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Anti-Obesity Effect of Rice Bran Extract on High-Fat Diet-Induced Obese Mice

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ABSTRACT: Obesity involving adipose tissue growth and development are associated with angiogenesis and extracellular matrix remodeling. Rice bran has antioxidant and cardioprotective properties, and can act as a food supplement with potential health benefits, such as lowering blood pressure, hepatic steatosis, and inflammation. Therefore, we hypothesized that rice bran extract (RBE) can regulate adipose tissue growth and obesity. Male Institute of Cancer Research mice were fed with a high-fat diet (HFD) for 8 weeks and then supplemented with 220 and 1,100 mg/kg/d RBE while the low-fat diet group (control) were not. In addition to body weight, adipose tissue mass, and vessel density, we evaluated the mRNA expression of angiogenic factors such as matrix metalloproteinases, *Mmp-2*, *Mmp-9*, and the vascular endothelial growth factor (*Vegf*) in visceral and subcutaneous adipose tissues using real-time polymerase chain reaction. Administration of RBE to HFD-induced obese mice reduced the body weight and adipose tissue mass compared with untreated mice. It also decreased blood vessel density in the adipose tissue. Furthermore, RBE downregulated *Vegf* and *Mmp-2* mRNA levels in visceral fat tissue. These results demonstrate that RBE, at high concentrations, significantly reduces adipose tissue mass and prevents obesity development in HFD-induced obese mice, which might be partly mediated via an anti-angiogenic mechanism.

Keywords: anti-obesity, angiogenesis, high-fat diet, rice bran extract

INTRODUCTION

Obesity is a major public health challenge in modern medicine (Mendonça and Soares, 2015). The World Health Organization (2020) defines obesity as a body mass index equal or greater than 25 kg/m^2 in Asian countries. Obesity is associated with several diseases, including metabolic syndrome, cardiovascular diseases, diabetes mellitus, fatty liver diseases, and cancer, leading to associated morbidity and mortality. Recently, adipose tissues, especially in the visceral (VSC) compartment, have been identified not only as an energy depository tissue, but also as an active endocrine organ releasing biologically active molecules (Marseglia et al., 2014) associated with these diseases.

Obesity involves adipose tissue growth due to an increase in fat cell numbers (hyperplasia) and/or size (hypertrophy) accompanied with remodeling of the adipose tissue extracellular matrix (ECM) (Lee et al., 2013). Matrix metalloproteinases (MMPs) are critical during the growth of adipose tissue and its microvasculature (Lijnen et al., 2002; Visse and Nagase, 2003). While normally maintained at minimal levels, they are expressed and secreted during tissue remodeling involving adipogenesis (Woo et al., 2016). MMP-2 and MMP-9 control tissue development and microvessel maturation by modulating the ECM (Bouloumié et al., 2001; Lijnen et al., 2002). Their activities indirectly induce angiogenesis as MMP inhibitors, both synthetic and endogenous, can inhibit angiogenic responses both *in vivo* and *in vitro* (Quintero-Fabián et al., 2019; Bergers et al., 2000).

Recently, anti-obesity drugs have been withdrawn due to high incidence of adverse effects (Derosa and Maffioli, 2012; Onakpoya et al., 2018; Chao et al., 2020). Therefore, traditional herbal medicines are being increasingly studied as natural alternatives to weight loss therapeutics. Rice bran extract (RBE) contains antioxidants (γ-oryzanol, tocopherols, tocotrienols, and ferulic acid) and

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can be used as a food supplement to lower systemic blood pressure and cholesterol, and regulate pancreatic function (Nhung et al., 2016; Nurrahma et al., 2018; Ardiansyah et al., 2019; Duansak et al., 2020). However, the anti-obesity effect of RBE is still unclear. Therefore, we aimed to evaluate the effect of RBE on adipose tissue mass in diet-induced obese mice and its possible anti-angiogenic mechanism.

