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Effects of Red Rice Bran Extract on High-Fat Diet-Induced Obesity and Insulin Resistance in Mice

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ABSTRACT: Insulin resistance is a salient player in the pathogenesis of obesity and its related abnormal glucose-insulin homeostasis. Red rice bran extract (RRBE) demonstrates several bioactive phytochemicals with anti-diabetic properties. However, little is known about its molecular mechanisms. Therefore, the present study was designed to investigate the anti-insulin resistant mechanisms of RRBE in a model of high-fat diet (HFD)-induced insulin resistance. In this study, mice were randomly divided into four groups: low-fat diet with distilled water (Group L), HFD with distilled water (Group H), HFD with 0.5 g/kg RRBE, and HFD with 1 g/kg RRBE. Metabolic parameters, histological changes in the pancreas, and gene expression levels were evaluated after treating HFD-fed mice with RRBE for six weeks. Mice from Group H exhibited significantly higher blood glucose levels prior to and after an oral glucose tolerance test, fasting serum insulin levels, islet size, pancreatic insulin expression levels, and lower skeletal muscle insulin-degrading enzyme (IDE) expression levels compared to Group L. In contrast, these were all significantly restored in the RRBE-treated groups. Also, RRBE treatment was found to upregulate the expression of insulin receptor substrate (IRS) and glucose transporter (GLUT) genes in the adipose tissues and GLUT genes in the muscles and livers of HFD-fed mice. According to our results, RRBE may ameliorate abnormal glucose-insulin metabolism by modulating the expression of insulin, IDE, IRS, and GLUT genes in the major metabolic target tissues of mice after being fed with HFD.

Keywords: hyperglycemia, hyperinsulinemia, insulin resistance, obesity, red rice bran

INTRODUCTION

Obesity and its associated metabolic complications emerged as a global epidemic (Bray et al., 2018). Insulin resistance, defined as the inability of target cells or tissues to respond to insulin stimulation, is one of the primary causes of impaired metabolic homeostasis in obesity and type 2 diabetes mellitus (T2DM) (Fazakerley et al., 2019). Notably, insulin emerged as a key hormone regulating tissue-specific and systemic energy homeostasis, particularly glucose homeostasis (Petersen and Shulman, 2018). In pancreatic islet β-cells, the upregulation of the insulin gene in response to various stimuli, especially elevated blood glucose levels, leads to the production of insulin and its subsequent release into the bloodstream (Prentki and Nolan, 2006; Vasiljević et al., 2020). In general, insulin binds to the insulin receptor (INSR) on the target cells' cell membrane to phosphorylate insulin receptor substrates (IRS), such as IRS1 and IRS2, resulting in the activation of downstream mediators and subsequent propagation of the insulin response, including stimulation of glucose transporter (GLUT)4-mediated glucose uptake in muscle cells and adipocytes, activation of glycogen synthesis, and inhibition of gluconeogenesis in hepatocytes (Petersen and Shulman, 2018). Furthermore, GLUT2 functions as a glucose sensor in hepatocytes and plays a key role in glucose-insulin homeostasis by responding to changes in glycemia (Thorens, 2015). Accordingly, the impaired insulin-regulated glucose uptake in key metabolic target tissues, including adipose tissue, skeletal muscle, and liver, has been observed in the pathogenic mechanisms of insulin resistance and its con-

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sequences, such as hyperglycemia, glucose intolerance, and hyperinsulinemia (Petersen and Shulman, 2018; Fazakerley et al., 2019). In addition to the compensatory β-cell hypersecretion in an insulin-resistant state, decreased insulin-degrading enzyme (IDE) activity or expression levels and a resultant reduction in insulin clearance also contribute to the development of hyperinsulinemia (Najjar and Perdomo, 2019; González-Casimiro et al., 2021). Therefore, improving insulin signaling, glucose uptake, and insulin clearance pathways can be used as a potential preventive or therapeutic strategy for obesity and its complications.

