International Food Research Journal 20(2): 873-882 (2013)
Journal homepage: *http://www.ifrj.upm.edu.my*

Effect of Riceberry oil (deep purple oil; *Oryza sativa* Indica) supplementation on hyperglycemia and change in lipid profile in Streptozotocin (STZ)-induced diabetic rats fed a high fat diet

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Article history

Received: 29 June 2012
Received in revised form: 13 September 2012
Accepted: 17 September

Keywords

Riceberry oil
diabetes
GLUT 4
blood glucose
lipid profile
rat
rice bran extract

Abstract

Riceberry oil (RBBO) has been described as having a high level of antioxidants and functional properties. The aim of the present study was to determine the effects of RBBO on changes in blood glucose, insulin levels, and GLUT4 transporter as well as lipid profiles in Streptozotocin (STZ) - induced hyperglycemic rats fed a high-fat diet. Seventy male Sprague-Dawley rats, aged six weeks and weighing 196.09 ± 10.46 g, were randomly divided into two groups: the first group of 20 rats was fed with a basal diet and another group of 50 rats were fed with a high fat (HF) diet. After two weeks, rats fed the HF-diet were induced to hyperglycemia by two doses of STZ injections (20 and 30 mg/kg; i.p.). Normal rats were divided into two groups: one group fed with basal diet (NC) and another group fed with basal diet with the oil source replaced with 5% RBBO (NR). Diabetic rats were randomized into five groups of 10 rats each as follows: untreated diabetic rats (DMC) fed a high fat (HF) diet alone; and 4 treated groups fed with high fat + 5% RBBO (DMRL); HF + 7.5% RBBO (DMRM); HF + 15% RBBO (DMRH); and HF + metformin 300mg/kg BW (DM-MET), respectively. All rats were given free access to their diet and water for 12 weeks. After 12 weeks of supplementation, significant improvement of blood glucose, insulin, HbA1C, intraperitoneal glucose tolerance and GLUT 4 transporter level were observed in the RBBO supplemented groups compared to the DMC group. Significant reductions in TC, LDL-cholesterol, TG and TG/HDL ratio were also shown in rats fed with RBBO when compared to those of diabetic rats. Findings in the present study demonstrate that RBBO, a nutraceutical food, may be useful as an alternative food supplement for the alleviation of hyperglycemia and dyslipidemia conditions.

Introduction

The prevalence of chronic non-communicable diseases is rapidly emerging as a serious public health problem (Amos *et al*., 1997; Hossain *et al*., 2007). At least 170 million globally have diabetes and this figure probably will become more than double by 2030 (Wild *et al*., 2004). Type 2 diabetes mellitus (T2DM) is a chronic disease characterized by absolute insufficiency of insulin action and abnormalities in insulin production or insulin resistance (Alberti *et al*., 1998). The disorders in carbohydrate, fat and protein metabolism in diabetes contribute to the insufficiency of insulin on target cells (Expert Committee, 2004; Kuzuya *et al*., 2002). The chronic hyperglycemia is associated with adverse effects resulting in the dysfunction and failure of various organs, especially the microvascular and nervous systems leading to long-term complications in the retina, kidney, brain and central nervous system (Alberti *et al*., 1998; Sahin *et al*., 2007; Kuhad *et al*., 2009).

Rice bran is a byproduct from the rice milling process and a good source of vitamins, minerals and other bioactive compounds (Nakornriab *et al*., 2008). Riceberry oil used in this study was obtained from the bran part of a new breeding line named "Riceberry rice" (deep purple grain; *Oryza sativa* L.), a cross-bred strain from the Chao Hom Nin Rice variety which is well known as containing high antioxidant properties and KhaoDawk Mali 105 which is well known as a fragrant rice. Riceberry oil was obtained from the extraction of the bran part from the Riceberry grain

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variety by a cold pressure technique; it contains high antioxidant contents such as γ-oryzanol, α-tocopherol, γ-tocotrienol, β-carotene, lutein, co-enzyme Q10 (Vanavichit and Kongkachuichai, 2007), phenolic compounds, quercetin, isorhamnetin and other active compounds (Nakornriab *et al*., 2008; Leardkamolkarn *et al*., 2011). Several studies have indicated that rice bran oil (RBO) contains tocotrienols, ferulic acids and phenolic acid fractions and that supplementation of RBO to diabetic rats could significantly improve serum total cholesterol (TC), TC/HDL-C ratio, non-esterified fatty acid (NEFA), triglycerides (TG), very low density lipoprotein (VLDL), and increased area under the curve of insulin, as well as improve glycemic control and reduce postprandial glycemia (Chou *et al*., 2009; Kuhad *et al*., 2009; Kuhad and Chopra, 2009; Siddiqui *et al*., 2010; Choi *et al*., 2010; Judy *et al*., 2010). There has been a suggestion that γ-oryzanol and ferulic acid in RBO have a strong effect against LDL oxidation and that α-tocopherol could inhibit lipid peroxidation (Mäkynen *et al*., 2010). In addition, γ-oryzanol and γ-tocotrienol in RBO suppress hyperlipidemic and hyperinsulinemic response in diabetic rats (Chen and Cheng, 2006). Few studies have determined the effect of RBO in diabetic rats (Hsieh *et al*., 2005). Since there has not been a previous report to demonstrate the potential effect of Riceberry oil consumption on glycemic control and dyslipidemia, therefore, this study aimed to investigate the effects of consumption of cold pressed Riceberry oil on hyperglycemia, lipid profiles, and skeletal muscle glucose transporter 4 (GLUT4) protein expression in Streptozotocin (STZ) - induced diabetic rats fed a high fat (HF) diet.

