; (3) increased lipogenesis; and (4) impaired lipid oxidation [2, 5, 6]. Excess lipids may lead to the overproduction of lipotoxic intermediates that can mediate many detrimental effects such as insulin resistance, oxidative stress and apoptosis. This phenomenon is also known as cardiolipotoxicity.

Cardiac metabolic disturbances are believed to be a consequence of impairment in energy regulation involved with several regulators. Among these, peroxisome proliferator-activated receptor α (PPARα) is a well-established member of the nuclear receptor family of transcription factors and is highly expressed in the myocardium [7]. The activation of this receptor enhances FA catabolic pathways such as FA β-oxidation, and regulates FA anabolic pathways such as FA storage. AMP-activated protein kinase (AMPK) is one of the many regulatory enzymes that have been suggested to play a key role in cardiac lipid and glucose homeostasis [8]. Indeed, the activation of AMPK in the cardiac tissue promotes FA oxidation and glucose uptake by inactivating the acetyl-CoA carboxylase (ACC) and translocating the intracellular glucose transporter 4 (GLUT4) to the sarcolemma respectively. AMPKα2 is the predominant catalytic isoform found in the heart. As PPARα and AMPK are considered to be the main energy sensors, their down-regulation can lead to metabolic disturbances in the heart [4, 6, 9-11]. Additionally, elevated expression of FA transporters, such as a cluster of differentiation 36 (CD36), contributes to the excessive uptake and accumulation of lipids in the heart [12]. Moreover, lipogenesis is considered to be an important pathway for lipid synthesis in both human and animal hearts, which is associated with the up-regulation of a lipogenic transcription factor known as sterol response element-binding protein-1c (SREBP-1c) [2, 6]. In addition to the abnormal fat metabolism, the defects in glucose uptake and oxidation have also been observed in the hearts of the patients and animal model with metabolic syndrome [11, 13]. High-fat diet (HFD) feeding has been recently reported to reduce the expression of AMPK and GLUT4 in the hearts of mice, thereby impairing glucose metabolism [11]. Moreover, transgenic mice with overexpression of GLUT4 could improve insulin sensitivity and hypertriglyceridemia in the setting of an HFD intake [14].

It has been proposed that increased oxidative stress is associated with the development of cardiac diseases [15]. Under oxidative stress condition, the reactive oxygen species (ROS) can promote oxidative damage to biomolecules such as lipids. Numerous studies demonstrated malondialdehyde (MDA) as a significant marker for lipid peroxidative damage to patients and animals with metabolic and cardiovascular diseases [16, 17]. ROS can be detoxified in the heart by activation of antioxidant transcription factors and enzymes such as nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) [18]. Contrarily, several oxidative stress-responsive transcription factors, including nuclear factor-kappa B (NF-κB), can also induce oxidative stress in the heart by producing ROS [19, 20].

Maejo Int. J. Sci. Technol. **2019**, *13*(02), 96-109

Rice bran, a by-product from rice milling, contains many phytonutrients also known as phytochemicals that can be used for the prevention of impaired lipid and glucose metabolism in patients with diabetes mellitus [21, 22]. In previous studies the RBE intake has been reported to decrease hyperlipidemia and oxidative stress in association with a reduction in vascular dysfunction in animals with metabolic syndrome [23-25]. Moreover, the treatment with RBE from Khao Dawk Mali 105 or KDML105 variety (*Oryza sativa* Linn.) resulted in decreases in insulin resistance, expression of the lipogenic gene, and fat deposition in the pancreas [26], strongly suggesting that RBE treatment could be useful for preventing cardiac metabolic derangement and oxidative damage. However, the mechanisms of the cardioprotective effect of RBE have yet to be explained. Thus, in the present study the effects of RBE on lipid accumulation and oxidative damage, and their possible molecular mechanisms are examined in the hearts of rats fed an HFD.

MATERIALS AND METHODS

Experimental Diets and Preparation of RBE

An HFD was prepared as previously described [24]. The main ingredients of the HFD included margarine, pork belly, pork liver, whole hen egg, sugar, wheat flour and standard rodent chow (C.P., Thailand). The HFD (5.12 kcal/g) contained 65% of calories derived from fat while the standard chow diet (3.04 kcal/g) contained 13% of calories derived from fat.

