Original paper

Blueberry (*Vaccinium virgatum* Aiton) Leaf Infusion Ameliorates Insulin Resistance in Mice Fed a High-fat, High-sucrose Diet

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Blueberry (*Vaccinium virgatum* Aiton) leaf extract (BLEx) has attracted attention as a beneficial food component. Here, we examined the effect of dietary BLEx on glucose and lipid metabolism in mice fed a high-fat, high-sucrose diet (HFHSD). Growth parameter data showed that 3% BLEx slightly reduced body weight and adipose tissue weight accompanied by inhibition of HFHSD-induced enlargement of adipocytes. Liver weight was significantly reduced in the 3% BLEx group secondary to reduction of lipid accumulation in the hepatocytes. HFHSD-induced augmentation of fasting serum glucose levels was ameliorated by 3% BLEx. HFHSD-induced increase of fasting serum insulin level, Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) and Quantitative Insulin Sensitivity Check Index (QUICKI) also tended to be ameliorated. Oral glucose tolerance tests (OGTTs) revealed that 3% BLEx normalized HFHSD-induced elevation of the area under the curve for serum glucose, whereas dietary BLEx, even for the 3% dose, did not have a suppressive effect on the serum glucose change after OGTT. Taken together, these observations suggest that BLEx is a promising agent for the prevention of HFHSD-induced insulin resistance.

Keywords: high-fat, high-sucrose diet, insulin resistance, blueberry leaf, glucose

Introduction

Type II diabetes is an emerging subject in terms of human health and medical care worldwide. Because type II diabetes is mainly a lifestyle disease, exercise and dietary modification to increase intake of functional foods are recommended in the early stages of the disease. In the context of functional food, several studies on polyphenol-rich, plant-derived materials have demonstrated the efficacy of these foods in improving abnormal

glucose metabolism associated with obesity (Wang *et al.* 2014, Bao *et al.* 2014). Blueberry is one of the notable materials for the prevention and amelioration of obesity and obesity-related diseases including insulin resistance (Khanal *et al.* 2012, Seymour *et al.* 2011, Prior *et al.* 2010, DeFuria *et al.* 2009). It is native to southeastern North America and belongs to the *Ericaceae* plant family. Recent studies have revealed that not only the fruit but also blueberry leaves have various beneficial effects on human health

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(Takeshita *et al.* 2009, Kai *et al.* 2011). Blueberry leaf infusion has been used as a folk medicine for lifestyle-related diseases in Europe, but scientific evidence has not been fully established. On the other hand, because there is sufficient evidence for the safety of blueberry leaf infusion in humans, blueberry leaf is now processed into a beverage and is available commercially in Japan. According to these cultural and commercial backgrounds, blueberry leaf could be developed as a functional food. Blueberry leaf extract (BLEx) has a slight sour and astringent taste because it contains organic acids such as quinic acid and is rich in various polyphenols, especially rutin and chlorogenic acid (Piljac-Zegarac *et al.* 2009, Kim *et al.* 2010). Although these are not necessarily characteristic components in leaves, leaf tissues have a higher oxygen radical absorbance capacity with higher total phenolic contents than those in fruit tissues (Ehlenfeldt and Prior 2009). It has recently been reported that BLEx has an inhibitory effect on angiotensin-converting enzyme activity (Sakaida *et al.* 2007) and reduces plasma glucose and triglyceride levels in streptozotocin-diabetic rats (Cignarella *et al.* 1996). These reports support the use of BLEx as a medicine for lifestyle-related diseases. Here we focused on the effect of a hot-water blueberry (*Vaccinium virgatum* Alton) leaf extract on insulin resistance in mice with diet-induced obesity, with the goal of utilizing blueberry leaf in tea form for the prevention of metabolic dysfunction.