Red-colored rice varieties (*Oryza sativa* Linn.) and their bran extracts contain several bioactive compounds that reportedly possess antioxidant (Surarit et al., 2015), anti-inflammatory (Limtrakul et al., 2016), and anti-diabetic properties (Boue et al., 2016; Tantipaiboonwong et al., 2017). Consistent with our preliminary study, a previous *in vitro* study showed that the bran extract of red rice (Red Hawm or Hawm Dowk Mali Deang) exhibits the highest antioxidant activities and total phenolic contents among all the rice bran extracts (Surarit et al., 2015). In addition to its antioxidant capacity, treatment with red rice bran extract (RRBE) markedly increased glucose uptake associated with the upregulated expression of insulin-signaling components and GLUTs in adipocytes (Boue et al., 2016), suggesting that RRBE treatment may prevent the development of insulin resistance in insulin-sensitive cells or tissues. However, its molecular mechanism in ameliorating obesity-related insulin resistance has not yet been fully elucidated, particularly *in vivo*. Therefore, in the present study, we investigated the anti-insulin-resistant effects and mechanisms of action of RRBE in high-fat diet (HFD)-fed mouse models, which mimic the important features of obesity and insulin resistance in humans.

MATERIALS AND METHODS

Preparation of RRBE

One thousand grams of red bran from Red Hawm rice variety (Mae Chai Agricultural Cooperative Ltd., Phayao, Thailand) was mixed in 6,000 mL of 50% ethanol solution for three days at approximately 25°C. Then, the extractant solution was filtered through Whatman filter paper number 1 (GE Healthcare UK Ltd., Buckinghamshire, UK) using vacuum filtration apparatus. After filtration, the sample residue was re-extracted twice, following the same procedure, and the collected filtrates were concentrated in a rotary evaporator (R-100, BUCHI Labortechnik AG, Flawil, Switzerland). Next, the concentrated extracts were lyophilized to obtain RRBE, which was finally stored at −20°C prior to further experiments. The extraction yield of RRBE was 7.59%.

Induction of obesity and insulin resistance in mice

Twenty-four male Institute of Cancer Research (ICR) mice (four weeks old; Nomura Siam International Co., Ltd., Bangkok, Thailand) were housed in a standard environment (22±25°C, 60% humidity, 12-h light/dark cycle). Following the seven-day acclimation, six mice were fed a low-fat diet (LFD; 10% kcal from fat, Research Diets Inc., Brunswick, NJ, USA) (Group L) throughout the duration of the study. The remaining mice were fed an HFD (45% kcal from fat, Research Diets Inc.) for six weeks to induce obesity and hyperglycemia, a sign of insulin resistance. When the body weights and fasting blood glucose (FBG) levels of the HFD-fed mice were significantly different from their LFD-fed counterparts, they were considered obese and hyperglycemic. Subsequently, the HFD-fed mice were randomly assigned to the following groups (n=6/group), according to their body weight and FBG levels, and were orally administered different doses of RRBE daily for six weeks: HFD+ distilled water group (Group H), HFD+0.5 g/kg RRBE group (Group HR0.5), and HFD+1 g/kg RRBE group (Group HR1). Mice in the H group were used as the obese insulin resistant group, while mice in the L group served as the control group. The induction of obesity-associated insulin resistance by HFD-feeding in ICR mice has been previously reported (Naowaboot et al., 2018a; Naowaboot et al., 2018b). The body weights of the animals were evaluated on a weekly basis. The animals were fasted overnight and were euthanized using inhalant isoflurane for blood and tissue collection after 12 weeks. The blood and tissue samples were used for determining glucose-insulin homeostasis parameters and for histological and gene expression analyses (see below). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Phayao, Phayao, Thailand (no. 59 01 04 0037).

Determination of blood glucose-insulin homeostasis parameters

At baseline (0-week), after the mice were fasted overnight (15 h), their FBG levels were determined from a tail tip bleed using a glucometer (Roche Diabetes Care GmbH, Mannheim, Germany) after 6 weeks and after 12 weeks. On day 81 of the experiment, oral glucose tolerance test (OGTT) was performed on all the mice after fasting for 15 h with a three-day interval for recovery. Blood samples were collected via tail prick at 0 (before glucose load), 30, 60, 90, and 120 min after oral administration of 50% glucose solution (2 g/kg body weight). Blood glucose was measured using a glucometer, and the area under the curve (AUC) for blood glucose was calculated. After sacrifice, fasting serum insulin was determined using the rat/mouse insulin enzyme-linked immunosorbent assay kit (Merck KGaA, Darmstadt, Germany), in accordance

with the manufacturer's protocol.

Histopathological analysis of pancreatic tissue

At the end of the study, the pancreas was weighed, immediately placed in 10% neutrally buffered formalin, embedded in paraffin, cut into 5-μm thick sections using a microtome, and stained with hematoxylin and eosin. Representative images were captured at ×100 and ×400 magnification using a light microscope (Olympus BX53, Olympus Corp., Tokyo, Japan) equipped with a digital camera (Olympus DP21, Olympus Corp.). The area of the pancreatic islets was measured in ×100 images using AxioVision AC microscopy software (Carl Zeiss, Jena, Germany).