Materials and Methods

Preparation of Riceberry oil (RBBO)

Riceberry oil (RBBO) was obtained from cold press extraction of deep purple bran of the Riceberry rice variety. Analysis methods of phytochemical contents and antioxidant activity of deep purple oil (RBBO) and other refined rice bran oil used in the present study were: vitamin E by following the method of Amaral *et al*. (2005); γ-tocotrienol by following the method of Narayan *et al*. (2006); β-carotene and lutein by following the method of Speek *et al*. (1985); polyphenol by following the method of Brune *et al*. (1991); co-enzyme Q10 by following the method of Okamoto *et al*. (1988); and ORAC activity by following the method of Prior *et al*. (2003). The analysis data revealed that RBBO contains greater amounts of phytochemical contents and antioxidant activity than the refined rice bran oil such as α-tocopherol and γ-tocotrienol (10.84 and 42.81 vs 9.47 and 25.18 mg/100g), γ-oryzanol (2.07 vs 0.46 g/100g), β-carotene and lutein (0.43 and 1.56 mg/100g vs not detected). While co-enzyme Q10 in RBBO was 2.00 mg/100g and the refined rice bran oil was 1.52 mg/100g (Jarathsuthitham, 2004). Polyphenol content and ORAC activity of RBBO were 49.90 mg/100g and 68,000 μmole TE/100g while there were no data of these contents available for other rice bran oil.

Animals and diet

All protocols of animal experiments in the present study were approved by the Experimental Animal Care and the Animal Ethics Committee, Faculty of Science, Mahidol University, Thailand. Seventy male Sprague-Dawley rats aged 5 weeks with average body weight of 145.36 ± 9.89 grams were purchased from The National Laboratory Animal Centre, Salaya Campus, Mahidol University, Nakorn-Pathom, Thailand. Rats were individually housed in stainless steel cages in an air-conditioned room (23±2°C, 40-50% relative humidity) with 12/12-hour light/dark cycle controlled room at the Laboratory Animal Unit, Faculty of Science, Payathai Campus, Mahidol University, Thailand. All the animals were provided free access to a commercial pellet diet (C.P., Thailand) and water ad libitum for 1 week in order to facilitate an adaptation to a new environment. The composition of the basal diet and the experimental diets formulated in conformity with AIN-76 (Reeves, 1997) are shown in Table 1.

Induction of diabetes in rats

All rats, 6 weeks of age with average body weight of 196.09 ± 10.46 grams, were randomly divided into two groups; the first group of 20 rats was fed with basal diet and another group of 50 rats was fed with a high fat (HF) diet for 2 weeks. The high fat diet (HF) was a modified AIN-76 diet containing 200 g of casein, 30 g of corn starch, 107.5 g of sucrose, 50 g of cellulose, 280 g of corn oil which provided 67% of total energy. DL-methionine, vitamin and mineral mixtures were added as described in AIN-76 (Reeves, 1997). After two weeks of feeding with the high fat diet (HF), rats were deprived of food for 12-16 h and induced to diabetes condition by two doses of intra-peritoneal (i.p) injection of Streptozotocin (STZ) in 10.0 mM citrate buffer (pH 4.5) at 20 and 30 mg/kg body weight twice in a week period as modified from the Zhang method (Zhang *et al*., 2010). Non-diabetic rats (normal group) were injected only with citrate buffer. After one week of STZ injection, diabetic rats were confirmed by determination of non-fasting

Table 1. Composition of basal and experimental diet for normal and STZ-induced diabetic rats

Ingredients	Normal control (NC)	Normal +5%RBBO (NR)	Diabetes control (DMC)	Diabetes+5 % RBBO (DMR_L)	Diabetes+7.5 %RBBO (DMR_M)	Diabetes+15% RBBO (DMR_H)	Diabetes+ Metformin (DM-MET)
Casein	20.0	20.0	20.0	20.0	20.0	20.0	20.0
[c]Corn starch	15.0	15.0	3.0	3.0	3.0	3.0	3.0
[c]Sucrose	50.0	50.0	10.8	10.8	10.8	10.8	10.8
Fiber (Cellulose)	5.0	5.0	5.0	5.0	5.0	5.0	5.0
DL-methionine	0.3	0.3	0.3	0.3	0.3	0.3	0.3
[a]Mineral mixture	3.5	3.5	3.5	3.5	3.5	3.5	3.5
[b]Vitamin mixture	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Corn oil	5.0	-	28.0	23.0	20.5	13.0	28.0
Riceberry oil	-	5.0	-	5.0	7.5	15.0	-
Water	-	-	28.2	28.2	28.2	28.2	28.2
[d]Metformin	-	-	-	-	-	-	300 mg/kg body weight rat
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[a]AIN-76 mineral mixture obtained from MP Biomedicals, LLC, lllkirch, France. [b]AIN-76 vitamin mixture obtained from MP Biomedicals, LLC, lllkirch, France. [c]Sucrose and corn starch obtained from Nichiku Medical Industries,Ltd.,Yokohama, Japan. [d]Meformin obtained from Merck Sorono,London,UK

