

An extract of chokeberry attenuates weight gain and modulates insulin, adipogenic and inflammatory signalling pathways in epididymal adipose tissue of rats fed a fructose-rich diet

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Abstract

Chokeberries are a rich source of anthocyanins, which may contribute to the prevention of obesity and the metabolic syndrome. The aim of the present study was to determine if an extract from chokeberries would reduce weight gain in rats fed a fructose-rich diet (FRD) and to explore the potential mechanisms related to insulin signalling, adipogenesis and inflammatory-related pathways. Wistar rats were fed a FRD for 6 weeks to induce insulin resistance, with or without chokeberry extract (CBE) added to the drinking-water (100 and 200 mg/kg body weight, daily: CBE100 and CBE200). Both doses of CBE consumption lowered epididymal fat, blood glucose, TAG, cholesterol and LDL-cholesterol. CBE consumption also elevated plasma adiponectin levels and inhibited plasma TNF- α and IL6, compared with the control group. There were increases in the mRNA expression for *Irs1*, *Irs2*, *Pi3k*, *Glut1*, *Glut4* and *Gys1*, and decreases in mRNA levels of *Gsk3 β* . The protein and gene expression of adiponectin and *Ppar γ* mRNA levels were up-regulated and *Fabp4*, *Fas* and *Lpl* mRNA levels were inhibited. The levels of gene expression of inflammatory cytokines, such as *Il1 β* , *Il6* and *Tnfa* were lowered, and protein and gene expression of ZFP36 (zinc finger protein) were enhanced in the epididymal adipose tissue of the rats that consumed the CBE200 extract. In summary, these results suggest that the CBE decreased risk factors related to insulin resistance by modulating multiple pathways associated with insulin signalling, adipogenesis and inflammation.

Key words: Chokeberry extract; Insulin signalling; Adipogenesis; Inflammation

Chokeberry, known as *Aronia melanocarpa*, is found in the eastern parts of North America, as well as Northern and Eastern Europe. Although usually consumed as a fruit, it has also been used in traditional medicine to treat hypertension and atherosclerosis in Russia and Eastern European countries⁽¹⁾. Chokeberry has attracted scientific interest because of its high content of phenolic phytochemicals. The active compounds found in chokeberry include anthocyanins and flavonoids, some at concentrations over five times greater than those found in cranberries^(2,3). A comparative *in vitro* study has shown that chokeberries display higher antioxidant activity with the oxygen radical absorption capacity assay than that obtained with blueberries, cranberries or lingonberries⁽⁴⁾.

Anthocyanins and anthocyanin-rich extracts exhibit diverse potential health benefits in animal and human studies, including cardioprotective⁽⁵⁾, anti-diabetic^(6,7) and anti-inflammatory

properties⁽⁸⁾. Although it has been reported that anthocyanins are poorly absorbed and circulate in the blood exclusively as unmetabolised parent glycosides⁽⁹⁾, Kay *et al.*⁽¹⁰⁾ observed that in human subjects, anthocyanins exist in the circulation primarily as metabolites, and cyanidin 3-glycosides are absorbed and transported in human serum and urine primarily as glucuronide and methyl glucuronide derivatives. Moreover, recent studies in rodents have shown that anthocyanins are rapidly absorbed from both the stomach and small intestine⁽¹¹⁾, with derivatives found in multiple organs, including adipose tissue⁽¹²⁾.

Adipocyte and adipose tissue dysfunction are primary defects in obesity and may link obesity to several health problems, including increased risk of type 2 diabetes, hypertension, dyslipidaemia and atherosclerosis^(13,14). In cultured adipocytes, anthocyanins enhance adiponectin secretion⁽¹⁵⁾,

Abbreviations: CBE, chokeberry extract; CBE100, group fed chokeberry extract at 100 mg/kg body weight daily added to drinking water; CBE200, group fed chokeberry extract at 200 mg/kg body weight daily added to drinking water; CON, group fed with water alone; EAT, epididymal adipose tissues; FABP4, fatty acid-binding protein 4; FAS, fatty acid synthase; FRD, fructose-rich diet; Irs, insulin receptor substrate; LPL, lipoprotein lipase; ZFP36, zinc protein finger 36.