Rice bran from the organic KDML105 was obtained from a local mill in Surin province, Thailand. As previously described with some modifications [22, 24], the stabilised rice bran (2,000 g) was extracted with distilled water (8 L) at 70 °C for 1 h. The supernatant was collected by centrifugation at 8,000 rpm for 10 min., then re-centrifuged and lyophilised. An approximate 18.3% yield of RBE was obtained. Consistent with previous reports [24, 26], our preliminary study showed that the treatment with RBE at a dose of 2,205 mg/kg/day was highly effective in ameliorating the impaired fat and glucose metabolism in HFD-fed rats. Therefore, this dose was chosen for the current study. For animal treatment, RBE was dissolved in distilled water.

Animal Treatments

Male outbred Sprague-Dawley rats (6−8 weeks old; National Laboratory Animal Center, Thailand) were housed under controlled conditions for temperature, relative humidity and light. After a week of adaptation, the rats were assigned to the following three groups (n = 8/group):

(1) Control (C) group: rats received standard chow;

(2) HFD group: rats received HFD;

(3) HFD + RBE group: rats received HFD and 2,205 mg RBE/kg/day via oral gavage.

The body weight and food intake of the rats were recorded every day. After four weeks of feeding, all rats were euthanised, and their blood was drawn by a cardiac puncture. Rat serum and hearts were harvested for further studies. All animal protocols were approved by the Animal Ethics Committee of the Faculty of Medicine, Thammasat University, Thailand (Permission No. AE 002/2013).

Measurement of Lipids and Cardiac Markers in Serum

The concentrations of FFA, TG and total cholesterol (TC) were measured using enzymatic colorimetric reagent kits (FFA assay kit, Wako, Japan, and Fluitest lipid assay kits, Analyticon Biotechnologies AG, Germany). The serum levels of creatine kinase muscle-brain fraction (CK-

Maejo Int. J. Sci. Technol. **2019**, *13*(02), 96-109

MB) and lactate dehydrogenase were determined using Abbott AxSYM analyser (Abbott Laboratories, USA) and COBAS INTEGRA 400 Plus (Roche Diagnostics, Switzerland) respectively.

Measurement of Lipids in Heart

The lipids were isolated from the tissues as previously described [27]. A total of 50 mg of the left ventricle was homogenised in 1 mL of isopropanol. After centrifugation, the levels of TG and TC in the supernatant were measured with the commercial kits from Analyticon Biotechnologies AG (Germany) and expressed as mg/g tissue.

Histological Analysis

The sections of the left ventricles were stained with hematoxylin and eosin. Images were detected under a light microscope (Olympus CX31, Olympus, Japan) equipped with a microscope camera (Olympus DP20). The average cross-sectional area of fifty cardiomyocytes were measured for each rat (n = 3/group) by AxioVision software (Carl Zeiss, Germany).

Measurement of mRNA Expression in Heart

Total RNA was extracted from left ventricular tissue using TRIzol reagent (Invitrogen, USA) and its concentrations were measured by NanoDrop instrument (Thermo Scientific, USA). An equal amount of RNA was then reverse transcribed using cDNA synthesis kit (Applied Biosystems, USA). Finally, the transcript levels were quantified by real-time polymerase chain reaction instrument and TaqMan reagents (Applied Biosystems, USA). The relative expression levels of CD36, PPARα, AMPKα, SREBP-1c and GLUT4 were determined by comparative cycle threshold method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene, and its levels were used to normalise the expression levels of target genes.

Measurement of Protein Expression in Heart

The total protein was extracted from the homogenised left ventricles using a cell lysis buffer (Cell Signaling Technology, USA) and its concentrations were determined by Bradford assay reagents (Bio-Rad, USA). Fifty micrograms of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membrane. The membranes were then incubated in blocking buffer (LI-COR Bioscience, USA) for 1 h at room temperature. The blocked membranes were then incubated at 4°C overnight with the primary antibodies as follows: anti-PPARα (BioVision, Inc., USA), anti-CD36, anti-AMPKα, anti-SREBP-1, anti-Nrf2 anti-HO-1 (Santa Cruz Biotechnology, USA), anti-GLUT4, anti-NF-κB p65 and anti-GAPDH (Cell Signaling Technology, USA). After washing with Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with DyLight 680 conjugated antibodies (Cell Signaling Technology, USA) for 1 h in the dark at room temperature. Finally, band intensities were quantified using Odyssey Fc Imaging system (LI-COR Bioscience, USA). GAPDH was used as a reference protein and its levels were used to normalise the expression levels of target proteins.