MATERIALS AND METHODS

Animals and experimental model

The animal protocols were approved by the Animal Ethics Committee of Thammasat University, Pathum Thani, Thailand (AE 002/2559). Male Institute of Cancer Research (ICR) mice (20～25 g) were obtained from the National Laboratory Animal Center of Mahidol University (Nakhon Pathom, Thailand) and were housed at 25±2°C with a 12-h light/dark cycle. They were fed standard rodent chow and water *ad libitum*. The control mice were fed for 8 weeks with a diet containing 70% kcal carbohydrate, 20% kcal protein, and 10% kcal fat (0.72 mg of cholesterol per gram of lard), with a total energy of 3.85 kcal/g. Mice in the high-fat diet (HFD) group were fed an HFD for 8 weeks containing 20% kcal carbohydrate, 20% kcal protein, and 60% kcal fat (0.72 mg cholesterol per gram of lard), with a total energy of 5.24 kcal/g. Mice in the HFD+RBE group received HFD with RBE (220 or 1,100 mg/kg/d). All treatments were administered orally by feeding tube every day for 8 weeks. After the treatment, mice were fasted for 6 h and then anesthetized with 2% isoflurane by inhalation. Subcutaneous (SC) and VSC fat pads were removed, weighed, snap-frozen in liquid nitrogen, and stored at −80°C until use. Portions of the fat pads were prepared for histological study.

Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The HFD and normal diets were purchased from Research Diets (New Brunswick, NJ, USA).

Preparation and characterization of RBE

The bran (Khao Dawk Mali 105 rice variety) was purchased from a local mill in Surin province, Thailand. The organically grown rice used was approved by the Organic Agriculture Certification of the Department of Agricultural Extension (Bangkok, Thailand). Freshly milled rice bran was stabilized at 130°C for 90 s. About 2,000 g of stabilized rice bran was boiled in 8,000 mL of distilled water for 1 h at 70°C. After centrifugation at 6,583 *g* for 10 min, the supernatant was freeze-dried into powdered extract using a freeze dryer (Lyophilization Systems Inc., Kingston, NY, USA). The preparation procedure has been described previously (Qureshi et al., 2002). Folin-Ciocalteu method, approved by the Association of Official Analytical Chemists, was applied for proximate analysis, determination of total phenolic compounds, and γ-oryzanol contents of the RBE using high-performance liquid chromatography (Butsat and Siriamornpun, 2010). The crude extract yield was 18% and the contents of total phenolic compounds were 4.6 mg gallic acid equivalents/g extract and 4.6 μg γ-oryzanols/g extract.

Real-time quantitative polymerase chain reaction (PCR)

Total RNA from the SC and VSC adipose tissues from individual animal in each group was extracted using GenUP™ Total RNA Kit (Biotechrabbit GmbH, Berlin, Germany) and was reverse-transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The cDNA was quantified by real-time PCR using SYBR Green PCR Master Mix (QPCR Green Master Mix, Biotechrabbit GmbH) on a StepOnePlus Real-Time PCR System (Applied Biosystems Foster, Waltham, MA, USA). The PCR reaction consisted of 10 μL 2×SYBR Green PCR Master Mix, 1 μL each of forward and reverse primers (final concentration 0.5 μM), 4 μL cDNA template (40 ng), and 5 μL nuclease-free water. The PCR conditions included a denaturing step at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, and 63～65°C for 30 s. Following the PCR, melting curve analysis was performed to access whether a single, specific PCR product was obtained. The specificity of the PCR product was also analyzed using 1.5% (w/v) agarose gel electrophoresis with ethidium bromide. The PCR data were normalized to the internal control, β-actin (*Actb*). The fold change of expression (treated vs. control) was calculated using a $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Table 1 lists all the PCR primers used for gene expression analysis (Qiao et al., 2015; Lee et al., 2016).