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regulate the expression of multiple adipocyte-specific genes^(15,16) and also reverse TNF- α -induced insulin resistance⁽¹⁷⁾. In addition, dietary anthocyanins were shown to suppress obesity when administered to mice fed high-fat diets^(18,19).

The aim of the present study was to investigate whether feeding an extract of chokeberry improved metabolic parameters in rats fed a fructose-rich diet (FRD) to induce insulin resistance. Effects on body weight gain and epididymal fat accumulation and the underlying molecular mechanisms of action on the expression of the adipose genes involved in insulin signalling, adipogenic and inflammation pathways were evaluated.

Materials and methods

According to the manufacturer, the chokeberry extract (CBE) used was prepared from frozen Aronia berries from Northern Europe. Berries were stirred at room temperature with 4-fold excess by weight of 60% ethanol–water for 3–4 h. The mixture was then centrifuged and the supernatant was spray-dried (CellBerry[®], the dried CBE, was provided by Integrity Nutraceuticals International (Spring Hill, TN, USA)). This extract contained at least 10% anthocyanins based on HPLC and MS analyses (lot no. TGB-071020).

Animals

Male Wistar rats (5 weeks old) were housed in a temperature-controlled room according to the Guidelines for Animal Care of the Beltsville Area Animal Care and Use Committee. After a 1-week acclimatisation period, rats were assigned randomly to receive either the CBE at 100 or 200 mg/kg body weight/d (n 6 for each group: CBE100 and CBE200) added to drinking-water or water alone (CON) group. All rats were placed on a FRD for 6 weeks. The diet contained (g/kg diet): casein, 207; DL-methionine, 3.0; fructose, 600; lard, 50; cellulose, 79.8; AIN mineral mix, 50.0; zinc carbonate, 0.04; AIN vitamin mix 10.0; and green food colour 0.15 (89 247-Teklad Animal Diets, Madison, WI, USA). During the experimental period, the consumption of food and fluid and body weight were monitored every other day. At the termination of the feeding experiment, following an overnight fast, blood glucose levels were tested from blood collected from the tail vein. Rats were then anaesthetised and blood was collected from the portal vein in pre-cooled tubes containing EDTA and centrifuged at 5000 rpm for 15 min at 4°C. The epididymal adipose tissues (EAT) were carefully

removed and weighed before being snap-frozen in liquid N₂ and stored at –80°C until analysed.

Immunoblotting

For the immunoanalysis of adipose tissue, approximately 100 mg of EAT were homogenised at 4°C for 30 s in lysis buffer containing 20 mM-Tris (pH 7.4), 2 mM-EDTA, 50 mM-NaF, 200 μ M-Na₃VO₄, 250 μ M-phenylmethylsulfonyl fluoride, 1 μ M-leupeptin, 1 μ M-pepstatin and 0.36 μ M-aprotinin. Protein concentrations were determined by a commercial assay (Bio-Rad D_c protein assay; Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard. Rabbit polyclonal antibody against adiponectin was purchased from ProSci (Poway, CA, USA). Rabbit polyclonal antibody against zinc finger protein 36 (ZFP36) was purchased from Genway (San Diego, CA, USA).

Gene expression in epididymal adipose tissues

Total RNA was isolated from EAT using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentrations and integrity were determined using RNA 6000 Nano Assay Kit and the Bioanalyzer 2100, according to the manufacturer's instructions (Agilent, Santa Clara, CA, USA). The complementary DNA were synthesised from total RNA using SuperScript II RT (Invitrogen). The primers used are described in our previous study⁽²⁰⁾ and included in Table 1. Real-time quantitative PCR was performed using SYBR Green PCR Master Mix (ABI, Foster, CA, USA). The expression of the housekeeping gene, peptidylprolyl isomerase A, was used to normalise the expression of target genes.

