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Goat milk kefir with black rice extract reduced insulin resistance through suppressing RBP4 expression in diabetic rats

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Abstract.
BACKGROUND: High anthocyanin in black rice ameliorates hyperglycemia and insulin resistance through improving GLUT4-RBP4 system and lipid profile. Activity of anthocyanin will increase during the fermentation process.
OBJECTIVES: To evaluate the effect of goat milk kefir supplemented with black rice extract on blood glucose, lipid profile, HOMA-IR and RBP4 gene expression in diabetic rats.
METHOD: Twenty five male Spargue Dawley rats were divided into 5 groups: 1) normal rats; 2) diabetic rats; 3) diabetic rats +1 mL of kefir/200 g body weight (BW); 4) diabetic rats +2 mL of kefir/200 g BW; and 5) diabetic rats +4 mL of kefir/200 g BW. Blood glucose levels were measured before and after intervention, whereas lipid profile, HOMA-IR and RBP4 gene expression in white adipose tissue were analyzed at the end of intervention.
RESULTS: The blood glucose, lipid profile, HOMA-IR, and RBP4 gene expression in diabetic rats and diabetic rats with kefir were significantly different ($p < 0.05$). The diabetic rats with kefir had lowered blood glucose, total cholesterol, triglyceride, LDL, RBP4 gene expression, and HOMA-IR, and conversely HDL levels were higher than in diabetic rats.
CONCLUSIONS: Goat milk kefir supplemented with black rice extract reduced insulin resistance through improving lipid profile and suppressing RBP4 expression in diabetic rats.

Keywords: Type 2 diabetes, insulin resistance, RBP4 gene expression, goat milk kefir, black rice extract

1. Background

Retinol-binding protein 4 (RBP4) is related with the development of insulin resistance and diabetes mellitus (DM). Li et al. [1] investigated the serum levels of RBP4 in type 2 diabetes mellitus (T2DM) with and without retinopathy, and suggested that RBP4 may be involved in the process of diabetic retinopathy and may be a

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novel biomarker for its diagnosis and treatment in diabetic patients. RBP4 is a identified adipokine secreted by adipocytes and the liver. According to Mazaki-Toviet et al. [2] RBP4 is a 21 kDa protein predominantly synthesizeds in the liver and has a role as the major blood carrier of retinol and is involved in the regulation of systemic insulin sensitivity. High RBP4 in the serum occurs before the development of diabetes and appears to identify insulin resistance and is associated with cardiovascular risk factors [3]. It may cause insulin resistance by contributing to the development of an inflammatory state in adipose tissue through activation of proinflammatory cytokines in macrophages [4]. Cho et al. [5] found plasma RBP4 levels to be elevated in subjects with impaired glucose tolerance (IGT) or T2DM and to be related to various clinical parameters known to be associated with insulin resistance. Yang et al. [6] showed that serum RBP4 levels are elevated in insulin-resistant mice and humans with obesity and T2DM. Broch et al. [7] suggested that RBP4 is associated negatively with weight loss, and the decrease of RBP4 was related to the reduction in the levels of triglycerides and with the increase in HDL-cholesterol. A mechanism linking high-fat-diet-induced obesity with insulin resistance has been proposed to be related to endoplasmic reticulum stress. This stress may be a common molecular pathway for insulin resistance and β-cell loss that can cause T2DM. Insulin secretion disorder and β-cell apoptosis, resulting from the lipotoxic effects, may contribute to the loss of β-cell function in the pathogenesis of T2DM. The molecular mechanisms of lipotoxicity probably involve oxidative stress in endoplasmic reticulum [8]. Therefore, in addition to controlling blood glucose levels, coping with oxidative stress is also very important for diabetic patients.