Measurement of Oxidative Stress Markers in Heart

MDA assay was done as previously described [28] with some modifications. In brief, 200 µL of cardiac supernatant or tetraethoxypropane standard (Sigma-Aldrich, USA) were mixed with 1

Maejo Int. J. Sci. Technol. **2019**, *13*(02), 96-109

mL of 10% trichloroacetic acid solution (Merck, Germany). After boiling, cooling, and centrifugation, 800 μL of the supernatant was treated with 400 μL of 0.67% thiobarbituric acid (Sigma-Aldrich, USA). After re-cooling, the absorbance of the supernatant was measured at 532 nm. Total protein levels in cardiac tissues were determined by Bradford assay reagents (Bio-Rad, USA). The results are expressed as nM/mg of protein.

The total antioxidant capacity of cardiac tissue was evaluated by the per cent inhibition of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) as described by previously published method [29]. Briefly, 10 μL of cardiac supernatant or Trolox standard was added to 1 mL of $ABTS^{\bullet+}$ solution (Sigma-Aldrich, USA). The absorbance was assayed spectrophotometrically at 734 nm. The $ABTS^{\bullet+}$ radical-scavenging activity was then calculated with following equation: % inhibition = $[(A0-A1/A0] \times 100$, where A0 is absorbance of initial $ABTS^{\bullet+}$ at t = 0 min and A1 is absorbance of $ABTS^{\bullet+}$ with sample or standard at t = 1 min. The results are expressed as μM Trolox/mg of protein.

Statistical Analysis

All results were analysed using one-way analysis of variance (ANOVA), followed by the least significant difference's (LSD) post hoc test (SPSS version 16.0, SPSS Inc., USA). A p value of less than 0.05 was considered statistically different. Data are presented as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

It is well established that rodent models of metabolic syndrome, including the high-calorie diet-fed rodents, are involved in the development of cardiac lipid deposition and oxidative damage [5,16]. Consistent with these observations, we demonstrated that the overaccumulation of cardiac TG and TC, and oxidative damage were developed after the rats were fed an HFD (Figures 1A and 4A). These elevations were found to coincide with increased serum levels of FFA, TG and TC (Figure 2). Moreover, abdominal obesity, hepatic steatosis, hyperglycemia and glucose intolerance were observed in rats fed HFD alone (data not shown). Food and energy intake was not significantly different between the HFD and HFD + RBE groups. Compared with the controls, the energy intake was significantly increased in all HFD-fed groups (data not shown). Unfortunately, the cardiac hypertrophy and markers were found to be unaffected in the rats fed short-term HFD for 4 weeks (Figures 1B and 1C). There were no significant differences in the relative heart weight or the mean cross-sectional area of left ventricular myocytes among the animal groups. In parallel, hematoxylin- and eosin-stained sections from all rats revealed normal left ventricular structure (Figure 1D). In addition, the serum levels of creatine kinase-MB and lactate dehydrogenase were not different between the groups (data not shown). Our results suggest that the abnormalities of lipid, glucose and redox homeostasis, rather than the abnormalities of cardiac histology and markers, are probably the early pathological events in rats fed on short-term HFD. Thus, these HFD-fed rats could be used as a suitable model for studying the effects of RBE on cardiac metabolic and oxidative stress, as described below.