Biochemistry

Plasma adiponectin was determined with a rat ultrasensitive EIA (Phoenix Pharm, Burlingame, CA, USA). Plasma NEFA were measured using a colorimetric assay (Wako, Richmond, VA, USA). Serum TNF- α and IL-6 were determined with a rat ultrasensitive EIA (Alpco, Salem, NH, USA). Measurement of blood glucose, insulin, TAG and cholesterol were performed as described^(20,21).

Statistical analyses

Data were analysed by one-way ANOVA followed by the least square difference (LSD) test. P values < 0.05 were considered significant.

Table 1. Real-time PCR primers

Gene	Sequence (5' to 3' forward)	Sequence (5' to 3' reverse)
<i>AdipoQ</i>	AGGAAACTTGTGCAGGTTGGA	GAACACCTGCGTCTCCCTTCT
<i>Lpl</i>	TGGAGCCCATGCTGCTG	CAAGCCAGTAATTCTATTGACCTTCTT
<i>Fas</i>	GCCTCACTCCGAGGAACAAACA	CCCGGCATTTCAGAAATAGTGGCA
<i>Fabp4</i>	GAAGTGGGAGTGGGCTTT	TTATGGTGTCTTGACTTTCCT

AdipoQ, adiponectin; *Lpl*, lipoprotein lipase; *Fas*, fatty acid synthase; *Fabp4*, fatty acid-binding protein 4.

Table 2. Effects of chokeberry extract (CBE) on body weight and epididymal pad weight (Mean values with their standard errors for six rats)

	CON		CBE100		CBE200	
	Mean	SE	Mean	SE	Mean	SE
Initial body weight (g)	181 ^a	3	183 ^a	2	180 ^a	1
Final body weight (g)	416 ^a	6	402 ^a	5	398 ^a	5
Body weight gain (g)	236 ^a	5	219 ^b	5	217 ^b	4
Epididymal adipose weight (g)	9.2 ^a	0.2	8.3 ^b	0.2	8.0 ^a	0.2

CON, group fed with water alone; CBE100, group fed CBE at 100 mg/kg body weight daily added to drinking water; CBE200, group fed CBE at 200 mg/kg body weight daily added to drinking water.

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

Results

General observations and plasma biochemistry

Food intake and water consumed did not differ among the three groups. Body weight gain and epididymal adipose weight were reduced at both levels of CBE intake (Table 2, $P < 0.05$). Fasting blood glucose and plasma insulin, TAG, total cholesterol, LDL-cholesterol and plasma NEFA levels were all reduced in animals consuming both levels of CBE. CBE consumption increased the plasma adiponectin and HDL-cholesterol levels (Table 3). In addition, a significant reduction in the plasma IL-6 and TNF- α occurred in both the CBE groups (Table 3).

Chokeberry extract altered mRNA expression in the insulin signalling pathway and glucose uptake in epididymal adipose tissues

As shown in Fig. 1(a), CBE consumption (CBE200) with the FRD enhanced mRNA levels of components of the insulin signalling pathway, including increases in mRNA levels of insulin receptor substrate 1 (*Irs1*) (2.3-fold), *Irs2* (1.8-fold) and phosphatidylinositol 3 kinase regulatory subunit 1 (*Pi3kr1*) (1.5-fold), and inhibited phosphatase and tensin homolog (*Pten*) mRNA levels (0.61-fold), compared with the FRD-fed control rats.

As shown in Fig. 1(b), CBE consumption (CBE200) induced an increase in *Glut1* (1.6-fold), *Glut4* (1.5-fold) and glycogen

synthase (*Gys*) (1.5-fold) mRNA expression, and inhibited glycogen synthase kinase 3 β (*Gsk3 β*) mRNA levels (0.62-fold), compared with the FRD-fed control rats. Similar trends were observed in the CBE100 group but values were not significant (data not shown).