Many researchers suggested that anthocyanin can significantly ameliorate hyperglycemia and insulin resistance in type 2 diabetic mice. Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. A research demonstrates that dietary anthocyanin-rich bilberry extract reduces blood glucose levels and enhances insulin sensitivity in type 2 diabetic mice [9]. Sasaki et al. [10] reported that cyanidin 3-glucoside (C3G) ameliorates hyperglycemia and insulin resistance due to downregulation of RBP4 expression in diabetic mice. C3G significantly upregulated the glucose transporter 4 (GLUT4) and downregulated RBP4 in the white adipose tissue, which indicates the anti-diabetic effect of C3G via the regulation of GLUT4-RBP4 system.

Some researchers suggested that anthocyanin improved lipid profile. Guo et al. [11] showed that anthocyanin-rich extract from black rice prevented metabolic syndrome by improving lipid profile and increasing insulin sensitivity in fructose-fed rats. The fructose-fed rats were treated by anthocyanin-rich extract from black rice for 8 weeks had lower levels of triglyceride, total cholesterol, low density lipoprotein (LDL), and increased high density lipoprotein (HDL) than the non-treatment group. Tsuda et al. [12] reported that dietary cyanidin 3-glucoside-rich purple-colored corn may ameliorate insulin resistance through suppressing the mRNA levels of enzymes involved in fatty acid and triacylglycerol synthesis and lowering the sterol regulatory element binding protein-1 (SREBP-1) mRNA level in white adipose tissue in high-fat diet–induced mice. According to Juan et al. [13] activity of anthocyanin is known to increase during the fermentation process although it is found to be sensitive during the pasteurization. Previous study showed that the combination kefir of goat milk and black rice extract has DPPH-scavenging activity higher than the goat milk kefir [14]. Therefore in this study we investigated the effect of goat milk kefir supplemented with black rice extract in different doses on blood glucose, lipid profile, HOMA IR and RBP4 gene expression in diabetic rats.

2. Material and methods

2.1. Black rice extract and kefir preparation

Kefir grains were obtained from Center for Livestock Training (Malang-East Java, Indonesia). Local black rice and milk of Ettawah Crossed bred goat were taken from Yogyakarta, Indonesia. The black rice extract was prepared according to Sirait and Jelena [15] 500 g of local black rice was left in 1 L of distillated water for 24 hour, and then it was blended, and filtered with a cotton sheet. Kefir preparation was done according to Chen et al. [16] with slight modification. Kefir was prepared from black rice extract and goat milk (1 : 1). Black rice extract

and raw goat milk were pasteurized at 90°C for 30 min in a water bath and cooled to inoculation temperature. The heat-treated milk was inoculated with 3% of kefir grains and incubated at room temperature for 18 hours. After the grains separated, then the kefir was stirred, and stored at 4°C before used.

2.2. Animals

Twenty five (25) male Spargue Dawley rats (180–200 g, 8 weeks) were used. They were housed individually in cages and maintained under standard conditions (12 : 12-h light/dark cycle and 22–25°C room temperature). They were acclimatized, for 5 days by a semi-purified diet formula for rats by Wostmann [17] with slight modification (whole wheat flour was substituted by cornstarch). The diet consisted of 24% casein, 0.30% DL-methionine, 61% cornstarch, 1% vitamin mix, 3.5% mineral mix, 0.2% choline chloride, 5% alpha cell, and 5% corn oil (Percentages in diet composition means total % in 100 g diet). This study was approved by Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

2.3. Induction of diabetes

Induction of diabetes was done according to Shirwaikar et al. [18]. Diabetes was induced after overnight fasting by intraperitoneal injection of 60 mg/kg BW streptozotocin (STZ) (NacalaiTesque, Japan), 15 minutes after intraperitoneal injection of 120 g/kg BW nicotinamide (NA) (Sigma Aldrich, USA). The STZ was dissolved in citrate buffer (pH 4.5) and the NA was dissolved in normal saline. After 5 days of induction, the rats with blood glucose level $\geq$200 mg/dL were considered as diabetic rats.