blood glucose (NFBG) from the tail vein of each rat using a portable glucometer (Accu-chek Performa®, Roche Diagnostics Ltd.,Thailand). Rats with NFBG values ≥ 300 mg/dL were considered diabetic (Islam *et al*., 2008).

Experimental design

The STZ-induced diabetic rats fed the high fat diet were randomly assigned into five groups of 10 rats each with approximate mean body weight 326.44 ± 21.43 grams and non-fasting blood glucose value 384.98 ± 45.54 mg/dL, while non-diabetic rats (control) were randomly assigned into two groups of 10 rats each with approximate mean body weight 323.29 ± 22.52 grams and non-fasting blood glucose value 122.70 ± 17.12 mg/dL. Two groups of non-diabetic rats were fed the basal diet containing 5% lipid from corn oil (NC) or 5% Riceberry oil (NR). The STZ-induced diabetic rats were continuously fed with the high fat diet. While the untreated diabetic rats (DMC) were fed the high fat diet (HF) containing 28% corn oil, three groups of diabetic rats were treated with Riceberry oil (RBBO) at three levels: the high fat diet containing 23% corn oil and 5% RBBO for a low dose (DMRL); 20.5% corn oil and 7.5% RBBO for a medium dose (DMRM); 13% corn oil and 15% RBBO for a high dose treatment (DMRH). The last group of the diabetic rat was fed the high fat diet containing 28% corn oil and administered metformin at 300 mg/kg body weight rat (Okamoto *et al*., 2008). The dosage of metformin used in the present study was 300 mg/kg body weight rat and this dosage was according to the study of Okamoto *et al*. (2008). All animals were allowed free access to their diet and water for 12 weeks. During the experimental period, body weight and food intake were recorded 3 times each week and non-fasting blood glucose was determined weekly. At the end of the 12th week, rats were deprived of food for 12-16 h and anesthetized with Xylazine® 5 mg/kg BW rat and Zoletil® 20 mg/kg BW rat. Blood was drawn from the abdominal vena cava into a normal tube or a heparin coated tube and centrifuged at 1000 x g for 15 min at 4°C to obtain serum and plasma. The body organs such as heart, pancreas, liver, kidney, spleen and hindlimb muscles (soleus, gastrocnemius; right side) were removed, cleaned with ice phosphate buffer saline, immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Groups of experimental design:

Group I: Non diabetic rats fed the basal diet (AIN-76A) with 5% lipid from corn oil (NC)

Group II: Non diabetic rats fed the basal diet (AIN-76A) with 5% lipid from RBBO (NR)

Group III: STZ-induced diabetic rats fed the high fat diet containing 28% corn oil (DMC)

Group IV: STZ-induced diabetic rats fed the high fat diet containing 23% corn oil and 5% RBBO (DMRL)

Group V: STZ-induced diabetic rats fed the high fat diet containing 20.5% corn oil and 7.5% RBBO (DMRM)

Group VI: STZ-induced diabetic rats fed the high fat diet containing 13% corn oil and 15% RBBO (DMRH)

Group VII: STZ-induced diabetic rats fed the high fat diet containing 28% corn oil and administered metformin 300 mg/kg BW rat (DM-MET) (Okamoto *et al*., 2008)

Chemicals and reagents

Chemicals and reagents used throughout this study were analytical grade. Streptozotocin was purchased from Merck KGaA (Lot # D00085400, Darmstadt, Germany). Casein was purchased from Erie Foods

International, Inc (Illinois, USA). DL-methionine was purchased from Sigma-Aldrich Co.LLC (Missouri, USA) and choline bitartrate salt was purchased from Sigma-Aldrich Co.LLC (Missouri, USA). AIN-76 mineral mixture and vitamin mixture were purchased from MP Biomedicals, LLC (lllkirch, France). Corn starch and sucrose were obtained from Nichiku Medical Industries, Ltd., Yokohama, Japan. Metformin was purchased from Merck Sorono (London, UK). Cellulose was purchased from Solka-Floc (Cat. No. CB41476572, Solka-Floc 200, New York, USA). Citrate buffer and normal saline (0.9 %) were purchased from the Government Pharmaceutical Organization (Bangkok, Thailand).