Consumption of chokeberry extract modulated the expression of genes and proteins involved in epididymal adipose adipogenesis

As shown in Fig. 2(a) and (b), CBE consumption (CBE200) caused a significant increase in adiponectin (*AdipoQ*) mRNA levels (2.1-fold) and adiponectin protein levels (169%), compared with the FRD-fed control rats ($P < 0.05$, both). CBE also induced *Ppar γ* mRNA expression (1.6-fold) and inhibited fatty acid binding protein 4 (*Fabp4*) (0.7-fold), fatty acid synthase (*Fas*) (0.63-fold) and *Lpl* (0.65-fold) mRNA expression (Fig. 2(c)), but did not significantly affect fatty acid translocase (*Cd36*) mRNA expression. Changes in the CBE100 group were not significant (data not shown).

Chokeberry extract inhibited the epididymal adipose inflammation gene expression and induced ZFP36 expression

As shown in Fig. 3(a), CBE consumption (CBE200) caused a significant decrease in *Tnf α* (0.52-fold), *Il β* (0.38-fold) and *Il6* (0.45-fold) mRNA levels, compared with the FRD-fed

Table 3. Effects of chokeberry extract (CBE) on blood and plasma parameters in the fasted state (Mean values with their standard errors for six rats)

	CON		CBE100		CBE200	
	Mean	SE	Mean	SE	Mean	SE
Blood glucose (mmol/l)	4.9 ^a	0.2	4.4 ^b	0.2	4.3 ^b	0.3
Insulin (ng/ml)	1.3 ^a	0.12	0.95 ^b	0.06	0.91 ^b	0.09
TAG (mmol/l)	1.92 ^a	0.15	1.25 ^b	0.11	1.15 ^b	0.13
Cholesterol (mmol/l)	2.35 ^a	0.19	1.65 ^b	0.18	1.58 ^b	0.14
LDL-C (mmol/l)	1.18 ^a	0.13	0.83 ^b	0.13	0.75 ^b	0.13
HDL-C (mmol/l)	0.46 ^a	0.03	0.62 ^b	0.04	0.70 ^b	0.02
NEFA (mmol/l)	0.75 ^a	0.07	0.48 ^b	0.03	0.43 ^b	0.05
Adiponectin (μ g/ml)	19.8 ^a	2.1	27.3 ^b	2.4	29.8 ^b	2.6
IL-6 (pg/ml)	220 ^a	10	158 ^b	7	81 ^c	5
TNF- α (pg/ml)	353 ^a	9	190 ^b	10	103 ^c	6

CON, group fed with water alone; CBE100, group fed CBE at 100 mg/kg body weight daily added to drinking water; CBE200, group fed CBE at 200 mg/kg body weight daily added to drinking water; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

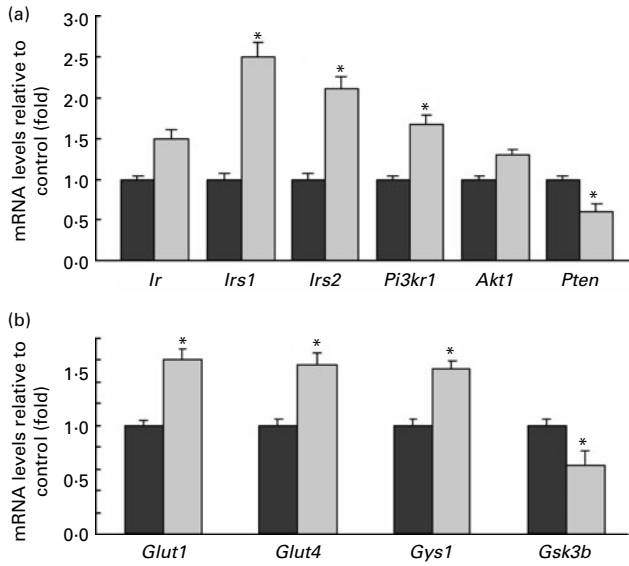


Fig. 1. Effects of feeding a chokeberry extract (CBE) on the mRNA levels related to insulin signalling, GLUT and glycogen synthesis in epididymal adipose tissue. ■, Control group; □, group fed CBE at 200 mg/kg body weight daily (CBE200). (a) *Ir*, *Irs1*, *Irs2*, *Pi3kr1*, *Akt1* and *Pten* and (b) *Glut1*, *Glut4*, *Gys1* and *Gsk3b*. Values are means with their standard errors and are presented as fold of control (n 5–6). *Mean values were significantly different from those of the control group ($P < 0.05$).

control rats. In contrast to these inflammatory factors, CBE consumption enhanced *Zfp36* mRNA and ZFP36 protein expression (Fig. 3(b) and (c), 1.45-fold and 139%). Changes in the CBE100 group were not significant (data not shown).