Table 1. Primer sequences used for real-time polymerase chain reaction analysis

Gene	Primer sequences (5′→3′)	Size (bp)
Actb		
Forward	CATCCGTAAAGACCTCTATGCCAAC	171
Reverse	ATGGAGCCACCGATCCACA	
Vegf		
Forward	CTTGTTCAGAGCGGAGAAAGC	125
Reverse	ACATCTGCAAGTACGTTCGTT	
Mmp-2		
Forward	AGATCTTCTTCTTCAAGGACCGGTT	225
Reverse	GGCTGGTCAGTGGCTTGGGGTA	
Mmp-9		
Forward	GAGCTGTGCGTCTTCCCCTTC	204
Reverse	GGAATGATCTAAGCCCAGTGC	

Immunohistochemistry

SC and VSC adipose tissues were fixed in 10% formalin, paraffin embedded and sectioned for immunohistochemistry. For quantitative comparisons between groups, the sections were immunolabeled using a standardized procedure with the anti-von Willebrand factor (vWF) rabbit polyclonal antibody (phospho S536 antibody, 100 μL, Abcam, Cambridge, UK) at 4°C overnight. Sections were visualized after binding of secondary antibody conjugated to peroxidase activity with diaminobenzidine substrate (Vector NovaRED, Vector Laboratories, Inc., Burlingame, CA, USA). Buffer alone or nonspecific purified rabbit immunoglobulin G served as controls. After immunolabeling of the vWF, light microscopy images of the SC and VSC sections were obtained (DAS-Fi2, Nikon, Tokyo, Japan; digital camera, Olympus, Tokyo, Japan). The vWF labeling density was measured by light intensity values [using Image J software (National Institutes of Health, Bethesda, MD, USA) with digital units between 0=white and 255=black] on at least 10 randomly selected windows (100 μm×100 μm) per section. For each window, the digital light intensity was determined as the sum of the light intensities of all pixels divided by the number of pixels. Four random sections per organ were used to determine the mean optical densities for each animal. All measurements were carried out under standardized light microscopy settings.

Histological analysis

Portions of SC and VSC were fixed in 10% formalin, paraffin embedded, sectioned, stained with hematoxylin and eosin (H&E), and examined using light microscopy (Olympus). The adipocyte sizes were analyzed using Image J software.

Statistical analysis

Values are presented as mean±standard error of the mean. A normality test was applied to the data, and multiple comparisons were done using one-way analysis of variance followed by Tukey's post hoc test. $P<0.05$ was considered statistically significant. Data were analyzed using SPSS software version 25 (IBM Corp., Armonk, NY, USA).

RESULTS

Effects of RBE on body weight, adipose tissue mass, and adipocyte size

Mice fed on a HFD for 8 weeks exhibited an average increase of 22% body weight compared with control mice (Fig. 1). In contrast, after 8 weeks, mice fed with HFD supplemented with RBE had significantly lower body weight ($P<0.05$; Table 2); body weights were 12% and 15% lower in the 220 and 1,100 mg/kg/d group, respectively, compared with only HFD-fed mice. Consistent with the body weight decrease, we observed a significant decrease in the average adipose tissue mass by 28% and 35% in the HFD mice supplemented with 220 and 1,100 mg/kg/d RBE, respectively, compared with HFD only mice (Table 2).

Analysis of H&E-stained adipose tissue sections revealed that the adipocytes in both VSC and SC tissues of mice fed with HFD supplemented with 1,100 mg/kg/d RBE were significantly smaller than those fed with HFD only (Fig. 2). We confirmed that increase in fat cell size (hypertrophy) contributed to increase in adipose tissue mass in the obese mice.