Materials and Methods

Animals BLEx was prepared as a hot water extract by Bizen Chemical Co. Ltd (Okayama, Japan). Briefly, blueberry powder was extracted in 16 parts of hot water (95 – 100°C) for 30 min twice. Then, the extract was filtered and heat sterilized. Finally, the extract was dried with a spray dryer, producing a powder. The composition of BLEx was analyzed by Japan Food Research Laboratories (Tokyo, Japan) and is shown in Table 1. In addition, BLEx contained 368 mg eq. procyanidin B1/g proanthocyanidin (MASIS Inc., Food & Drug Nano Analysis, Aomori, Japan), 73.0 mg/g chlorogenic acid and 187 mg/g quinic acid (Japan Food Research Laboratories). Total polyphenol was 403 mg eq. tannic acid/g. The animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals at the University of Miyazaki and in compliance with the Law Concerning the Protection and Control of Animals (Japan Law No. 105), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification No. 88 of the Ministry of the Environment, Japan), and the Guidelines for Animal Experimentation (the Japanese Association for Laboratory Animal Science). Room temperature was maintained at 22 – 24°C with a 12-h light/dark cycle (0800 – 2000). C57/BL6J mice (male, 6-week-old) were purchased from Japan SLC (Hamamatsu, Japan) and acclimatized for 3 weeks. Mice were divided into 2 groups: normal diet (ND) and high-fat, high-sucrose diet (HFHSD) groups. Diets were prepared according to AIN-93G with slight modification

Table 1. Composition of BLEx

Component (g / 100 g)	
Moisture	5.1
Protein	1.1
Fat	0.4
Ash	5.6
Others	87.8

as shown in Table 1 (Reeves *et al.* 1993, Suzuki *et al.* 2011). Mice were fed ND or HFHSD for 4 weeks. Then, the HFHSD group was subdivided into control, 1% BLEx, and 3% BLEx groups, and each group was assigned to the respective experimental diets shown in Table 2 for 4 weeks. At the end of the feeding period, the mice were sacrificed under Somnopentyl anesthesia after 12 h of fasting (Kyoritsu Seiyaku Corporation, Tokyo).

Histological analysis Adipose and liver tissues were excised immediately after sacrifice and dipped into a 4% paraformaldehyde solution. Embedding, section preparation and hematoxylin-eosin staining were outsourced to Sapporo General Pathology Laboratory (Sapporo, Japan).

Serum parameters Serum glucose, triglycerides, and cholesterol were measured using the Lab AssayTM Glucose, Wako L-type TG M, and Wako Cholesterol E commercial kits, respectively (Wako, Osaka, Japan). Serum insulin was measured using an ELISA-based commercial kit (Morinaga, Yokohama, Japan). These experiments were performed according to the manufacturer's protocols.

Analysis of liver lipids To quantify liver triglycerides and cholesterol, a liver homogenate was prepared using Micro Smash MS-100R (Tomy Seiko, Tokyo, Japan) then total lipids were extracted from the homogenate by the Folch method (Folch *et al.* 1957) using a chloroform/methanol (2:1) solution. The solvent was dried under a N₂ stream and extracted lipids were re-solubilized in 5% Tween-20 isopropanol. Triglycerides and total cholesterol were measured using the Wako L-type TG M and Wako Cholesterol E commercial kits (Wako).

Oral glucose tolerance test Two weeks after initiation of BLEx-containing feedings, oral glucose tolerance test (OGTT) was performed. After 12 h of fasting, blood was collected from the tail vein for the base line data. A glucose solution was administered orally at 1.5 g/g body weight and blood was collected from the tail vein at 30, 60, and 120 min after administration. Serum was prepared and stored at –30°C before analysis.