Discussion

Consumption of a FRD contributes to insulin resistance, hyperinsulinaemia, dyslipidaemia and hypertension in animal models^(22–24). Furthermore, consumption of a FRD leads to abdominal adipose tissue endocrine dysfunction in normal rats, and increased adipose tissue mass and adipocyte size⁽²⁵⁾. Growing evidence suggests that adipose tissue plays a crucial role in the regulation of systemic energy homeostasis, insulin sensitivity and lipid/carbohydrate metabolism⁽²⁶⁾. In the present study, we used the FRD-fed rat model to investigate the effects of a CBE rich in anthocyanins. The present data indicated that CBE consumption reduced weight gain, epididymal fat accumulation and improved systemic glucose/lipid metabolism. We also demonstrated that CBE consumption increased plasma adiponectin levels and decreased plasma TNF- α and IL-6 levels. In addition, we demonstrated that consumption of CBE regulated gene expression in multiple pathways involved in insulin signalling, adipogenesis and inflammation.

FRD-induced adipose tissue dysfunction in studies of high-polyphenol extracts of other plants caused alterations in the production of many adipocyte-derived factors such as NEFA and adiponectin⁽²⁴⁾. These dysregulated factors induced local and systemic insulin resistance, which is a major contributor to the pathogenesis of type 2 diabetes and plays a key role in associated metabolic abnormalities, such as obesity, dyslipidaemia and hypertension⁽²⁷⁾. Evidence from human

and animal studies demonstrated that loss of body weight is associated with an increase of insulin sensitivity^(6,28,29). In the present study, we found that consumption of a CBE, high in polyphenols, reduced weight gain and epididymal fat accumulation, and improved systemic insulin sensitivity-related factors, such as fasting glucose, plasma insulin and lipids. At the molecular level, although the FRD impaired insulin signalling pathways in multiple tissues, such as liver⁽³⁰⁾, skeletal muscle⁽²³⁾ and adipose tissue⁽²⁴⁾, the consumption of CBE improved the impaired gene expression related to insulin signalling and glucose uptake in EAT, and up-regulated the decreased *Irs1*, *Irs2* and *Pi3k* mRNA expression and the expression of other genes related to carbohydrate metabolism, such as *Glut1*, *Glut4* and *Gys1*. CBE also inhibited the expression of *Pten* and *Gsk3 β* mRNA, which are increased in insulin-resistant adipose tissue^(31,32) (Fig. 1).

In contrast to many other factors derived from adipose tissue, circulating adiponectin and adipose adiponectin expression are decreased in insulin resistance^(33,34). In most clinical reports, primate studies and genetic models, serum adiponectin levels have been reported to be negatively correlated with body weight, visceral fat mass and resting insulin levels^(35–37). Transgenic mice overexpressing *AdipoQ* have increased insulin sensitivity and improved glucose tolerance and TAG clearance⁽³⁸⁾. We have reported previously that FRD feeding significantly decreased plasma adiponectin and *AdipoQ* mRNA expression⁽²⁴⁾. In the present study, we observed that feeding CBE significantly increased plasma adiponectin levels and the mRNA and protein expression of adipose adiponectin of FRD-fed rats (Fig. 2). These results support the previous finding in human subjects that combination therapy of statins with an extract of chokeberry