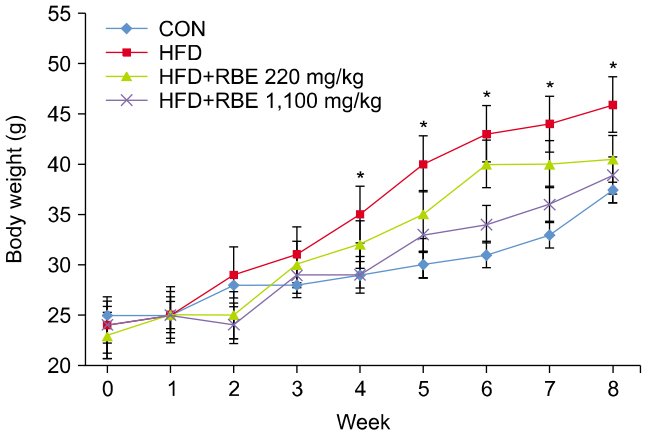

Fig. 1. Effect of rice bran extract (RBE) on body weight in high-fat diet-induced obese mice. Data are presented as mean±SEM. *$P<0.05$ vs. CON. CON, control diet; HFD, high-fat diet.

Table 2. Effect of RBE on body weight, white adipose tissue weight, food intake, and energy intake

Variable	CON	HFD	HFD+RBE 220 mg/kg	HFD+RBE 1,100 mg/kg
Initial weight (g)	25±0.5	24±0.8	23±1.2	24±0.5
Final weight (g)	37.5±0.84	45.9±0.76*	40.5±0.42*	38.9±0.56#
Food intake (g/d)	3.1±0.1	3.6±0.1	3.7±0.2*	3.8±0.5*
Energy intake (kcal/d)	11.9±0.2	18.9±0.5*	19.4±0.5*	20.0±2.6*
Adipose tissue weight (g)	0.29±0.11	0.74±0.11*	0.53±0.27*#	0.48±0.23*#

Data are presented as mean±SEM.
*$P<0.05$ vs. CON and #$P<0.05$ vs. HFD group.
RBE, rice bran extract; CON, control diet; HFD, high-fat diet.

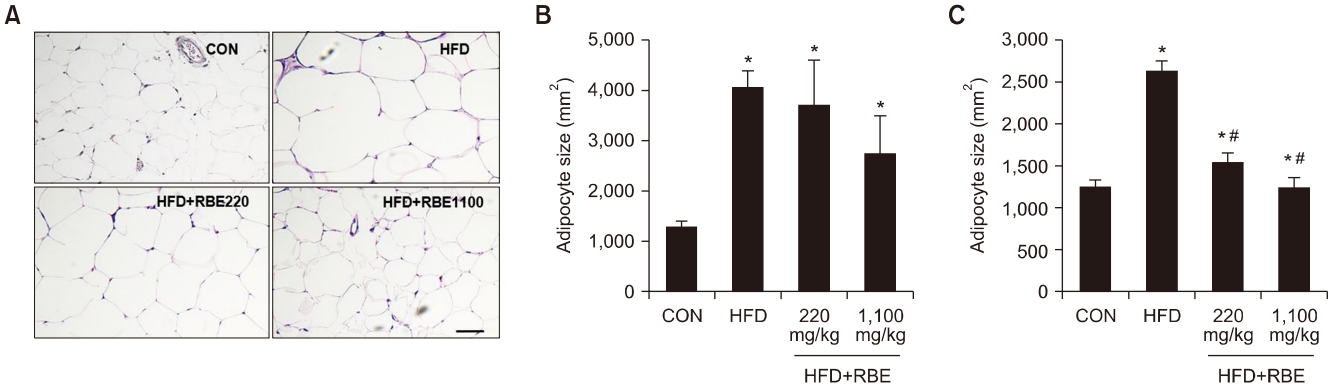

Fig. 2. Effect of rice bran extract (RBE) on the size of fat cells in high-fat diet (HFD)-induced obese mice. (A) Visceral adipose tissues were stained with hematoxylin-eosin and visualized at ×400 magnification. Length bar=50 μm. RBE was administered for 8 weeks, which reduced the fat cells. Light microscopic analysis of the adipocytes sizes (area, mm^2) in subcutaneous (B) and visceral (C) in fixed areas. Institute of Cancer Research mice were fed a control diet (CON), HFD or HFD supplemented with RBE (220 or 1,100 mg/kg) for 8 weeks. Data are presented as mean±SEM. $^*P<0.05$ vs. CON and $^{\#}P<0.05$ vs. HFD group.