Gene expression analysis

For total RNA isolation, frozen tissue samples of the pancreas, epididymal adipose tissue, skeletal muscle, and liver were homogenized in TRIzol reagent (Ambion by Life Technologies, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. Using the High-Capacity Complementary DNA (cDNA) Reverse Transcription kit (Applied Biosystems™, Thermo Fisher Scientific Inc., Waltham, MA, USA), 800 ng total RNA was reverse-transcribed into cDNA. Real-time polymerase chain reaction (RT-PCR) was performed using TaqMan Gene Expression assays and Master Mix (Applied Biosystems™) with the CFX96 Touch RT-PCR System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The expression levels of each target mRNA were normalized to the levels of the reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and were calculated using the $2^{-\Delta\Delta CT}$ method. The TaqMan probes (Applied Biosystems™) used for the gene expression assays were as follows: insulin (Mm00731595_gH), IDE (Mm00473077_m1), INSR (Mm01211875_m1), IRS1 (Mm01278327_m1), IRS2 (Mm03038438_m1), GLUT2 (Mm00446229_m1), GLUT4 (Mm00436615_m1) and GAPDH (Mm99999915_g1).

Statistical analysis

IBM SPSS software version 20 (IBM Corp., Armonk, NY, USA) was used to perform all statistical analyses. Values are presented as the mean±standard error of the mean of the six mice in each group for each metabolic parameter, and n=4 for the histological and gene expression analyses. The statistical significance among the four groups evaluated by one-way analysis of variance (ANOVA) and Tukey's post hoc test was considered at $P<0.05$.

RESULTS

Effect of RRBE on the general characteristics and systemic glucose-insulin homeostasis of HFD-fed mice

No significant differences were found in the week 0 values of body weights and FBG (Fig. 1A and 1B, respectively) between the groups. Body weights were significantly higher in Groups H, HR0.5, and HR1 than in Group L after six and 12 weeks, but no differences were noted between Group H and the RRBE-treated groups (Fig. 1A). In addition, no significant difference was seen between the caloric intake of Group H and the RRBE-treated groups (data not shown). The group fed with HFD alone showed significant increases not only in FBG compared to the group fed with LFD at 6 and 12 weeks (Fig. 1B) but also in blood glucose levels at 0, 30, 60, 90, and 120 min and in the corresponding AUC during the OGTT (Fig. 1C and 1D, respectively). FBG, blood glucose levels at all-time points, and AUC of glucose were significantly decreased in both Groups HR0.5 and HR1 compared with Group H.

Non-treated HFD-fed mice were found to exhibit significantly greater fasting serum insulin levels compared to LFD-fed and RRBE-treated mice (Fig. 2A). The percentages of pancreas weight/body weight were not significantly different between the four groups of mice (Fig. 2B); however, pancreases from untreated HFD-fed mice exhibited higher islet size compared to LFD-fed and RRBE-treated mice (Fig. 2C and 2D). Insulin mRNA levels were significantly upregulated in the pancreas (Fig. 2E), while IDE mRNA levels were significantly downregulated in skeletal muscles (Fig. 2F) from HFD-fed mice compared with LFD-fed mice. In contrast, RRBE treatment significantly downregulated the pancreatic mRNA expression of insulin but upregulated muscle IDE mRNA expression in HFD-fed mice, compared to mice fed with HFD alone.

Effect of RRBE on the expression of genes involved in insulin signaling and glucose uptake in white adipose tissue, skeletal muscle and liver

IRS1, IRS2, and GLUT4 mRNA levels were significantly decreased in both epididymal adipose tissues (Fig. 3A) and gastrocnemius muscles (Fig. 3B) of HFD-induced insulin-resistant mice compared with LFD-fed mice. Meanwhile, no change was found in the expression of INSR mRNA in these tissues. Liver tissues from mice fed with a HFD demonstrated significantly reduced expression levels of INSR, IRS1, IRS2, and GLUT2 mRNA compared with control mice (Fig. 3C). Adipose tissue from HFD-fed mice treated with 0.5 and 1 g/kg RRBE demonstrated significantly higher IRS1, IRS2, and GLUT4 mRNA levels than mice in Group H, with INSR mRNA levels remaining unchanged. GLUT4 mRNA levels in skeletal muscle and GLUT2 mRNA levels in the liver were significantly