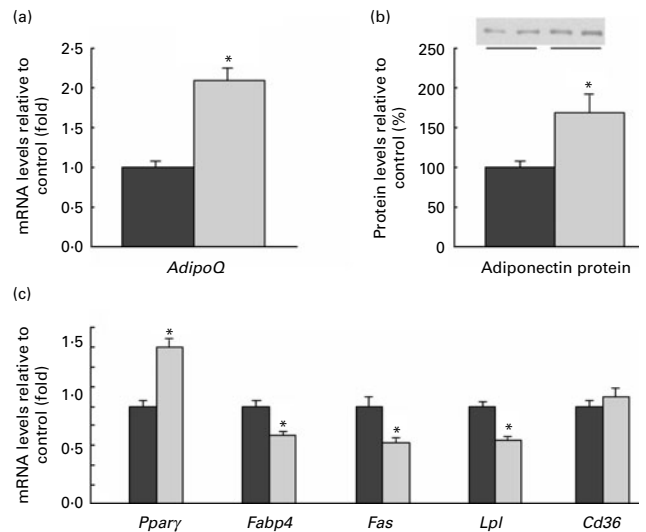


Fig. 2. Effects of feeding a chokeberry extract (CBE) on the expression of genes and proteins involved in adipogenesis in epididymal adipose tissue. ■, Control group; □, group fed CBE at 200 mg/kg body weight daily (CBE200). (a) *AdipoQ* mRNA, (b) adiponectin protein and (c) *Ppar γ* , *Fabp4*, *Fas*, *Lpl* and *Cd36* mRNA. (b) Representative experiments on the immunoblots of adiponectin were analysed using densitometry. Values are means with their standard errors and are presented as percentage of control (n 5–6). *Mean values were significantly different from those of the control group ($P < 0.05$).

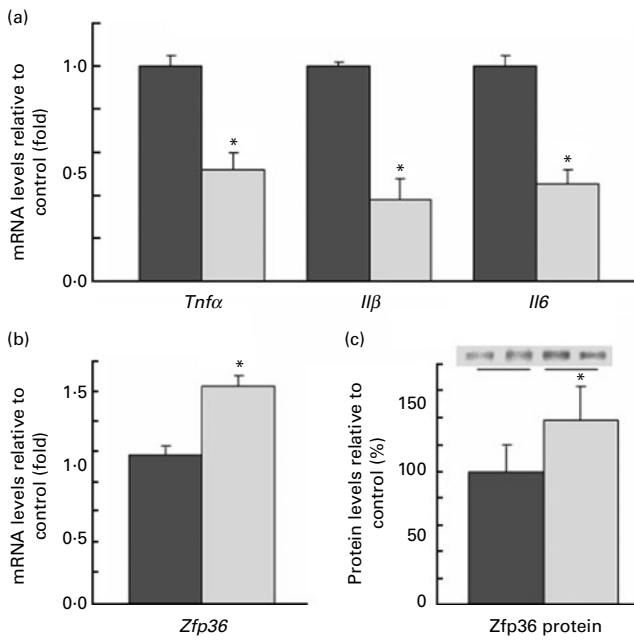


Fig. 3. Effects of feeding a chokeberry extract (CBE) on the expression of genes and proteins involved in inflammation in epididymal adipose tissue. ■, Control group; □, group fed CBE at 200 mg/kg body weight daily (CBE200). (a) *Tnfa*, *Il1b* and *Il6* mRNA, (b) *Zfp36* mRNA and (c) ZFP36 protein. (c) Representative experiments on the immunoblots of ZFP36 were analysed using densitometry. Values are means with their standard errors and are presented as fold of control ($n = 5-6$). * Mean values were significantly different from those of the control group ($P < 0.05$).

increased plasma adiponectin levels in patients after myocardial infarction⁽⁸⁾. Adiponectin possesses insulin sensitising and anti-atherogenic properties⁽³⁵⁾.