Maejo Int. J. Sci. Technol. **2019**, *13*(02), 96-109

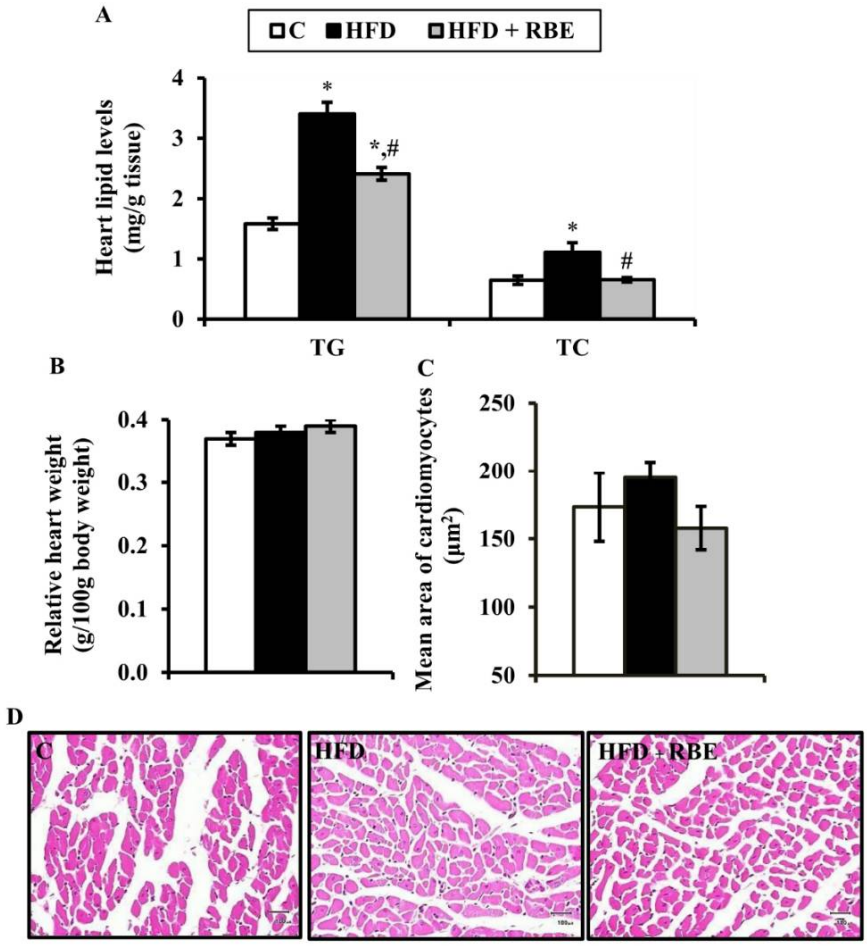

Figure 1. Left ventricular lipid contents (A), relative heart weight (B) and left ventricular cardiomyocyte size (C) and histology (400×; bar = 100 μm) (D) of the experimental rats. Values are mean ± SEM (n = 8 for heart weight and lipid levels and n = 3 for histology). $^{*}p < 0.05$ compared with C group; $^{\#}p < 0.05$ compared with HFD group.

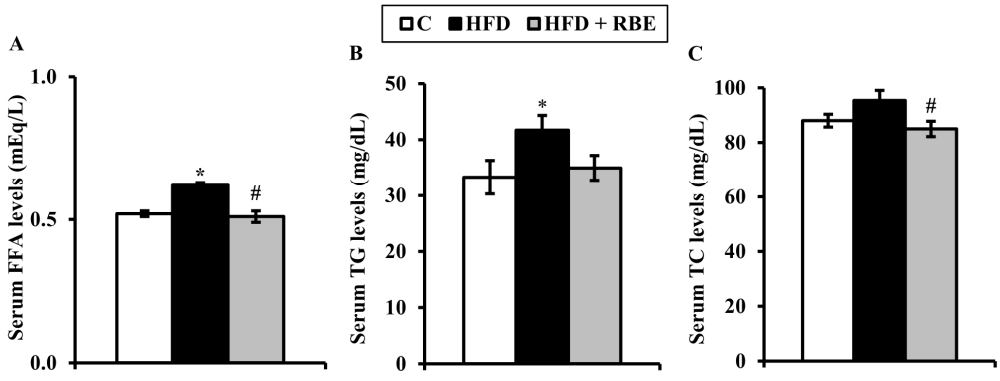

Figure 2. FFA (A), TG (B) and TC (C) levels in the serum of the experimental rats. Values are mean ± SEM (n = 8). $^{*}p < 0.05$ compared with C group; $^{\#}p < 0.05$ compared with HFD group.