Effects of RBE on mRNA expression of *Vegf*, *Mmp-2*, and *Mmp-9* in adipose tissues

In SC adipose tissues, there was significant reduced in the *Vegf* mRNA level in HFD mice. However, there was no difference between RBE-treated and untreated mice (Fig. 3A). There was no significant difference in the *Mmp-2* mRNA levels between the RBE treated and untreated HFD-fed mice (Fig. 3B). Contrastingly, *Mmp-9* mRNA levels were significantly decreased in the HFD-fed mice (Fig. 3C), while it was significantly increased, by 67% and 30%, in the mice treated with 220 and 1,100 mg/kg/d RBE, respectively. In VSC adipose tissues, consistent with the *Vegf* mRNA levels, *Mmp-9* mRNA levels were lowered by 44% and 37%, respectively, compared

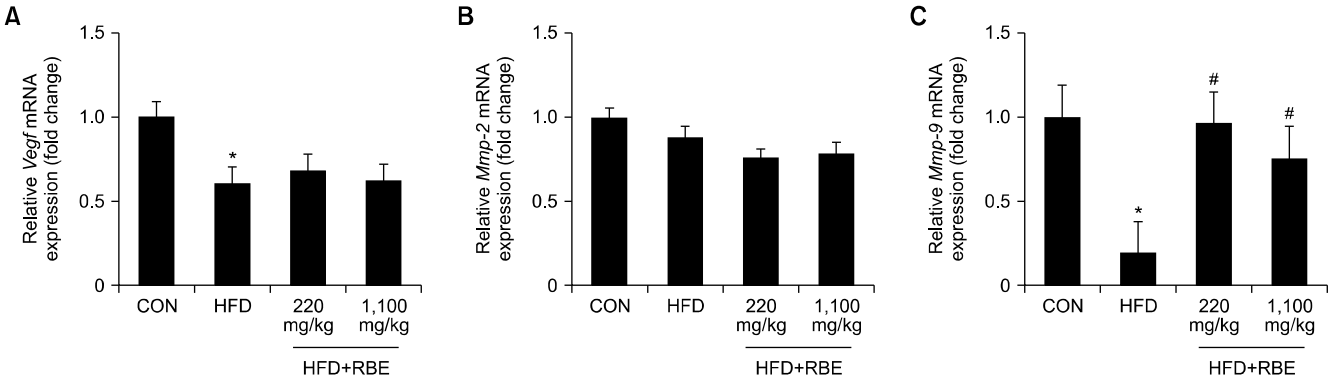

Fig. 3. Ratio of mRNA expression in subcutaneous (SC) adipose tissue. mRNA levels of *Vegf* (A), *Mmp-2* (B), and *Mmp-9* (C) in SC adipose tissue. Results are expressed as fold change over the CON group. Data are presented as mean±SEM. $^*P<0.05$ vs. CON and $^{\#}P<0.05$ vs. HFD group. CON, control diet; HFD, high-fat diet; RBE, rice bran extract.