Blood and tissue biochemical parameters

The concentration of serum insulin was measured using an enzyme-linked immunosorbent (ELISA) rat insulin assay kit (Shibayagi Co. Ltd., Shibukawa, Japan). Fasting and non-fasting blood glucose values were measured using a portable glucometer (Accu-check Performa®, Roche Diagnosis Ltd., Thailand). Whole blood was measured for Glycosylated hemoglobin (HbA1c) using an enzymatic colorimetric assay method (Shibata *et al*., 2000) Plasma was separated and subsequently analyzed for total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL-C) and high density lipoprotein cholesterol (HDL-C) using enzymatic methods (Shibata *et al*., 2000). Rats then were sacrificed by opening the rat diaphragm.

Intraperitoneal glucose tolerance test (IPGTT)

At week 11 of the experimental period, 12-h fasted rats (treatment and control) were i.p. injected with 2 g/kg BW glucose solution (50% (w/v) in normal saline) (Islam *et al*., 2008). Blood glucose concentration was measured just before injection (t = 0) and subsequently at 15, 30, 60, 120, and 180 min. Blood glucose levels were plotted against time to determine area under the curve (AUC).

Determination of glucose transporter type 4 protein expression (GLUT 4)

A hindlimb muscle (gastrocnemous and soleus) of rats was used to evaluate glucose transporter type 4 protein expression (GLUT 4). Briefly, 1.0 gram of hindlimb muscles (gastrocnemous and soleus) was homogenized in ice cold RIPA buffer (lysis buffer containing 25 mM Tris HCL pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) using a Polytron homogenizer (T25 digital Ultra-Turrax® Ika-Labortechnik, North Carolina, USA). Homogenized muscle was centrifuged at 10,000 g for 20 minute at 4^{o}C. The supernatant was collected and stored at -80^{o}C until analysis. Protein concentration in the supernatant was determined by bicinchoninic acid (BCA) protein assay kit (Cat No. 71285-3, Novagen, Madison WI, USA). Sample containing 50 µg of protein was loaded and separated in 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). Protein then was transferred to a nitrocellulose membrane (GE Healthcare, New Jersey, USA), washed with 0.1% Tween 20 in Tris-buffered saline (TBST) and blocked with 5% (W/V) nonfat skim milk powder in TBST for 90 minutes at room temperature. The blotting membrane was incubated in 5% (W/V) nonfat skim milk dissolved in TBST containing 1:1000 dilution of GLUT4 mouse mAb (Cell Signaling Technology, Boston, USA) overnight at 4^{o}C. The blot was then incubated with 1:1500 dilution of horseradish peroxidase conjugated anti mouse antibody (Invitrogen, New York, USA) in TBST for 90 minutes at room temperature. Then each membrane was visualized for GLUT4 protein using ECL Prime Western Blotting Detection Reagent (GE Healthcare, New Jersey, USA) and then exposed to the hyperfilm (GE Healthcare, New Jersey, USA). Band intensity was quantified using the National Institute of Health (NIH) image program. The method has been developed in our laboratory by modifying from Tremblay *et al*., 2001. 1:1000 dilution of β-actin (Santa Cruz Biotechnology, Inc., California, USA) in 5% (W/V) nonfat skim milk dissolved in TBST was used as loading control, therefore the results were reported relative to control which was arbitrarily set to 1.

Statistical analysis

All data were analyzed using SPSS 13.0 for Windows. Data are presented as means ± SEM. One – way analysis of variance (ANOVA) with Tukey post hoc test was applied to compare mean difference in body weight, food intake and biochemical parameters among groups. Significant mean difference was defined when p-value was less than 0.05 at 95% confidence interval. Additionally, percentage differences (%) between the mean of treated and non-treated diabetes rats were compared using unpaired Student's t-Test. P-values of less than 0.05 were considered to be significantly different.

Results

Body weight gain and food intake

During the feeding period, it was found that the daily food intakes of all diabetic rats (DMC, DMRL, DMRM, DMRH, and DM-MET) were significantly higher than those of both normal groups (NC and

Table 2. Effect of RBBO and metformin on body weight, food intake, NFBG, FBG, $HbA1_c$, serum insulin of normal (NC, NR) and diabetes (DMC, DMR_L, DMR_M, DMR_H, and DM-MET) groups after 12 weeks of the experimental period