2.4. Experimental study

After induction of diabetes, the rats were divided into five groups: 1) normal rats (N); 2) diabetic rats (DM); 3) diabetic rats with 1 mL of kefir/200 g body weight (KF1); 4) diabetic rats with 2 mL of kefir/200 g body weight (KF2), and 5) diabetic rats with 4 mL of kefir/200 g body weight (KF4). Before and after administration of kefir via gavage for 4 weeks, the blood glucose was measured. At the end of the study, the blood was taken under anesthesia to measure HOMA-IR. White adipose tissue was also taken to measure RBP4 gene expressions. After being snap-frozen in liquid nitrogen, tissues for RNA preparation were stored in freezer at –80°C. The collecting of samples was conducted after overnight fasted.

2.5. Biochemical analysis

Blood glucose and lipid profile were enzymatically analyzed using a commercial kit (Diasys, Holzheim, Germany). The HOMA-IR value was calculated as follows: fasting insulin (μU/mL) $\times$ fasting glucose (mmol/L)/ 22.5 [19].

2.6. Isolation of RNA and quantitative polymerase chain reaction (q-PCR)

Total RNA was extracted from frozen adipose tissue using the TRIzol reagent (Invitrogen, USA). Reverse transcription of 1 μg was done according to the protocol from Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The q-PCR was used with SsoFastEvaGreenSupermix (Bio-rad, United Kingdom) and total reaction for q-PCR was 10 μL. The results were normalized against beta actin. The analysis used primer sequences as follows (Table 1). The thermocycling conditions were : 5 min at 95°C, 1 min at 95°C, followed by 1 min at 57.4°C and 40 cycles.

Table 1
Primer sequences that were used in q-PCR for RBP4 gene expression

Gene	Primer
RBP4 (rat)	Forward 5- GGTGAGCAGCTTCAGAGTC-3
	Reverse 5- ACCATGTCTGCACACACTTC-3
Beta actin (rat)	Forward 5'- ACGGTCAGGTCATCACTATCG-3'
	Reverse 5'- GGCATAGAGGTCTTTACGGATG-3'

2.7. Statistical analysis

All values are presented as mean ± standard deviation. One-way ANOVA was used to analyze the differences in glucose levels, HOMA-IR values, and RBP4 gene expressions between the groups. Paired *t*-test was used to evaluate glucose levels before and after administration of kefir. Differences were considered statistically significant at $p < 0.05$.

3. Result

Goat milk kefir supplemented with black rice extract reduced significantly the blood glucose of the diabetic rats ($p = <0.001$). The decrease of blood glucose was negatively correlated with kefir doses (Table 2).

After intervention of goat milk kefir supplemented with black rice extract, lipid profiles between rat groups were significantly different ($p = <0.001$) (Table 3). Although the rats that received kefir for 4 weeks had HDL levels the same as in the normal rats, the levels of cholesterol, triglycerides and LDL remained lower in the normal rats than those in the KF rat groups.

The results demonstrated that the goat milk kefir supplemented with black rice extract can ameliorate insulin resistance that showed in lower HOMA IR in KF rats than that in DM rats, and it was significantly different ($p = <0.001$) (Table 4).

The RBP4 gene expressions in white adipose tissue of diabetic rats after receiving the goat milk kefir supplemented with black rice extract are shown in Table 5. We found the highest expression of RBP4 in diabetic rats, and the lowest in KF1, although there were not significant differences between intervention groups.

Table 2
Blood glucose level in rats before and after administration of kefir

Group	Blood glucose (mg/dL)		Mean Difference	95% CI	p
	Before	After			
N	78.49 ± 1.32^{a}	80.52 ± 1.30^{a}	2.03	–1.34–2.70	0.001
DM	231.33 ± 9.57^{b}	237.76 ± 8.90^{b}	6.44	–2.82–10.05	0.008
KF1	231.68 ± 0.83^{b}	144.25 ± 1.14^{c}	–87.43	85.23–89.63	<0.001
KF2	231.40 ± 3.84^{b}	122.16 ± 3.96^{d}	–109.24	107.46–11.01	<0.001
KF4	$228.67 \pm 1.58^{b,c}$	116.72 ± 1.70^{d}	–111.95	110.77–113.14	<0.001
P	<0.001	<0.001			

N: normal rats; DM: diabetic rats: KF1: diabetic rats received kefir 1 mL/200 g BW; KF2: diabetic rats received kefir 2 mL/200 g BW; KF4: diabetic rats received kefir 4 mL/200 g BW. Values are presented as mean ± SD ($n = 5$).[a,b,c,d] Indicate $p < 0.05$ in One Way ANOVA test followed by Games-Howell test.[b,c] Indicate no difference either [b] nor [c]. P in row indicates the differences of plasma glucose before and after kefir administration in the same group. P in the last row indicates the differences of plasma glucose between group.