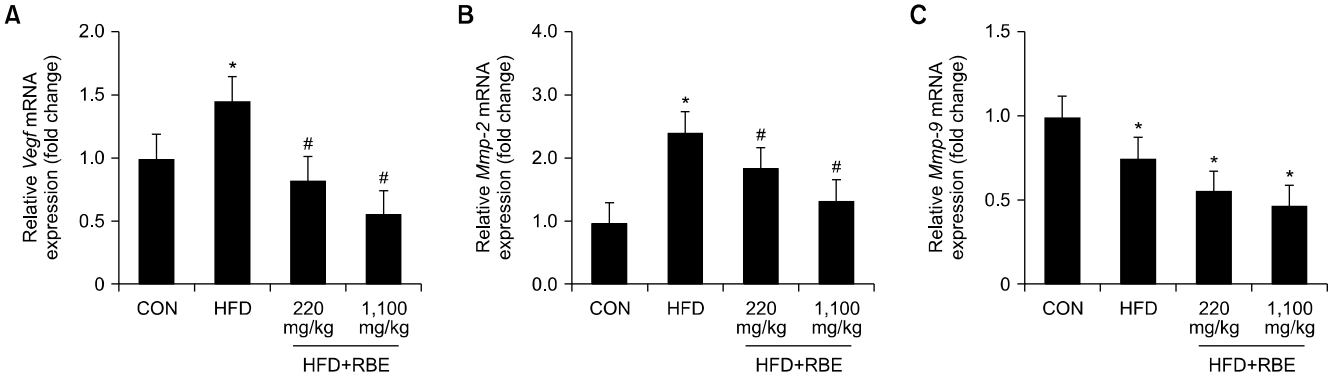

Fig. 4. Ratio of mRNA expression in visceral adipose tissue. mRNA levels of *Vegf* (A), *Mmp-2* (B), and *Mmp-9* (C) in visceral adipose tissue. Results are expressed as fold change over the CON group. Data are presented as mean±SEM. $^*P<0.05$ vs. CON and $^{\#}P<0.05$ vs. HFD group. CON, control diet; HFD, high-fat diet; RBE, rice bran extract.

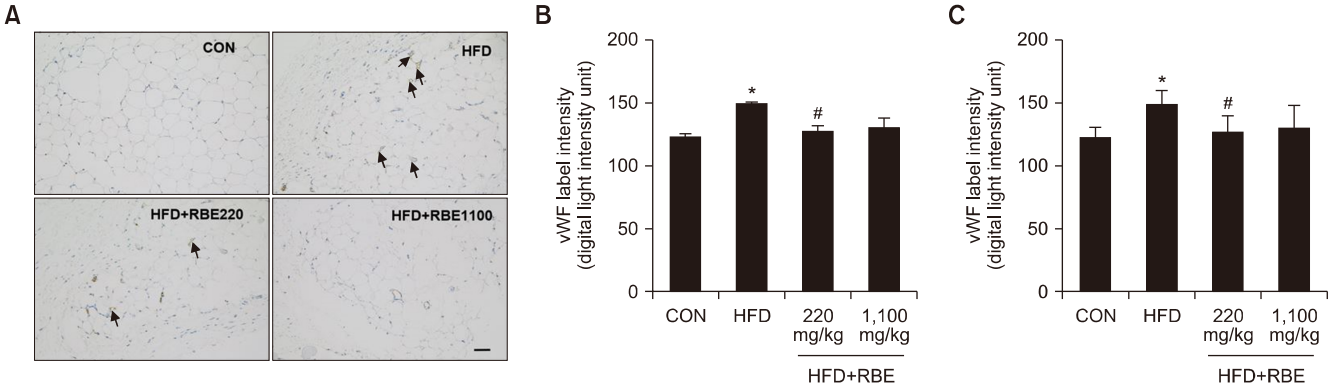

Fig. 5. (A) Immunohistological analysis of anti-von Willebrand factor (vWF) in adipose tissue (at ×200 magnification). Blood vessel density, indicated by the arrows. Length bar=50 μm. The light absorption of measurements expression as digital unit in subcutaneous (B) and visceral (C). Data are presented as mean±SEM. $^{*}P<0.05$ vs. CON and $^{\#}P<0.05$ vs. HFD group. CON, control diet; HFD, high-fat diet; RBE, rice bran extract.

to the untreated HFD group (Fig. 4A and 4C). The *Mmp-2* mRNA levels were significantly increased in HFD-fed mice by 120%, whereas in RBE treated mice, they were significantly decreased compared to HFD-untreated mice (Fig. 4B).