Maejo Int. J. Sci. Technol. **2019**, *13*(02), 96-109

Effects of RBE on Cardiac Lipid Accumulation and Metabolic Gene Expression in HFD-fed Rats

We first investigated the effects of RBE on the underlying mechanisms involved in cardiac lipid accumulation and glucose uptake. The increases in circulating FFA and TG have been reported in both animal and human studies on the setting of the metabolic syndrome [30,31]. This hyperlipidemia may also predispose the subjects to increased myocardial lipid delivery. In this view, CD36 is a major sarcolemmal FA transporter that facilitates the entry of long-chain FA into cardiac myocytes. In the models of metabolic syndrome, CD36 gene is up-regulated in the hearts of mouse and rat [5,12], and its inhibition in rat cardiomyocytes is associated with a reduction in TG accumulation and contractile dysfunction [32]. The present study shows that the treatment with RBE decreases cardiac TG and TC contents, thus demonstrating its lipid-lowering capacity (Figure 1A). Consistently, the treatment of HFD-fed rats with RBE prevents the increase in the serum levels of FFA, TG, and TC (Figure 2) as well as cardiac CD36 expression (Figures 3A and 3B). These results suggest that the RBE intake may attenuate the delivery of circulating lipids to the heart with a decrease in the lipid uptake via CD36, thus resulting in a reduction of lipid accumulation in the heart. These results are in agreement with previous reports which indicated that the RBE treatment caused a significant decrease in the circulating levels of lipid and pancreatic lipid in HFD-fed obese rats [25, 26], and that RBE can inhibit the expression of CD36 in the aortas of these obese rats [24]. Moreover, the treatment with protein hydrolysates, one of the important bioactive compounds in KDML105 rice bran, has been shown to have lipid-lowering effects in rats fed a high-energy diet [33].

PPARα, a lipid sensor, can be activated by FA and lipid metabolites. This activated PPARα can stimulate the expression of various metabolic genes, such as medium-chain acyl CoA dehydrogenase, further contributing to β-oxidation of FA [7]. Some *in vivo* and *in vitro* studies of metabolic syndrome have revealed that the down-regulation of PPARα and its target genes encoding FA oxidation enzymes results in impaired FA oxidation in the heart [4,5,9]. Furthermore, the treatment with PPARα agonist can reduce the myocardial TG contents in mice fed an HFD [34]. AMPK has emerged as an important sensor of cellular and whole-body energy homeostasis and is involved in the regulation of activities of FA oxidation-related enzymes such as ACC [8]. *In vivo* studies have revealed that cardiac AMPK expression and activity are perturbed during metabolic dysfunctions [10,11]. Interestingly, the treatment with AMPK activator has been reported to reduce the lipid accumulation through the up-regulation of PPARα in the cardiac cells [35]. The present results provide evidence of the failure of only HFD-fed rats in inducing PPARα and AMPKα expression (Figures 3A and 3B) and of their hearts with excessive accumulation of lipids. These down-regulations of both genes may contribute to the loss of energy-sensing pathways, consequently causing metabolic derangement in the heart. RBE treatment increases mRNA and protein expression levels of PPARα and AMPKα in the heart. A study by Ronis et al. [36] has revealed that the rice protein intake effectively increases the hepatic PPARα expression in rats fed HFD-high cholesterol diet. Thus, our findings suggest that the up-regulation of PPARα and AMPKα expressions by RBE treatment may be an early molecular compensation that helps to enhance the oxidation of the increased lipids in the heart.

One important mechanism that promotes the *de novo* lipogenesis is explained by the activation of SREBP-1c activity. The enhanced activity of SREBP-1c contributes to the up-regulation of various lipogenic genes such as fatty acid synthase gene, thus exacerbating the deposition of lipid within cells [37]. In parallel with elevated myocardial lipid content, SREBP-1c

Maejo Int. J. Sci. Technol. **2019**, *13*(02), 96-109

gene was significantly up-regulated in the hearts of patients and mice with metabolic syndrome [2,5]. These findings are coincident with our data. The results are presented in Figures 3A and 3B. Cardiac mRNA and protein levels of SREBP-1 are significantly higher in HFD-fed rats than in control rats, whereas RBE-fed rats have reduced SREBP-1 expression levels compared to rats fed HFD alone. These results are in agreement with previous studies which have demonstrated that the consumption of RBE and rice bran proteins decrease the expression of SREBP-1 in the pancreas and liver of rats with metabolic syndrome [26, 33]. Our results suggest that treatment with RBE may reduce lipogenesis in the heart by suppressing the expression of SREBP-1 gene.