Table 3
Lipid profile level after 4 weeks kefir administration

Group	Total Cholesterol (mg/dL)	Triglyceride (mg/dL)	LDL (mg/dL)	HDL (mg/dL)
N	104.65 ± 1.50^{a}	64.00 ± 1.15^{a}	52.94 ± 1.83^{a}	37.98 ± 1.62^{a}
DM	165.53 ± 2.84^{b}	131.28 ± 1.41^{b}	87.76 ± 1.28^{b}	18.09 ± 1.47^{b}
KF1	153.02 ± 2.15^{c}	109.01 ± 2.51^{c}	73.85 ± 1.39^{c}	26.99 ± 1.69^{c}
KF2	136.47 ± 2.93^{d}	92.45 ± 4.12^{d}	71.71 ± 3.32^{c}	31.94 ± 1.69^{d}
KF4	127.85 ± 3.58^{e}	84.83 ± 2.61^{e}	63.01 ± 2.81^{d}	37.32 ± 1.36^{a}
P	<0.001	<0.001	<0.001	<0.001

N: normal rats; DM: diabetic rats: KF1: diabetic rats received kefir 1 mL/200 g BW; KF2: diabetic rats received kefir 2 mL/200 g BW; KF4: diabetic rats received kefir 4 mL/200 g BW. Values are presented as mean ± SD ($n = 5$).[a,b,c,d,e] Indicate $p < 0.05$ in One Way ANOVA test followed by Tukey honest significant difference (HSD).

Table 4
HOMA IR value of diabetic rats after 4 weeks intervention of kefir

Group	HOMA IR
N	2.07 ± 0.75^{a}
DM	4.09 ± 1.24^{b}
KF1	$2.92 \pm 0.96^{a,b}$
KF2	1.86 ± 0.85^{a}
KF4	1.55 ± 0.51^{a}
P	0.002

N: normal rats; DM: diabetic rats: KF1: diabetic rats received kefir 1 mL/200 g BW; KF2: diabetic rats received kefir 2 mL/200 g BW; KF4: diabetic rats received kefir 4 mL/200 g BW. Values are presented as mean ± SD ($n = 5$).[a and b] Indicate $p < 0.05$ in One Way ANOVA test followed by Tukey honest significant difference (HSD). [a,b] Indicate no difference either [a] nor [b].

Table 5
RBP4 gene expression in white adipose tissue of diabetic rats after 4 weeks intervention of kefir

Group	RBP4 expressions in white adipose tissue
N	1.75 ± 0.05^{a}
DM	2.36 ± 0.04^{b}
KF1	1.65 ± 0.10^{a}
KF2	1.66 ± 0.05^{a}
KF4	1.67 ± 0.02^{a}
P	<0.001

N: normal rats; DM: diabetic rats: KF1: diabetic rats received kefir 1 mL/200 g BW; KF2: diabetic rats received kefir 2 mL/200 g BW; KF4: diabetic rats received kefir 4 mL/200 g BW. Values are presented as mean ± SD ($n = 5$).[a and b] Indicate $p < 0.05$ in One Way ANOVA test followed by Games-Howell test.