It is well known that increased adipose tissue mass and adipocyte dysfunction associated with obesity are linked to the abnormal regulation of adipogenesis⁽³⁹⁾. PPAR γ , a master regulator of adipogenesis⁽⁴⁰⁾, plays a critical role in glucose metabolism and energy homeostasis^(41,42). Fructose feeding induced a lower *Ppar γ* mRNA expression in white adipose tissue, and a PPAR ligand reversed the down-regulated expression of PPAR γ and systemic insulin resistance⁽⁴³⁾. In the present study, we observed that CBE consumption increased *Ppar γ* mRNA expression in EAT. PPAR γ regulates multiple genes in the adipose tissue regulating adipogenesis, including those encoding the adipocyte fatty acid-binding protein 4 (FABP4), fatty acid synthase (FAS) and lipoprotein lipase (LPL). FABP4 is postulated to be an early marker of the metabolic syndrome and the future onset of type 2 diabetes^(44,45). *Fabp4*-deficient mice are protected from insulin resistance, hyperglycaemia, dyslipidaemia and atherosclerosis^(46,47). We have reported that FRD feeding induced the overexpression of *Fabp4* mRNA⁽²⁴⁾. FAS is a key enzyme in *de novo* lipogenesis. Studies have reported that *Fas* mRNA and protein levels are elevated in obese Zucker rats⁽⁴⁸⁾, and polyphenols from cinnamon⁽²⁴⁾ and dietary green tea inhibit *Fas* mRNA expression in diet-induced insulin-resistant animals⁽⁴⁹⁾. LPL, the rate-limiting enzyme in TAG-rich lipoprotein catabolism, provides TAG-derived fatty acids to adipose tissue for storage.

Studies have shown that polyphenols from cinnamon and green tea also inhibit *Lpl* mRNA expression and other genes of lipogenesis^(24,49). In the present study, the data show that feeding polyphenols from chokeberry suppressed *Fabp4*, *Fas* and *Lpl* mRNA levels in EAT. CD36, referred to as fatty acid translocase, is a transmembrane protein present in many tissues that is believed to play a role in facilitating fatty acid transport⁽⁵⁰⁾. In *ob/ob* mice, and FRD-fed rats, CD36 mRNA⁽⁵¹⁾ and protein⁽²⁴⁾ levels in the adipose tissue were increased. CBE consumption did not affect CD36 mRNA expression in the adipose tissue.

Substantial evidence indicates that a state of low-grade chronic inflammation typically is associated with obesity, and the increased production of pro-inflammatory cytokines by adipose tissue plays a crucial role in the development of insulin resistance^(52,53). FRD feeding also induces the overexpression of plasma TNF- α and IL-6, which both contribute to the development of CVD by promoting insulin resistance, dyslipidaemia and endothelial dysfunction⁽⁵⁴⁾. TNF- α is known to be a potent negative regulator of adipogenesis and PPAR γ function⁽⁵⁵⁾. The present results suggest that consumption of CBE not only inhibited the plasma levels of TNF- α and IL-6 but also induced a decrease of mRNA expression of *Tnfa* and *Il1b* and *Il6*. This is in agreement with a human study in which chronic chokeberry consumption reduced the severity of plasma inflammation, increased anti-inflammation factor and increased plasma adiponectin levels⁽⁸⁾. Previous studies suggested that *Zfp36*, an anti-inflammatory protein, increased *Tnfa* mRNA degradation by binding to its 3' untranslated region⁽⁵⁶⁾, and that omental adipose *Zfp36* mRNA levels were correlated with insulin, insulin resistance index and adiponectinaemia in women⁽⁵⁷⁾. In the present study, we found that CBE consumption increased adipose *Zfp36* protein and mRNA expression, which is consistent with CBE feeding-induced decreases in TNF- α expression and increases in adiponectin expression in plasma and adipose tissue.

In summary, the present study provides evidence that an anthocyanin-rich extract of chokeberry reduced body weight gain and abdominal fat, improved the risk factors related to the metabolic syndrome in plasma and modulated multiple signalling pathways related to adipose dysfunction in an animal model. The present findings suggest that chokeberry or its extract might be beneficial in preventing or decreasing obesity and the metabolic syndrome. Further studies are needed in human volunteers at increased risks for diet-related chronic disease to ascertain the beneficial effects from consumption of polyphenols such as those found in chokeberry.

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