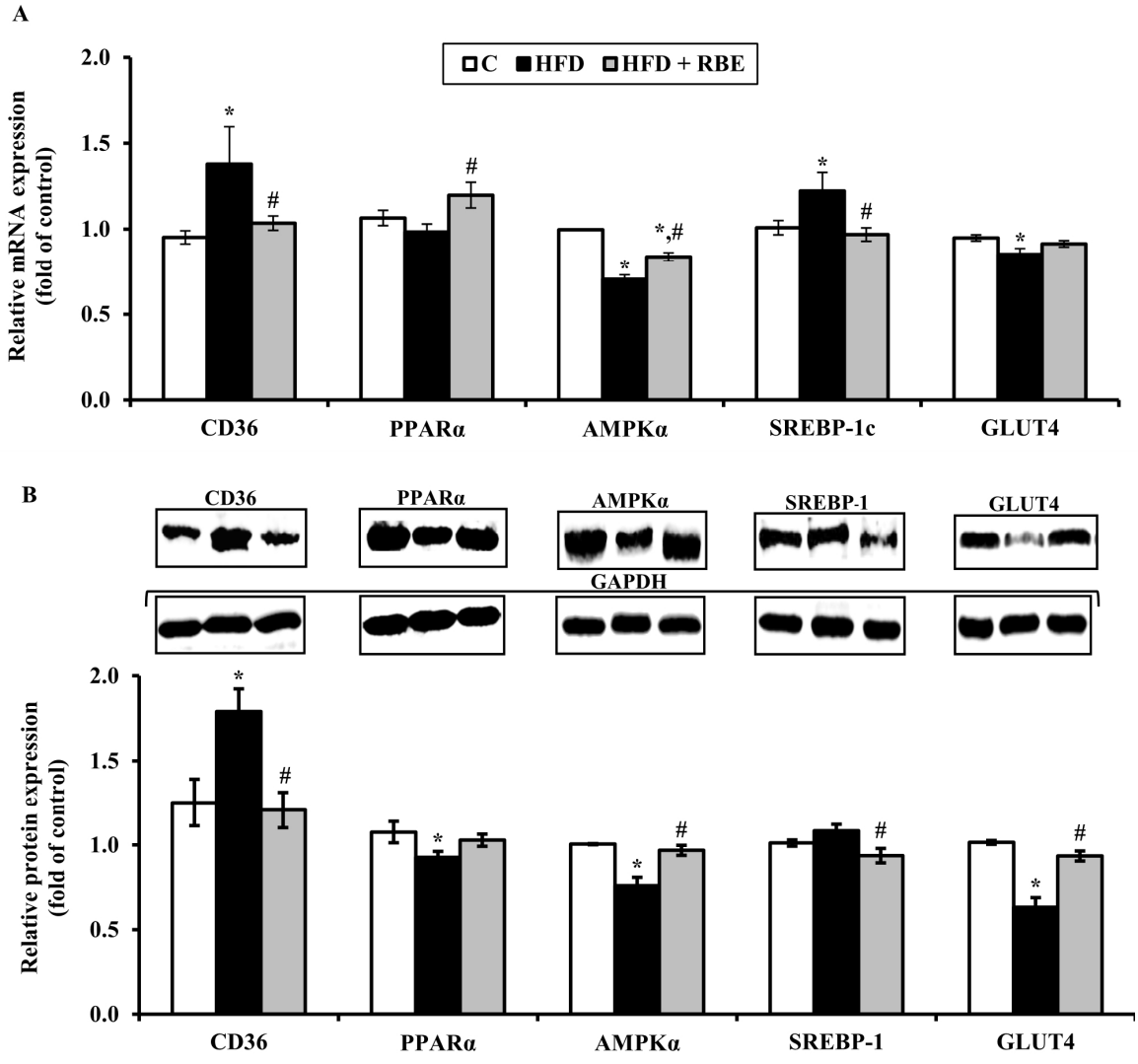

Figure 3. mRNA (A) and protein (B) expression levels of CD36, PPARα, AMPKα, SREBP-1 and GLUT4 in the left ventricles of the experimental rats. Values are mean ± SEM (n = 6). $^{*}p < 0.05$ compared with C group; $^{\#}p < 0.05$ compared with HFD group.