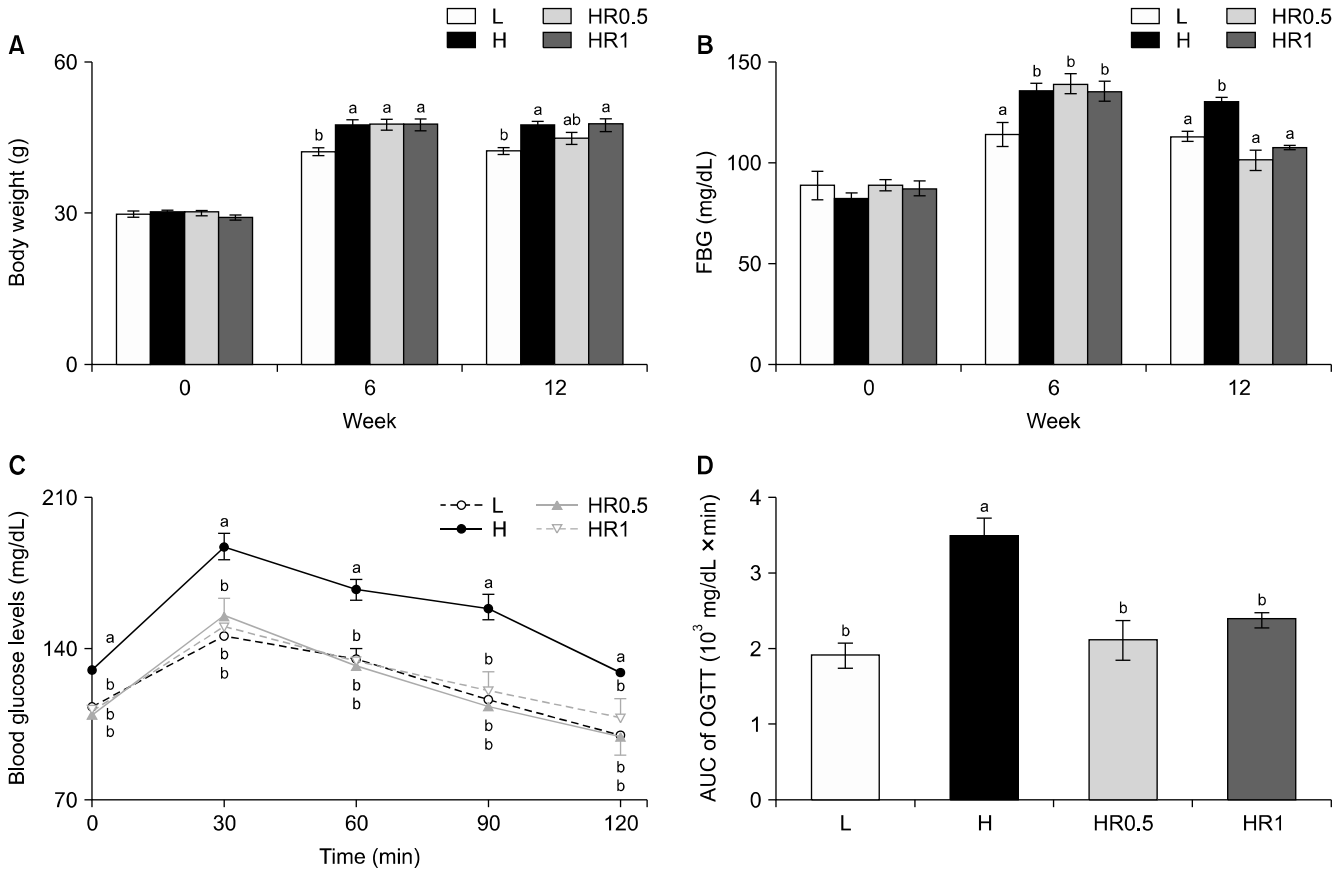

Fig. 1. Effect of red rice bran extract (RRBE) on body weight (A), fasting blood glucose (FBG) (B), blood glucose concentrations (C), and area under the curve (AUC) of the oral glucose tolerance test (OGTT) (D) in high-fat diet (HFD)-induced insulin-resistant mice. Values are denoted as mean±SEM (n=6). Different letters (a,b) indicate a significant difference at $P<0.05$. L, low-fat diet with distilled water; H, HFD with distilled water; HR0.5, HFD with 0.5 g/kg RRBE; HR1, HFD with 1 g/kg RRBE.

increased in both treatment groups, compared to Group H, without changes in INSR, IRS1, and IRS2 levels.