Effects of RBE on angiogenesis in adipose tissues

To investigate whether RBE-mediated decrease in adipose tissue mass resulted from inhibition of actual microvascular growth, we determined the blood vessel density in adipose tissue. vWF, a marker for blood vessel density, labeling in VSC and SC adipose tissue was higher in HFD-fed mice compared to the control mice. In contrast, 220 mg/kg/d RBE treatment decreased the blood vessel density in VSC adipose tissue of HFD-fed mice (Fig. 5).

DISCUSSION

Development and growth of adipose tissue are linked with angiogenesis and ECM remodeling (Lijnen et al., 2002; Rupnick et al., 2002; Cao, 2007) as they require formation of new blood vessels to provide oxygen and nutrients to adipocytes (Rupnick et al., 2002). Obesity can be reduced by inhibiting angiogenesis, which subsequently reduces adipogenesis. Several studies have demonstrated the anti-obesity effect of other rice cultivars and herbal medicines (Ho et al., 2012; Lim et al., 2016; Kim et al., 2019; Kim et al., 2021). Anthocyanin-enriched rice berry extract inhibits adipocyte formation and proliferation, and reduces the adipocyte numbers, while resulting in triglyceride accumulation in preadipocytes (3T3-L1 cells) (Kongthitilerd et al., 2020).

This current study suggests that RBE treatment can prevent obesity induced by HFD feeding by inhibiting adipogenesis and hypertrophy of adipocytes. Our data indicates that RBE could potentially be used for obesity management. Newly formed adipose tissues depend on continued angiogenesis for growth (Rupnick et al., 2002; Bråkenhielm et al., 2004). Hence, different angiogenesis inhibitors significantly reduce body weight and adipose tissue mass (Cao, 2007), indicating the involvement of angiogenesis in adipose tissue growth. Adipose tissue produces several angiogenic factors and inhibitors that regulate adipose angiogenesis. Angiogenesis factors, such as VEGF and fibroblast growth factor-2, promote proliferation and differentiation of endothelial cells within the fat tissue (Sanikommu et al., 2022) whereas thrombospondin-1 inhibits angiogenesis (Garside et al., 2010).

Serum analysis showed higher concentrations of growth factors in overweight and obese subjects compared to normal weight individuals (Silha et al., 2005). Consistently, studies using mice model reported that body weight gain and adipose tissue mass in obese animals are significantly reduced by several angiogenesis inhibitors, such as angiostatin, endostatin, TNP-470, TNP-470 analog CKD-732, and VEGF receptor 2-specific inhibitors (Bråkenhielm et al., 2004). Similarly, previous results showed that the anti-angiogenic herbal composition Ob-X reduces adipose tissue mass and body weight gain in obese mice (Kang et al., 2018), proving that adipose tissue growth and development may be prevented by inhibiting angiogenesis. Our present study showed that the blood vessel density of both VSC and SC adipose tissues were lower in RBE treated mice than in untreated obese mice. We suggest that RBE can regulate adipose tissue mass by inhibiting angiogenesis.

Adipocytes also produce MMPs and MMP inhibitors that are differentially expressed in adipose tissue in murine obese models (Bouloumié et al., 2001). A balance between MMPs and their inhibitors presumably controls adipose tissue development and maintenance. Recent studies suggested that MMPs are involved in tissue remodeling events associated with adipogenesis (Bauters et al., 2015). Moreover, endogenous and exogenous MMPs regulate adipogenesis and adipose tissue growth. MMP-2