The glucose uptake process, which is the rate-limiting step in the cardiomyocyte glucose metabolism, is mediated by members of GLUT protein family, especially the GLUT4 [38]. Decreased expression of GLUT4 was found in the cardiac tissue from both the diabetic rats and patients [12,13]. AMPK is considered to be a regulator not only of FA oxidation but also of glucose uptake and oxidation [9]. Hearts from animals with metabolic syndrome exhibit decreased GLUT4 expression due to the decreased levels and activities of AMPK [10,11], whereas treatment with

Maejo Int. J. Sci. Technol. **2019**, *13*(02), 96-109

AMPK activator increases GLUT4 translocation and glucose uptake [39]. From present results, RBE consumption markedly prevents the down-regulation of GLUT4 and AMPKα protein in the left ventricles of HFD-fed rats (Figure 3B). Thus, we speculate that the RBE consumption may improve the impairment of cardiac glucose uptake and metabolism by enhancing the up-regulation of GLUT4 and AMPKα genes. To support this notion, it has been shown in our previous and other studies that the treatment with RBE and rice bran protein prevents high-energy diet-induced impaired glucose metabolism in the rats [24,26,33]. In addition, the treatment of HFD-fed mice with rice bran and phytic acid has been shown to result in a marked increase in glucose metabolism by activating glucose utilisation [40].

Effects of RBE on Cardiac Oxidative Damage and Antioxidant Capacity in HFD-fed Rats

Herein, we also examined the antioxidant effects of RBE on cardiac oxidative damage. The pathological conditions such as hypercholesterolemia and high levels of FFA can induce oxidative stress in the cardiac tissue [41,42]. In the current study the HFD hearts exhibited increased MDA levels, which is a sign of oxidative stress via lipid peroxidative damage in comparison with the normal rat hearts (Figure 4A). In contrast, HFD-fed rats treated with RBE had a significant reduction in the MDA levels as compared to untreated HFD-fed rats. As shown in our previous report, the RBE administration was also marked by the reduction of MDA levels in the serum and aorta [24]. Although there was no significant difference in the total antioxidant capacity of the cardiac tissues between the C and HFD groups, administration of RBE resulted in an increased total antioxidant capacity when compared to the other groups, as determined by ABTS radical-scavenging assay (Figure 4B). In line with our results, previously conducted animal studies showed that the treatment with rice bran, phytic acid and rice bran peptides significantly reduced the levels of oxidative stress markers such as MDA [24,43,44]. The Nrf2 is an essential transcription factor that plays a major role in the prevention of ROS overproduction during oxidative stress [18]. The activation of the Nrf2 pathway can transactivate the expression of antioxidant genes such as HO-1. HO-1 is the rate-limiting enzyme of heme degradation that converts pro-oxidant heme to antioxidant by-products (e.g. carbon monoxide). In a rat model of heart failure the treatment with HO-1 activator has been shown to reduce lipid peroxidation and infarction in the heart [45]. As an oxidative stress-sensitive transcription factor, the activity of NF-κB p65 that was inhibited by NF-κB inhibitor could reduce ROS production in the left ventricular tissues [19]. In the present study the administration of RBE can improve HO-1 expression and reduce NF-κB p65 expression in the heart without restoring Nrf2 expression (Figure 4C). This protection is likely to be associated with the elevation of antioxidant defence capacity in the heart, leading to a decrease in oxidative injury.

Previous studies have shown many biological activities of rice bran protein and phytic acid such as hypolipidemic, hypoglycemic and antioxidant effects [33,40,43,44]. These studies lead us to hypothesise that protein and/or phytic acid may be bioactive compounds in our RBE for improving the cardiac energy metabolism and antioxidant capacity. However, the bioactive compounds in RBE are still unknown. Further studies are needed to elucidate the bioactive constituents in RBE.