4. Discussion

Our study showed that diabetic rats received the goat milk kefir supplemented with black rice extract had blood glucose levels lower than those without kefir ($p = <0.001$), and the blood glucose correlated with doses of the kefir (Table 2). The decrease of blood glucose level in diabetic rats with kefir may be the effect of reducing of insulin resistance, which was affected by the antioxidant, anthocyanin, in the black rice, and as a result the sensitivity of insulin and uptake of blood glucose increased. After the intervention, the lowest levels of the blood glucose and the HOMA-IR were found in diabetic rats with 4 mL kefir (Tables 2 and 4). Sasaki et al. [10] reported that anthocyanin (cyanidin 3-glucoside; C3G) ameliorates hyperglycemia and insulin resistance due to the reduction of RBP4 expression in type 2 diabetic mice. In this study, the RBP4 expressions in diabetic rats were higher than in those with kefir, although there were not differences among dose. Yang et al. [6] suggested that increasing the circulating levels of RBP4 results in glucose intolerance, and conversely, deleting the RBP4 gene in mice increases insulin sensitivity. In this study, the diabetic rats that received goat milk kefir supplemented with black rice extract also had low RBP4 gene expression and HOMA-IR. Therefore, we propose that the goat milk kefir supplemented with black rice extract reduced blood glucose through suppressing of RBP4 gene expression, and increased insulin sensitivity. These results are consistent with Möhlig et al. [20] who reported that the RBP4 was correlated with insulin resistance (HOMA).

The oxidative stress usually occurs in diabetic conditions, and it disturbs the insulin signaling that leads to insulin resistance. Reducing of oxidative stress will improve insulin sensitivity, so the glucose uptake also increases. Tangvarasittichai [21] suggested that oxidative stress leads to insulin resistance, dyslipidemia, β-cell dysfunction, impaired glucose tolerance, and therefore causes T2DM. In diabetes, increased free radicals level resulted from glucose oxidation, and non-enzymatic glycation of proteins. The high free radicals levels cause diminished antioxidant defense mechanisms that lead to damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance [22].

According to Liu et al. [23] there is a positive association between plasma RBP4 and total cholesterol, LDL cholesterol and triglycerides, but an inverse correlation with HDL cholesterol levels. In this research, the diabetic rats had higher levels of cholesterol, triglyceride, LDL and also RBP4 expression, and conversely, lower HDL than normal rats. The intervention of the kefir influenced the lipid profile of the diabetic rats. The diabetic rats that received kefir had significantly total cholesterol, triglyceride, LDL, and also RBP4 expressions lower than those that did not receive kefir, otherwise they had HDL higher than those without kefir ($p < 0.001$) (Tables 3 and 5). These results are in accordance with Liu's findings that the diabetic rats had high RPB4 expression and also higher total cholesterol, LDL cholesterol and triglycerides levels.

The effect of goat milk kefir supplemented with black rice extract corresponds with other reports. Guo et al. [11] reported that dietary anthocyanins-rich extract from black rice can prevent and ameliorate the hyperlipidemia and insulin resistance in fructose-fed rats. Anthocyanin-containing Haskap (*Lonicera caerulea* L.) fruit decreased postprandial blood lipids and blood glucose levels. The main anthocyanin pigment in Haskap is cyanidin-3-glucoside and contained 13.2% [24]. Cyanidin-3-glucoside (prime anthocyanin of black rice) suppresses the development of high-fat diet-induced obesity [12], and improves hyperglycemia and insulin sensitivity in type 2 diabetic mice via activation of AMP-activated protein kinase [9]. This improvement might be due to the antioxidant properties of anthocyanin. Although sensitive to pasteurization, previous reports showed that the fermentation process could increase the anthocyanin content [13], total phenolic, flavonoid, and antioxidant activity in black soya bean [25]. This increase might be due to microbial hydrolysis reaction during the fermentation process that increases in the number of flavonoids and phenolic compounds, thus resulting in enhancement of antioxidant activity [26]. This finding is also supported by our previous study that showed goat milk kefir supplemented with black rice extract had higher total phenolic content compared to goat milk kefir that was not supplemented with black rice extract [14]. Based on the result of this study, we recommend that the type 2 diabetic patients consume goat milk kefir supplemented with black rice extract to reduce insulin resistance and ameliorate lipid the profile.

5. Conclusion

Goat milk kefir supplemented with black rice extract reduced insulin resistance through improving lipid profile and suppressing RBP4 expression in diabetic rats.

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