Indices for insulin resistance As indices of insulin resistance, Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) and Quantitative Insulin Sensitivity Check Index (QUICKI) values were calculated as follows (Berglund *et al.* 2008). HOMA-IR = fasting insulin (μU/mL) x serum glucose (mg/dL)/405; QUICKI = 1/[log (fasting serum glucose (mg/dL)) + log (fasting insulin (μU/mL))]

Statistical analysis Data were analyzed using the Tukey–Kramer test to evaluate the significance of differences. Significance

Table 2. Dietary composition

Component (g / kg)	ND	HFHSD		
		Control	BLEx 1 %	BLEx 3 %
Vitamin mix	10	10	10	10
Mineral mix	35	35	35	35
Choline bitartrate	2.5	2.5	2.5	2.5
Cystine	3.0	3.8	3.8	3.8
Soybean oil	70	20	20	20
<i>t</i> -Butylhydroquinone	0.01	0.06	0.06	0.06
Sucrose	100	200	200	200
Casein	200	250	250	250
Corn starch	397.5	148.7	148.7	148.7
Pegelatinized corn starch	132	0	0	0
Cellulose	50	50	40	20
Tallow	0	140	140	140
Lard	0	140	140	140
BLEx	0	0	10	30

Diets were prepared on the basis of AIN-93G and slightly modified according to previous report (Suzuki *et al.*, 2013).

Table 3. Effect of dietary BLEx on growth parameters

	ND	HFHSD		
		Control	BLEx 1 %	BLEx 3 %
Body weight (g)	32.0±1.2	34.2±1.1	34.8±0.9 ^a	30.2±1.1 ^b
Dietary intake (g/d)	3.9±0.1	3.9±0.1	3.7±0.1	3.7±0.1
Water intake (g/d)	10.2±1.2	10.3±1.2	10.2±1.2	10.1±1.3
Epididymal fat (g)	1.05±0.18	1.39±0.17	1.62±0.11 ^a	0.92±0.18 ^b
Renal fat (g)	0.46±0.09	0.59±0.07	0.70±0.05 ^a	0.34±0.08 ^b
Liver (g)	1.08±0.03	1.13±0.06 ^a	1.13±0.04 ^a	0.94±0.05 ^b
Kidney (g)	0.32±0.01 ^a	0.33±0.01	0.35±0.01 ^a	0.32±0.01 ^b
Spleen (g)	0.10±0.01 ^a	0.09±0.003	0.08±0.003 ^b	0.07±0.004 ^b

Data are means ± SD for 6-8 mice. The values with different superscript letters differ significantly (P < 0.05).

was defined as p < 0.05.

Results

Growth parameters Dietary intake and water intake were comparable between all experimental groups (Table 3). Body weight was slightly lower in the 3% BLEx group than in the control group. Epididymal fat, renal fat, and liver weight tended to be higher in HFHSD control mice than in the ND group, and their weights were the lowest in the 3% BLEx group. Differences in weight between the 1% and 3% BLEx groups were statistically significant. Although significant differences were also observed in kidney and spleen weights, they were not prominent.

Histology and liver lipids As shown in Fig. 1A, adipocyte size in 3% BLEx fed mice was smaller than that of HFHSD control mice. Moreover, remarkable lipid accumulation was observed in

the livers of HFHSD control mice, whereas lipid droplets were inconspicuous in the livers of 3% BLEx fed mice. Liver triglycerides and total cholesterol are shown in Table 4. BLEx tended to decrease both triglycerides and total cholesterol in a dose-dependent manner.

Serum biochemical parameters Table 5 shows serum lipids, glucose and insulin levels at the end of the experimental period under fasting conditions. Triglyceride and total cholesterol levels were comparable between dietary groups. Fasting glucose and insulin levels increased in the HFHSD control group compared to the ND group. The 3% BLEx group showed inhibited augmentation of HFHSD-induced serum glucose and insulin and a significant difference was observed in the glucose levels of the HFHSD control group and 3% BLEx group.

Indices of insulin sensitivity As indices of insulin sensitivity,

Table 4. Effect of dietary BLEx on liver lipid levels

(mg/g Liver)	ND	HFHSD		
		Control	BLEx 1 %	BLEx 3 %
Triglycerides	14.1±1.7 ^a	31.1±2.6 ^b	27.1±2.7 ^b	25.3±3.0 ^b
Total cholesterol	2.1±0.2 ^a	3.4±0.3 ^b	3.0±0.2 ^b	2.8±0.1

Data are means ± SE for 6–8 mice. The values with different superscript letters differ significantly ($P < 0.05$).