and MMP-9 can remodel the ECMs of murine and human adipogenic cells to facilitate adipogenesis (Bouloumié et al., 2001) and regulate the bioavailability of adipocyte growth factors that are either sequestered as inactive molecules in the matrix or blocked by their binding proteins (Sadowski et al., 2003). Both synthetic and endogenous MMP inhibitors suppress angiogenic responses (Van Hul and Lijnen, 2011; Quintero-Fabián et al., 2019). Deletion of tissue inhibitor of metalloproteinase-1 (*Timp-1*), an endogenous MMP inhibitor, reduces obesity in nutritionally obese mice (Lijnen et al., 2003). Treatment of obese mice with galardin, a synthetic MMP inhibitor, significantly reduced fat mass (Lijnen et al., 2002).

MMP-2 activity is found in condition media of adipocytes, and proteases are also highly expressed in adipose tissues of obese animal models (Lijnen et al., 2001; Maquoi et al., 2002). An HFD meal involves the intestines and digestive enzymes, resulting in elevation of protease activity leaking into systemic circulation (Modestino et al., 2019). Several membrane receptors have extracellular domains that might be cleaved by proteases. They also enter the mesentery and peritoneum (Delano and Schmid-Schönbein, 2008), and therefore might elevate levels of inflammation and capillary rarefaction in the VSC tissue by cleavage of the VEGFR-2 receptor. As MMP-2 is involved in receptor cleavage in the HFD model, it clips the extracellular domain of the leptin receptor (Mazor et al., 2018), and may also clip the VEGFR receptor and thereby affect *Vegf* mRNA levels and its signaling activity. MMP-2 might cleave VEGFR2 and VEGF itself. The present study indicates that RBE treatment of HFD-induced obese mice decreased *Vegf* and *Mmp-2* mRNA levels, especially in VSC adipose tissue, which implies that RBE reduces adipose tissue mass and body weight, at least partially, by blocking angiogenesis and adipose tissue growth. RBE might specifically regulate genes involved in both angiogenesis and MMPs in adipose tissues. MMP-2 and MMP-9 indirectly stimulate angiogenesis (Bergers et al., 2000), suggesting that obesity may be regulated by the synergistic action of angiogenesis and MMPs. However, we found no effect of MMP-9 on adipose tissue development, further supporting our finding that, at least in ICR mice, adipogenesis is partially due to MMP-2 activity.

This study is consistent with a previous study which demonstrated that purple rice (*Oryza sativa* L.) extract and its constituents, cyanidin and peonidin, inhibit VEGF-induced angiogenesis (Tanaka et al., 2012). Additionally, phenolic compounds in black rice bran exhibited anti-angiogenic properties along with strong antioxidant properties (Christanto et al., 2021). Moreover, an anthocyanin-rich extract from black rice suppressed tumor growth and angiogenesis by suppressing the expression of angiogenesis factors MMP-9 and MMP-2, which has been reported in tumor tissues (Hui et al., 2010). The RBE used in this study contained phenolic compounds like gallic acid and γ-oryzanol. Our study also supports the antioxidant role of RBE, which is a rich source of γ-oryzanol, by showing its anti-obesity effect (Christanto et al., 2021). RBE might directly affect the intestine by suppressing the elevated intestinal permeability and leakage of digestive enzymes during HFD by inhibiting proteases and/or blocking inflammatory pathways.

In conclusion, our results indicate the beneficial effects of RBE, which inhibits adipose tissue growth and obesity in nutritionally induced obese mice. This effect might be partially mediated by inhibiting angiogenesis. RBE might be a potential therapeutic for controlling human obesity and its related disorders.

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AUTHOR DISCLOSURE STATEMENT

Dr. Geert W. Schmid-Schönbein is the science advisor for Leading Biosciences Inc., San Diego, California, and owns the founders stock. The other authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

ND conceptualized, conceived and designed the research. ND and US performed the experiments, and collected, analyzed data, and interpreted the study results. ND drafted the manuscript. US edited and revised the manuscript. GWSS approved the final version of the manuscript.

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