	NC	NR	DMC	DMR_L	DMR_M	DMR_H	DM-MET
Weight (g)	327.99 ± 6.00 ^{a}	328.30 ± 5.78 ^{a}	339.34 ± 5.39 ^{a}	338.57 ± 6.11 ^{a}	340.14 ± 5.41 ^{a}	338.81 ± 6.42 ^{a}	336.26 ± 3.56 ^{a}
Weight gain (g)	150.91 ± 5.66 ^{a}	154.80 ± 7.05 ^{a}	153.16 ± 10.11 ^{a}	154.14 ± 3.81 ^{a}	155.31 ± 10.58 ^{a}	158.30 ± 6.60 ^{a}	158.17 ± 6.16 ^{a}
Food intake (g/d)	18.20 ± 0.28^{a}	18.43 ± 0.23^{a}	22.81 ± 0.29^{c}	21.49 ± 0.41^{b}	21.89 ± 0.42^{bc}	21.85 ± 0.17^{bc}	21.32 ± 0.22^{b}
Energy intake (kcal/day)	67.60 ± 1.02^{a}	68.44 ± 0.84^{a}	85.17 ± 1.08^{c}	80.79 ± 1.57^{c}	81.73 ± 1.56^{c}	81.59 ± 0.64^{c}	79.62 ± 0.84^{b}
NFBG (mg/dL)	99.83 ± 2.05^{a}	95.63 ± 1.99^{a}	405.51 ± 21.26^{c}	176.97 ± 15.58^{b}	178.64 ± 7.72^{b}	184.22 ± 23.87^{b}	129.00 ± 4.91^{ab}
FBG (mg/dL)	93.10 ± 2.54^{a}	93.30 ± 2.90^{a}	212.56 ± 25.94^{b}	133.31 ± 6.38^{a}	135.70 ± 6.73^{a}	133.10 ± 10.70^{a}	123.00 ± 1.72^{a}
%FBG diff.	-	0.91± 3.97	-	-25.66 ± 11.09 ‡	-27.98 ± 8.25 ‡	-25.15 ± 14.39 ‡	-33.43 ± 8.32 ‡
HbA_{1c} (%)	4.0 ± 0.06^{a}	4.0 ± 0.05^{a}	6.2 ± 0.18^{c}	4.5 ± 0.16^{ab}	4.6 ± 0.09^{b}	4.7 ± 0.20^{b}	4.2 ± 0.10^{ab}
Serum insulin (pg/mL)	569.11 ± 71.23^{b}	579.63 ± 66.42^{b}	161.82 ± 20.75^{a}	219.57 ± 20.24^{a}	203.61 ± 20.57^{a}	205.58 ± 13.07^{a}	158.70 ± 3.77^{a}
% Insulin diff.	-	10.63 ± 16.57	-	62.56 ± 25.53‡	53.22 ± 28.63	50.01 ± 21.34 ‡	18.15 ± 18.83

Means ± SEM, n=10
[a-c]Values in the same row with different superscript letters were significantly among groups, at $p<0.05$ by ANOVA and post-hoc Tukey test

Table 3. Lipid profiles of normal (NC, NR) and diabetes (DMC, DMR_L, DMR_M, DMR_H, and DM-MET) groups after 12 weeks of the experimental feeding

	NC	**NR**	**DMC**	**DMR_L**	**DMR_M**	**DMR_H**	**DM-MET**
Cholesterol (mg/dL)	109.30 ± 1.56^{a}	109.11 ± 2.12^{a}	132.40 ± 4.64^{b}	121.89 ± 2.85^{ab}	122.60 ± 2.57^{ab}	124.89 ± 3.06^{b}	122.75 ± 3.60^{b}
Triglyceride (mg/dL)	87.10 ± 1.96^{a}	87.70 ± 2.45^{a}	128.00 ± 5.05^{c}	105.10 ± 2.68^{b}	107.11 ± 1.46^{b}	106.20 ± 2.27^{b}	102.20 ± 2.56^{b}
HDL-Cholesterol (mg/dL)	72.50 ± 3.32^{b}	73.00 ± 0.86^{b}	53.25 ± 2.90^{a}	54.96 ± 3.59^{a}	57.86 ± 2.83^{a}	62.86 ± 5.45^{ab}	56.25 ± 1.86^{a}
LDL-Cholesterol (mg/dL)	23.60 ± 0.62^{a}	23.90 ± 1.30^{a}	30.20 ± 1.10^{b}	23.90 ± 0.86^{a}	24.70 ± 0.98^{a}	25.10 ± 1.10^{a}	23.92 ± 1.55^{a}
TC/HDL-C*	1.43 ± 0.07^{a}	1.45 ± 0.05^{a}	2.56 ± 0.14^{c}	2.30 ± 0.13^{c}	2.21 ± 0.06^{c}	2.03 ± 0.19^{b}	2.17 ± 0.09^{c}
TG/HDL-C*	1.15 ± 0.07^{a}	1.16 ±0.03^{a}	2.57 ± 0.09^{c}	1.96 ± 0.12^{b}	1.89 ± 0.07^{b}	1.75 ± 0.17^{b}	1.88 ± 0.08^{b}

Means ± SEM, n=10
[a-c]Values in the same row with different superscript letters were significantly among groups, at $p<0.05$ by ANOVA and post-hoc Tukey test

NR) at $p<0.05$, whereas body weight gains of all groups were unaffected by the diet, even though the energy intakes of all the diabetic rats were greater than those of the control groups as shown in Table 2. No adverse effects such as diarrhea or death were observed among any rats fed the experimental diets throughout 12 weeks of the study period.