Fig. 1. Tissue histology

Adipose tissue (A; HFHSD control, B; 3% BLEx) and liver (C; HFHSD control, D; 3% BLEx) histology. Tissues are fixed with 4% paraformaldehyde, and thin sections are stained with hematoxylin and eosin.

HOMA-IR and QUICKI values were calculated. HOMA-IR increased and QUICKI decreased in the HFHSD control group compared to the ND group, indicating induction of insulin resistance through HFHSD feeding. Although these parameters tended to be ameliorated in the 3% BLEx groups (Table 6), no significant difference was detected among dietary groups.

OGTT After oral glucose administration, serum glucose levels started to increase and plateaued at 30 or 60 min. Serum glucose levels in the 3% BLEx group were significantly lower than in the HFHSD control group at 30, 60, and 120 min. The area under the curve (AUC) for serum glucose over time was significantly lower in the 3% BLEx group than the HFHSD control group (Fig. 2A, B). To evaluate the change in serum glucose after oral administration, values in all groups at 0 min were adjusted to 0 in Fig. 2C. Glucose change quickly returned to baseline in the ND group whereas values in the HFHSD control group remained high at 120 min. Moreover, BLEx did not have a prominent effect on the serum glucose change at any dose and the AUC values were comparable between HFHSD control and 1% and 3% BLEx.

Discussion

Histological data show hepatic lipid accumulation decreased in the 3% BLEx group compared to the HFHSD control group. Our data are consistent with those of previous studies in which a liver-fat-reducing effect was shown in rats fed a 1–5% BLEx diet (Inoue *et al.* 2011, Nagao *et al.* 2008, Yuji *et al.* 2013). In addition, the

present data reveal the efficacy of BLEx in HFHSD-induced fatty liver, suggesting its efficacy in non-alcoholic hepatic steatosis (NASH) because high-fat-diet fed C57Bl mice are considered models of NASH (Takahashi *et al.* 2012). Reduction of liver weight in the 3% BLEx group may be attributed to the reduction in liver fat. In previous studies, BLEx was fractionated to explore the active component(s) and data revealed that polyphenols rich in rutin and chlorogenic acid, flavonol glycoside flavan-3-ols, and a complex mixture of proanthocyanidins significantly reduced liver TG levels (Inoue *et al.* 2011). These data indicate that BLEx contains several active components in terms of the liver-fat-reducing effect.

In previous studies, blueberry leaf failed to regulate fasting serum glucose levels in spontaneously hypertensive rats and 4-week-old Otsuka Long-Evans Tokushima Fatty (OLETF) rats; this finding is inconsistent with the present data (Inoue *et al.* 2011, Nagao *et al.* 2008, Yuji *et al.* 2013). Although OLETF rats are considered a model of type II diabetes, the onset of hyperglycemia is recognized after 18 weeks of age (Kawano *et al.* 1994). This information indicates that intake of blueberry leaf carries a low risk of hypoglycemia. As shown in Tables 4 and 5, fasting glucose, insulin, and surrogate markers of insulin resistance (HOMA-IR and QUICKI) became abnormal in HFHSD control mice, suggesting induction of insulin resistance. As 3% BLEx clearly ameliorated these insulin resistance-related parameters, BLEx is expected to manipulate glucose metabolism in insulin-resistant states without side effects.

OGTTs are commonly used diagnostic tests for diabetes, and HFHSD fed mice showed a significantly higher AUC for serum glucose after oral glucose administration. Administration of 3% BLEx normalized the AUC, suggesting a substantial preventive effect on insulin resistance. However, although the blood glucose change after glucose administration was obvious, BLEx slightly suppressed glucose change (Fig. 2C) and the AUC was comparable between the HFHSD control and BLEx groups. These data imply that BLEx directly interferes with the absorption of glucose and other sugars, since OGTT was performed in a fasting state and BLEx was estimated to be excreted from the gastrointestinal tract during OGTT. Moreover, inhibition of glucose absorption was exhibited by a number of polyphenols, some of which were detected in BLEx (Deguchi *et al.* 2010, Pereira *et al.* 2011). Therefore, it is reasonable to consider the inhibitory effect of sugar absorption by BLEx.