DISCUSSION

Animal models of HFD-induced obesity are characterized by a state of insulin resistance at both the tissue and systemic levels that play a central role in the dysregulation of glucose and insulin metabolism, resulting in various obesity-related chronic diseases, such as T2DM and cardiovascular diseases (Naowaboot et al., 2018a; Naowaboot et al., 2018b). Consistent with previous findings (Hansotia et al., 2007; Naowaboot et al., 2018a; Naowaboot et al., 2018b; González-Casimiro et al., 2021), HFD-fed mice exhibited signs of obesity-related insulin resistance, including hyperglycemia, glucose intolerance, hyperinsulinemia, enlarged islet size, altered expression of insulin metabolism-associated genes in the pancreas and skeletal muscle, and decreased expression of insulin signaling and glucose uptake genes in adipose tissues, skeletal muscles, and the liver. These, in turn, indicated successful insulin resistance induction. Therefore, this animal model is appropriate for investigating the molecular mechanisms underlying the anti-insulin-resistant effects of RRBE.

Dysregulation of insulin metabolism in obesity and T2DM results from the combination of resistance to insulin action, inappropriate insulin secretion, and defective insulin clearance, culminating in hyperinsulinemia (Hansotia et al., 2007; Naowaboot et al., 2018a; Naowaboot et al., 2018b; González-Casimiro et al., 2021). Initially, the pancreas responds to hyperglycemia or insulin resistance by increasing insulin biosynthesis and secretion from β-cells due to upregulated insulin expression and by expanding β-cell mass to maintain homeostatic glucose levels, resulting in hyperinsulinemia (Prentki and Nolan, 2006; Vasiljević et al., 2020). In addition, hyperinsulinemia can be attributed to defective insulin clearance via downregulation of IDE, a key enzyme involved in the breakdown of insulin into several fragments in skeletal muscle cells and other cells (Najjar and Perdomo, 2019; González-Casimiro et al., 2021). The sustained increase in blood insulin and glucose may lead to insulin resistance, which constitutes a vicious cycle of metabolic dysregulation in the pathogenesis of obesity and diabetes (Hudish et al., 2019). Therefore, blood glucose, insulin, and islet integrity are common parameters for evaluating the efficacy insulin-resistant glucose metabolism inter-