Maejo Int. J. Sci. Technol. **2019**, *13*(02), 96-109

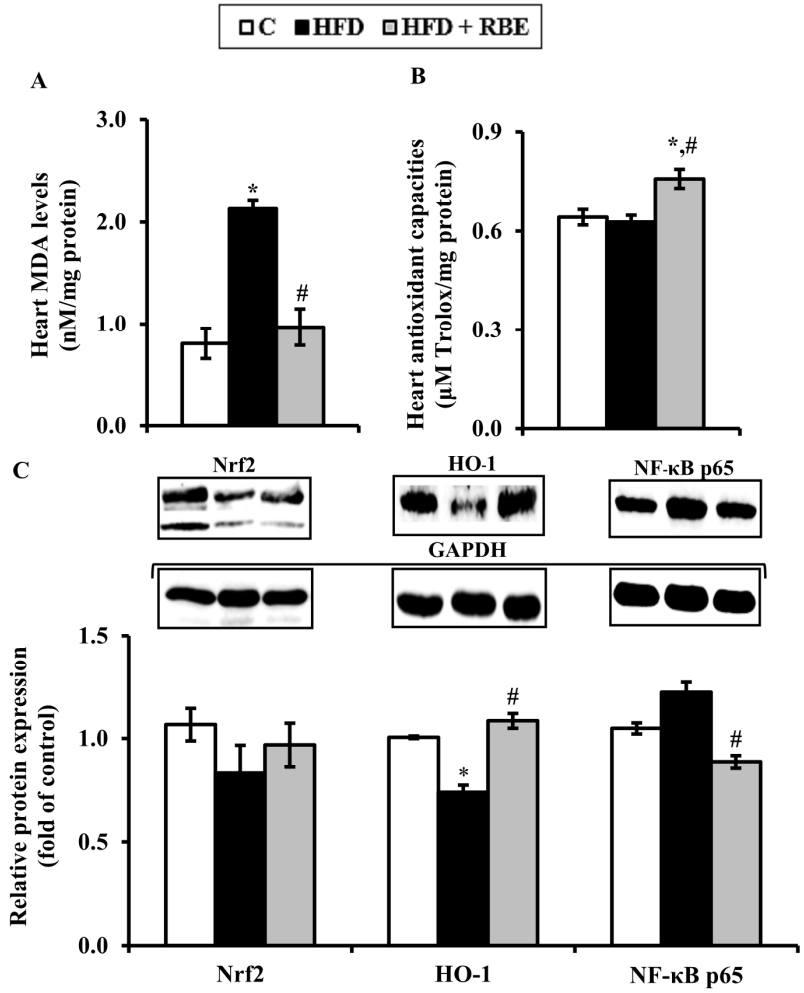

Figure 4. MDA levels (A), $ABTS^{\bullet+}$ radical-scavenging capacities (B) and protein expression levels of Nrf2, HO-1 and NF-κB p65 (C) in the left ventricles of the experimental rats. Values are mean ± SEM (n = 8 for MDA and ABTS assay and n = 6 for protein expression). $^{*}p < 0.05$ compared with C group; $^{\#}p < 0.05$ compared with HFD group.

CONCLUSIONS

The present results suggest that consumption of RBE can prevent the derangement of cardiac energy metabolism, partly by decreasing the myocardial lipid delivery as well as by improving the expression of genes involved in the lipid uptake (CD36), lipid oxidation (PPARα and AMPKα), lipogenesis (SREBP-1) and glucose uptake (GLUT4). In addition, consumption of RBE also exhibits preventive effects against HFD-induced oxidative damage to heart tissue, which may be associated with its antioxidant capacities. Therefore, RBE seems to be a potential food supplement for the prevention of cardiac abnormalities in the setting of metabolic syndrome. Additional studies are necessary to investigate its beneficial effects fully.

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Maejo Int. J. Sci. Technol. **2019**, *13*(02), 96-109

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Maejo Int. J. Sci. Technol. **2019**, *13*(02), 96-109

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