Plasma glucose, insulin concentration and Glycated hemoglobin (HbA1C) value

Non fasting blood glucose (NFBG), fasting blood glucose (FBG) and % HbA1c concentration are shown in Table 2. Significant increases in NFBG, FBG as well as % HbA1c were observed in the untreated diabetic group (DMC) compared to the control rats. The supplementation of RBBO for 12 weeks after STZ induction of diabetic rats reduced the NFBG, FBG and HbA1c level significantly in all the supplemented groups (176.97 to 184.22 mg/dL, 133.31 to 135.70 mg/dL and 4.5 to 4.7%, respectively) when compared to the untreated DMC group (405.51 mg/dL, 212.56 mg/dL and 6.2%), $p<0.05$. Although supplementation with RBBO reduced blood glucose and % HbA1c, the reduction of blood glucose value was not close to the non-diabetes groups (95.63 to 99.83 mg/dL, 93.10 to 93.30 mg/dL and 4.0%), $p < 0.05$. Nevertheless, FBG did not differ among RBBO supplemented groups when compared to NC and NR groups. Administration of metformin to diabetic rats significantly reduced the NFBG and FBG as well as % HbA1c (129.00 mg/dL, 123.00 mg/dL, 4.2%) to nearly that of the normal groups as shown in Table 2. Regarding the percentage changes in FBG values among diabetic rats supplemented with RBBO, metformin and DMC, the results demonstrated the significant reduction of FBG by about 25.15 to 27.98% for RBBO supplemented groups and 33.43% for metformin group, when compared to the DMC group as shown in Table 2.

Skeletal muscle glucose transporter 4 protein expression (GLUT4)

Western blot analysis of skeletal muscle GLUT4 levels relative to β-actin revealed significant increases in all the diabetic rats supplemented with 5%, 7.5% or 15% RBBO compared to the untreated

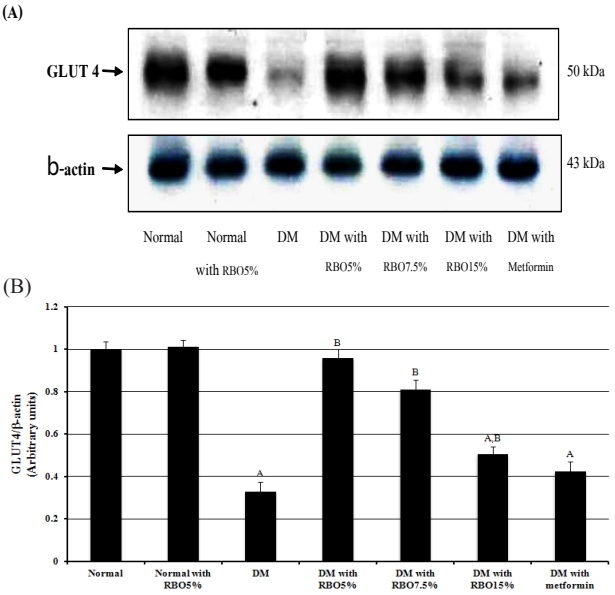

Figure 1. GLUT4 protein expression in skeleton muscle of STZ-induced diabetic (DM) rats and normal rats after 12 weeks of feeding experimental and basal diets. Dietary regimens are described in footnote of Table 1. The upper panels depict representative western blots of skeletal muscle GLUT4 and β-actin (for normalization of gel loading). The relative band intensity is reported in arbitrary units, with that of NC set at one. A, $p< 0.05$ compared to NC by unpaired Student's t-test; B, $p< 0.05$ compared to DM by unpaired Student's t-test.

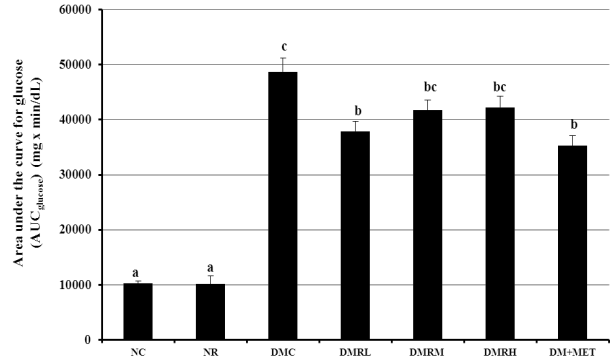

Figure 2. Area under the curve (AUC) for glucose tolerance test (mg x min/dL), after 12 weeks on experimental and basal diets feeding period. Dietary regimens are described in footnote of Table 1. Mean values with different superscript letters on the top of data bars were significantly different among groups, at $p<0.05$ by ANOVA and post-hoc Tukey test

DM rats (ranging from 51% for DMRH to 96% for DMRL) whereas a low level of GLUT 4 expression was found in DM-MET and untreated DM rats as shown in Figure 1. It should be noted that the greatest amount of GLUT 4 protein expression was observed in both normal rats and especially in the diabetic rats supplemented with 5% RBBO (DMRL) as shown in Figure 1.

Serum insulin concentration

Serum insulin concentrations in groups supplemented with RBBO tended to be higher than those of DMC and DM-MET but lower than the control groups (NC and NR), although there was no significant mean difference among groups. Also, no significant difference was observed between the normal control groups (569.11 ± 71.23 and 579.63 ± 66.42 pg/mL). These results indicated that diabetic rats supplemented with RBBO were unable to increase insulin concentrations to be equal to the normal groups (NC and NR). It might be that some beta cells which were damaged from STZ reagent could not completely recover. However, all diabetic rats supplemented with RBBO tended to increase insulin secretion whereas DM-MET (diabetes treated with metformin) and DMC groups showed the lowest amount of insulin concentrations (Table 2).