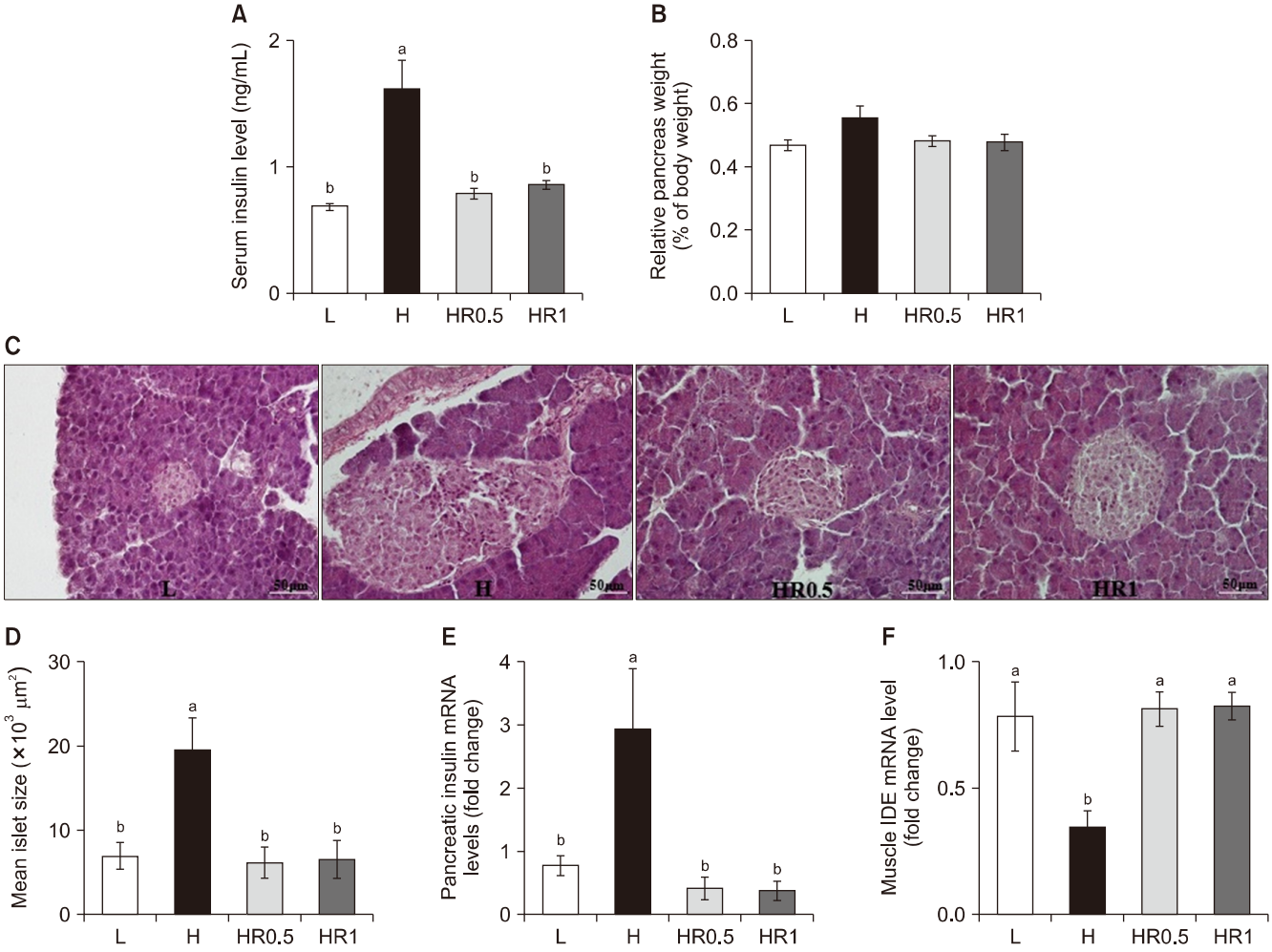

Fig. 2. Effect of red rice bran extract (RRBE) on fasting serum insulin (A), pancreas weight (B), pancreas histology (hematoxylin and eosin staining, magnification ×400, scale bar=50 μm) (C), pancreatic islet size (D), pancreatic insulin (E), and skeletal muscle insulin-degrading enzyme (IDE) mRNA expression (F) in high-fat diet (HFD)-induced insulin-resistant mice. Values are denoted as mean±SEM (n=4~6). Different letters (a,b) indicate a significant difference at $P<0.05$. L, low-fat diet with distilled water; H, HFD with distilled water; HR0.5, HFD with 0.5 g/kg RRBE; HR1, HFD with 1 g/kg RRBE.

ventions. Upon intervention with RRBE, FBG, and AUC values representing glucose tolerance were markedly reduced compared to non-treated HFD-fed mice, demonstrating its glucose-lowering and glucose disposal capacities, respectively. Consistent with these results, RRBE-fed mice demonstrated significantly reduced serum insulin levels, which were accompanied by significantly smaller islets of Langerhans, decreased pancreatic insulin gene expression, and increased muscular IDE gene expression compared to HFD-fed mice, indicating improved insulin metabolism. We concluded that RRBE exerts its beneficial effects by modulating the expression of insulin and IDE genes due to its anti-hyperglycemic and anti-hyperinsulinemic activities, which eventually improves glucose-insulin homeostasis. Concurrently, Tantipaiboonwong et al. (2017) demonstrated that red rice extract, which contains phenolic compounds, flavonoids, proanthocyanidins, and other bioactive compounds, effectively lowered hyperglycemia in streptozotocin-induced diabetic rats. Recent *in vitro* studies also documented that rice bran and colored rice phenolic extracts enhanced β-cell function by modulating insulin expression and secretion (Kang et al., 2020; Saji et al., 2020). Our preliminary phytochemical screening of RRBE confirmed the findings of these studies and demonstrated the presence of phenolics and flavonoids (unpublished results). Thus, it could be inferred that these compounds might be responsible for the potential benefits of the extract.

Next, the multifactorial pathogenic mechanisms of insulin resistance in metabolic syndrome are associated with impaired insulin signaling and gene expression, especially in key metabolic target tissues (Petersen and Shulman, 2018; Fazakerley et al., 2019). In insulin-resistant animal models and human subjects, the expression of insulin-signaling components, such as INSR, IRS, and GLUTs, were significantly decreased in adipose tissues (Arcidiacono et al., 2020), skeletal muscles (Qi et al., 2016), and livers (Wang et al., 2016). This indicates that the downregulation of insulin-signaling genes could be the cause of insulin resistance and glucose dysregulation.