Table 5. Effect of dietary BLEx on serum biochemical parameters

	ND	HFHSD		
		Control	BLEx 1 %	BLEx 3 %
Glucose (mg/dL)	180±18	236±33 ^a	206±15	153±10 ^b
Insulin (ng/mL)	0.22±0.07	0.60±0.13	0.70±0.18	0.48±0.08
Triglycerides (mg/dL)	80±8	71±4	92±4	86±10
Cholesterol (mg/dL)	106±4	134±10	150±4	126±15

Data are means ± SE for 6-8 mice. The values with different superscript letters differ significantly ($P < 0.05$).

Table 6. Effect of dietary BLEx on parameters in insulin resistance

	ND	HFHSD		
		Control	BLEx 1 %	BLEx 3 %
HOMA-IR index	2.90±0.96	10.64±3.30	9.35±1.95	4.95±0.67
QUICKI	0.355±0.037	0.296±0.016	0.290±0.010	0.314±0.014

Data are means ± SE for 6-8 mice. Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) and Quantitative Insulin Sensitivity Check Index (QUICKI) value were calculated as follows, respectively. $HOMA-IR = \text{Fasting insulin } (\mu\text{U/mL}) \times \text{serum glucose (mg/dL)} / 405$; $QUICKI = 1 / [\log (\text{fasting serum glucose (mg/dL)}) + \log (\text{fasting insulin } (\mu\text{U/mL}))]$

Fig. 2. Oral glucose administration test

Glucose (1.5 g/g body weight) was administered in fasting condition at 2 weeks after the initiation of the experimental diet period. (A) Monitoring of serum glucose concentration and (B) calculation of AUC value for glucose concentration versus time after administration. (C) Change in serum glucose level and (D) its AUC value. Data are represented as means ± SE for 2-6 mice. Asterisk marks shown in A denote significant difference from HFHSD control value. Values not sharing common alphabetic characters shown in B, D are significantly different from each other.

Lee *et al.* evaluated the effect of blueberry leaf extract (70% ethanol) on high-fat-diet induced obesity, and showed a moderate but insignificant reducing effect on serum glucose and insulin (Lee *et al.* 2014). Unfortunately, as this paper did not indicate the species of blueberry, discussion of differences in physiological function between this study and previous studies in terms of species is limited. A number of species of blueberry occur in nature and *Vaccinium virgatum* Aiton used in this study has potent antioxidant activity and contains high levels of polyphenols (Tsuda *et al.* 2013). Dietary polyphenols protect experimental animals from HFHSD-induced oxidative stress and prevent HFHSD-induced glucose metabolism disruption (Lee *et al.* 2009, Feillet-Coudray *et al.* 2009, Hanhineva *et al.* 2010). Therefore, as antioxidant capacity is important to exert anti-diabetic effects, it is reasonable to consider that the anti-diabetic potential of BLEx varies among species.

BLEx from *Vaccinium virgatum* Aiton is rich in polyphenols such as chlorogenic acid, rutin, and proanthocyanidins. Among them, proanthocyanidins are responsible for several beneficial physiological functions. Therefore, several candidates can be raised as active components to ameliorate glucose and lipid metabolism. For instance, chlorogenic acid and rutin ameliorate HFHSD-induced lipid and glucose metabolism (Takahashi *et al.* 2014, Panchal *et al.* 2011, 2012). Moreover, proanthocyanidin should be considered an active component because grape seed extract-derived proanthocyanidins showed potent antioxidant activity and prevented high-fructose diet-induced insulin resistance (Suwannaphet *et al.* 2010).

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