Intraperitoneal glucose tolerance test (IPGTT)

The results of intraperitoneal glucose tolerance test (IPGTT) are shown in Figure 2. The AUCglucose concentrations of diabetic rats fed with high fat diet containing RBBO supplement or metformin (DMRL, DMRM, DMRH, and DM-MET groups) declined and were significantly diminished after 120 min whereas the AUCglucose of the untreated diabetes (DMC group) continued to increase. In addition, no significant difference was observed in AUCglucose between the NC and NR groups. The estimation of area under the curve for glucose in the diabetes groups with RBBO or metformin administration showed no significant differences among treatment groups but the AUCglucose of diabetic rats fed RBBO and metformin were reduced by approximately 20.83%, 11.53%, 10.41%, and 25.58% respectively, when compared to the DMC group (Figure 2). Nevertheless, untreated diabetic rats (DMC) fed the high fat diet showed the greatest level of AUCglucose. As shown in Table 2 and Figure 2, all the relevant data indicated that the level of fasting blood glucose, HbA1c and intraperitoneal glucose tolerance test (IPGTT) had no statistical significant differences between RBBO supplemented groups and those in DM-MET group.

Plasma lipid profile concentrations

After 12 weeks of treatment, the responses to dietary supplement (RBBO) revealed that all the treatment groups with RBBO and DM-MET had improved lipid profiles such as plasma triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) concentrations which were significantly lower in all the RBBO and anti-diabetic drug supplemented groups than in the untreated DM group whereas total cholesterol (TC) and TG/HDL-cholesterol ratio (high density lipoprotein cholesterol) showed lower values than those of the untreated DM group as shown in Table 3.

Discussion

Type 2 Diabetes mellitus (T2DM) is a chronic disease characterized by hyperglycemia which results from the decrease in secretion and action of insulin (Alberti *et al*., 1998). The simulated experimental animal models were set up to examine the effect of RBBO on metabolic change in diabetic and dyslipidemic conditions. Currently, various investigators have demonstrated that feeding a high fat diet can induce insulin resistance and hyperinsulinemia in animal models (Sahin *et al*., 2007; Tanaka *et al*., 2007). Low dose of STZ has also been known to induce a mild impairment of insulin secretion in animals, the pathological condition which mimics the later stage of T2DM (Srinivasan *et al*., 2005; Ming *et al*., 2009; Zheng *et al*., 2012). Therefore, the combination of high fat diet feeding and multiple low doses of STZ injections were applied to induce hyperglycemia in the rat model of the present study. All the diabetic rats exhibited increased blood glucose as well as decreased insulin concentration compared to non-diabetic rats. The reduction of serum insulin concentration is due to a decline of insulin production in the pancreatic islets of diabetic rats which reflects a progressive loss of β-cell function by the action of STZ, leading to a reduction of insulin secretion and increasing serum blood glucose value (Ming *et al*., 2009). In addition, glycated hemoglobin (HbA1c) of diabetes rats was also increased up to 6.0%, indicating that the body could not control blood glucose, resulting in the elevation of blood glucose and reduced insulin secretion. The exposure of hemoglobin to the high levels of blood glucose could cause non-enzymatic glycation of hemoglobin resulting in higher percentage of glycosylated hemoglobin in all the diabetic rats (Siddiqui *et al*., 2010). After 3 months of treatment with experimental diets, all treatment groups of diabetic rats fed with RBBO, except for the metformin group, showed significant improvement on fasting blood glucose (FBG), non-fasting blood glucose (NFBG), glycated hemoglobin (HbA1c), and serum insulin concentration. Although the reduction of blood glucose and elevation of serum insulin level in RBBO supplemented groups did not reach the normal values, the STZ-induced diabetic rats supplemented with RBBO perhaps recovered and increased pancreatic β-cells and granulation to nearly normal condition which indicated by the improvement of hyperglycemia in RBBO supplemented diabetes rats. In addition, the greatest value of GLUT4 was observed in normal rats (NC; NR) and RBBO supplemented group compared to the DMC group, $p < 0.05$ (Figure 1). Therefore, this observation may indicate that RBBO supplement could improve insulin sensitivity, protecting pancreas islet β-cells as well as stimulating insulin secretion. Moreover, the improved serum insulin in RBBO supplemented groups indicated that bioactive compounds such as tocotrienol, γ-oryzanol or other active compounds, particularly ferulic acid, or β-carotene contained in Riceberry oil might stimulate insulin secretion from the remnant pancreatic β-cells and/or regenerated β-cells (Pari and Latha, 2002; Canas *et al*., 2012). The lowest serum insulin concentration found in the metformin group could be explained by metformin controlling or decreasing fasting blood glucose by limiting hepatic glucose production rather than promoting cells sensitivity to insulin (He *et al*., 2009). That study also was consistent with our data which found that insulin value in DM rats treated with metformin was significantly lower than those of the control and other experimental groups. In addition, Zhou *et al*., (2001) demonstrated that metformin enhanced phosphorylation and activation of adenosine monophosphate protein kinase (AMPK), which helped to regulate lipid and glucose metabolism. Metformin activated AMPK in hepatocytes, and thereby, the reduction of acetyl-CoA carboxylase (ACC) activity and the increase in fatty acid oxidation, resulting in decrease in hepatic glucose production. Results from IPGTT indicated that diabetic rats treated with metformin had the greatest reduction of AUC for glucose by -25.58% while the AUC of rats supplemented with RBBO ranged between -10.41% to -20.83% as compared to DM. The glycemic improvement might be due to the increased stimulation of uptake and utilization of glucose in the peripheral tissues, meanwhile metformin also acted to reduce hepatic glucose production (Zhou *et al*., 2001). This suggestion was consistent with our finding that diabetic rats treated with metformin had the lowest AUC for glucose as well as serum insulin level. Recently, Fang *et al*., (2010) investigated the mechanism of tocotrienols on reducing blood glucose by acting through peroxisome proliferator-activated receptor (PPAR) modulators. PPARs are transcriptional factors that regulate the expression of genes in lipid and carbohydrate metabolism. The binding of tocotrienols to PPAR will stimulate the expression of glucose transporter 4 (GLUT4) which contributes to lowering blood glucose by promoting glucose uptake into the peripheral cells (Aggarwal *et al*., 2010). The study of Fang *et al*., (2010) was consistent with our data of GLUT 4 protein transporter which found that all the RBBO supplemented groups significantly increased GLUT 4 protein transporter when compared