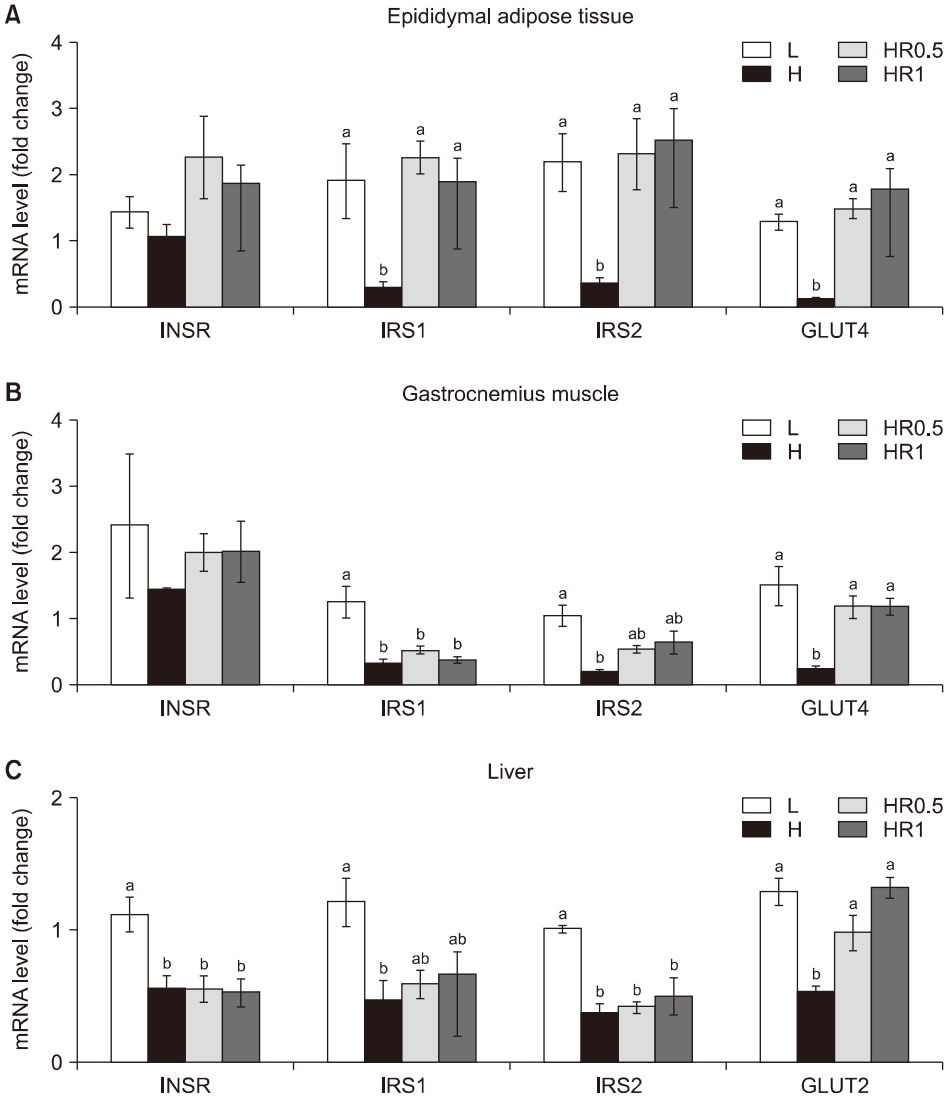

Fig. 3. Effect of red rice bran extract (RRBE) on the expression of genes associated with insulin signaling, namely, insulin receptor (INSR), insulin receptor substrate (IRS) 1, and IRS2, and glucose uptake, namely, glucose transporter (GLUT) 2 and GLUT4, in epididymal adipose tissue (A), gastrocnemius muscles (B), and liver (C) of high-fat diet (HFD)-induced insulin-resistant mice. Values are denoted as mean±SEM (n=4). Different letters (a,b) indicate a significant difference at $P<0.05$. L, low-fat diet with distilled water; H, HFD with distilled water; HR0.5, HFD with 0.5 g/kg RRBE; HR1, HFD with 1 g/kg RRBE.

RT-PCR was performed for insulin signaling and glucose uptake genes in the key insulin-sensitive tissues of obese mice to identify the molecular mechanisms responsible for the above-mentioned anti-insulin-resistant effects of RRBE. In this study, significantly lower adipose tissue IRS1, IRS2, and GLUT4 transcript levels were associated with the aforementioned systemic insulin resistance in HFD-fed mice. These were effectively reversed by RRBE administration. Furthermore, treatment with RRBE significantly reversed the HFD-induced reduction of GLUT4 and GLUT2 expression in skeletal muscles and livers, respectively. Taken together, we speculate that RRBE administration alleviates HFD-induced disturbances in glucose-insulin homeostasis, probably by increasing IRS and GLUT gene transcription. A major function of GLUT is to regulate the uptake of glucose across cell membranes. Additionally, hepatic GLUT2 functions as a glucose sensor, with its inactivation or deficiency contributing to the impairment of glucose-stimulated insulin secretion and glucose homeostasis (Thorens, 2015). RRBE increased GLUT2 expression in the liver, which suggests that RRBE may enhance hepatic glucose sensing to maintain glucose and insulin homeostasis under the HFD condition. However, INSR gene expression in all investigated tissues and IRS gene expression in both muscle and liver were not significantly affected by RRBE treatment, indicating that these genes are not involved in the improved insulin sensitivity in this study model. Many factors, such as hyperinsulinemia and reactive oxygen species, reportedly promoted insulin resistance in peripheral target cells or tissues (Fazakerley et al., 2019). For instance, chronic insulin treatment reduces not only INSR and GLUT4 expression in adipocytes (Thomson et al., 1997; Srivastava et al., 2018) and muscle cells (Yang et al., 2012; Turner et al., 2018) but also INSR and IRS1 expression in hepatoma cells (Yuan et al., 2002). These observations suggest that hyperinsulinemia could possibly be the cause or consequence of impaired insulin signaling. Therefore, the upregulation of insulin-signaling genes after treatment with RRBE is hypothesized to be attributed to its anti-hyperinsulinemic effects, which, in turn, may attenuate systemic insulin resistance and hyperinsulinemia. In line

with the previous study, RRBE treatment could promote glucose uptake in 3T3-L1 adipocytes and upregulate insulin signaling pathway-related genes, such as IRS1 and GLUT4 (Boue et al., 2016). This study suggested that the anti-diabetic effect could be attributed to its phenolic compounds, especially proanthocyanidins. Also, another study reported that procyanidins increased the amount of GLUT4 in the plasma membrane of both adipocytes (Pinent et al., 2004) and muscles (Yamashita et al., 2016) and that proanthocyanidins improved insulin sensitivity in the livers of rats fed with a high-fat fructose diet by activating the insulin-signaling pathway (Yogalakshmi et al., 2014). Moreover, treatment of β-cells with rice bran and red rice phenolic extracts increased the expression of GLUT2, resulting in increased β-cell function (Kang et al., 2020; Saji et al., 2020). Other rice bran compounds, such as ferulic acid and γ-oryzanol, have also been observed to demonstrate anti-diabetic activities, and the presence of these compounds in the RRBE cannot be ruled out (Mattei et al. 2021; Pereira et al., 2021). In the present study, the weight of the main tissues and organs, such as the adipose tissue and liver, were not significantly different in the RRBE-treated and HFD-fed groups (data not shown). Therefore, RRBE administration did not significantly decrease HFD-induced obesity in mice, indicating that its beneficial metabolic effects were not associated with changes in body weights.

In conclusion, RRBE treatment improves glucose-insulin homeostasis in HFD-induced insulin-resistant mice. The underlying mechanisms are likely to be associated with modulated expression of genes involved in insulin metabolism (insulin and IDE), insulin-signaling pathway (IRS1, IRS2 and GLUT4), and glucose sensing (GLUT2). However, conducting more studies in the future is necessary to determine the specific bioactive compounds in RRBE and to elucidate its molecular mechanisms at the posttranscriptional and/or functional levels, as well as other mechanisms involved in metabolic diseases and their associated complications.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: NM, JT, CT. Analysis and interpretation: NM, ATU, SP. Data collection: NM, ATU, SP. Writing the article: NM, ATU, SP, PH, NS, JN. Critical revision of the article: NM, PH, NS, JN. Final approval of the article: all authors. Statistical analysis: NM, ATU, SP. Obtained funding: NM. Overall responsibility: NM.

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