to untreated diabetic group, $p<0.05$, especially at low dose; 5% RBBO was similar to the values of normal control groups as shown in Figure 1. The increment in GLUT 4 protein level in RBBO supplemented groups, especially at low dose of RBBO might be explained by the action of various bioactive compounds contained in Riceberry oil, particularly tocotrienol, tran-β-carotene, and especially γ-oryzanol which is composed of phytosterols esters of trans-ferulic acid or other plant sterols; triterpenes that enhanced cellular insulin dependent glucose uptake. In the case of insulin resistance or insulin deficiency, it causes low response in signaling GLUT4 to take up glucose (Huang and Czech, 2007). This was consistent with our study which found that the untreated diabetic group had the lowest GLUT 4 value. However, our results did not agree with another previous study which showed that plasma insulin concentration was lower in rats fed the rice bran oil (RBO) diet than that of the diabetes control group and the plasma glucose value did not differ among the experimental groups (Chen and Cheng, 2006). The difference in the results of the present study with the previous study might be due to the differences in rice varieties, some bioactive compounds or the extraction process of the rice bran oil. Generally, STZ-induced diabetes rats had increased blood glucose, hemoglobin A1c, total cholesterol, triglyceride, LDL-C, and decreased HDL-C (Rajasekaran *et al*., 2006). After supplementation of diabetic rats with RBBO or metformin, there was improvement in serum lipid profile compared to untreated diabetic rats. All RBBO supplemented groups or metformin treated groups had lower serum total cholesterol, LDL-cholesterol and TG concentration than the untreated diabetes group (DMC). That might be due to the effect of various bioactive compounds contained in RBBO such as γ-oryzanol, coenzyme Q10, tocopherol, tocotrienols, polyphenol, and some phenolic acids including ferulic acid, p-coumaric acid and diferulate (Lin and Lai, 2011; Yawadio *et al*., 2007). A study by Chen and Cheng (2006) demonstrated that feeding of 10% and 15% rice bran oil to type 2 diabetic rats for 4 weeks significantly reduced serum triglyceride and LDL-C. Several studies reported that γ-tocotrienol in rice bran oil could improve the lipid profile through the increases in LDL-receptor and HMG-CoA reductase mRNA expression (Chen and Cheng, 2006; Chou *et al*., 2009). Bioactive compounds in rice bran oil such as γ-oryzanol and ferulic acids or other anti-oxidative compounds might be active in lowering lipid level by reduction of cholesterol in plasma and in liver; Son *et al*., (2010) demonstrated that feeding γ-oryzanol and ferulic acids to hypercholesterolemic mice for 7 weeks significantly lowered plasma and hepatic lipid profile by increasing fecal cholesterol and triglyceride excretion (Yawadio *et al*., 2007; Son *et al*., 2010; Guo *et al*., 2007). Sakatani *et al*. (2005) indicated that diabetes is associated with alterations in lipid profile such as the regulation of plasma or tissue lipid levels which led to an increase in the risk of micro or macrovascular diseases and related diabetes complications (Pushparaj *et al*., 2007; Zhang *et a*l., 2010). Therefore, those reports are consistent with our results that RBBO might be helpful to prevent diabetic complications through improving dyslipidemia.

Conclusion

The present study indicated that the supplementation with RBBO could improve hyperglycemia and lipid profiles in STZ-induced diabetes rats fed a high fat diet. Further studies need to identify the active ingredient in Riceberry oil and by what mechanism it could play an important role in diminishing diabetic complications.

Acknowledgements

This research was supported by the National Research Council of Thailand.

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