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RESEARCH PAPER

Cyanidin and malvidin in aqueous extracts of black carrots fermented with *Aspergillus oryzae* prevent the impairment of energy, lipid and glucose metabolism in estrogen-deficient rats by AMPK activation

Sunmin Park · Suna Kang · Do-Youn Jeong · Seong-Yeop Jeong · Jae Jung Park · Ho Sik Yun

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Abstract Black carrots (*Daucus carota L.*) are rich in anthocyanins which contribute many health benefits, but are limited by bioavailability and instability when exposed to oxygen, heat and light. Fermenting black carrots may improve the stability, absorption and bioactivity of its anthocyanins. Here, we examined whether and by what mechanisms the long-term consumption of unfermented black carrot extract (BC) and its extracts fermented with *Lactobacillus plantarum* (BCLP) or *Aspergillus oryzae* (BCAO) might prevent menopausal symptoms including impaired energy, glucose and lipid metabolism in estrogen-deficient animals with diet-induced obesity. Ovariectomized (OVX) rats were fed four different high-fat diets containing 2 % dextrin (OVX-control), 2 % BC, 2 % BCLP, or 2 % BCAO for 12 weeks. Sham rats were fed high-fat diets containing 2 % dextrin. The contents of total anthocyanins increased in BCAO compared to BC and BCLP, whereas the contents of cyanidin-3-rutinosides, malvidin-3,5-diglycosides and delphine-3-glucoside were lower and cyanidin and malvidin were much higher in BCLP and BCAO than BC. Fat mass and weight gain were lower in descending order of OVX-control > BC and BCLP > BCAO due to increased energy expenditure and fat oxidation. However, BC, BCLP and especially BCAO all normalized HOMA-IR, an indicator of insulin resistance and glucose intolerance, in OVX rats. OVX increased serum total and LDL cholesterol and triglycerides, but BC, BCLP and BCAO significantly prevented the increases. BCAO markedly decreased hepatic triglyceride levels by increasing gene expressions of CPT-1 and PPAR-α, which are involved in fatty acid oxidation, and decreasing mRNA expressions of FAS and SREBP-1c, which are associated with fatty acid synthesis. This was related to increased pAMPK → pACC signaling and improved hepatic insulin signaling (pAkt → pFOXO-1). Cyanidin and malvidin markedly decreased fat accumulation in 3T3-L1 adipocytes by increasing CPT-1 and decreasing FAS and SREBP-1c expression in comparison with cyanidin-3-rutinoside and malvidin-3,5-diglycosides. In conclusion, with increasing cyanidin and malvidin, BCAO prevented the exacerbation of lipid and glucose metabolism by activating hepatic insulin signaling and AMPK activation by in OVX rats.

Keywords Black carrot · Fermentation · Menopause · Glucose tolerance · Dyslipidemia · Body composition

Abbreviations

HRT	Hormone replacement therapy
PPAR	Peroxisome proliferator-activated receptor
OVX	Ovariectomized
En %	Percent energy
BC	Unfermented black carrot extracts
BCAO	Black carrot fermented with *Asperillus oryzae*

S. Park · S. Kang
Department of Food and Nutrition, Obesity/Diabetes Center, Asan, Korea

S. Park (✉)
Department of Food and Nutrition, Hoseo University, 165 Sechul-Ri, BaeBang-Yup, Asan-Si, ChungNam-Do 336-795, South Korea
e-mail: smpark@hoseo.edu

D.-Y. Jeong · S.-Y. Jeong
Department of R&D, Sungchang Research Center for Fermentation Microbes, Sunchang, Korea

J. J. Park · H. S. Yun
WellRun B&F, Cheonan-Si, Chungnam-Do, Korea

BCLP	Black carrot fermented with *Lactobacillus plantarum*
OGTT	Oral glucose tolerance test
AUC	Area under the curve
HOMA-IR	Homeostasis model assessment for insulin resistance index
AMPK	AMP kinase
RQ	Respiratory quotient
DEXA	Dual-energy X-ray absorptiometry
PCR	Polymerase chain reaction
SREBP-1c	Sterol regulatory element-binding protein-1c
FAS	Fatty acid synthase
CPT-1	Carnitine palmitoyltransferase-1
PKB or Akt	Protein kinase B
FOXO-1	Forkhead box protein O-1
ACC	Acetyl CoA carboxylase

Introduction

The prevalence of metabolic syndrome in postmenopausal women is higher than in pre-menopausal women (Ainy et al. 2007; Chedraui et al. 2013). Furthermore, osteoporosis is more prevalent in older women over the age of 50 (Gourlay et al. 2014). This is, at least in part, due to estrogen deficiency which impairs energy, glucose, lipid and bone metabolism in postmenopausal women (Baños et al. 2011; Lizcano and Guzmán 2014). Estrogen deficiency promotes a positive energy balance by increasing food intake and decreasing energy expenditure through its interaction with orexigenic and anorexigenic hormones and changes in body fat distribution characterized by greater visceral fat mass (Butera 2010).

Genistein administration (0.15 % supplemented diet) was shown to decrease both food intake and parametrial, inguinal and retroperitoneal fat pads in OVX mice (Kim et al. 2006). Increased visceral fat mass results in the release of cytokines that induce inflammation (Tchernof and Després 2013). The accumulation of intra-abdominal fat has emerged as a risk factor for metabolic syndrome which increases the risk of diabetes, dyslipidemia, hypertension and cardiovascular disease (Björntorp 1996; Tchernof and Després 2013). However, genistein administration increases uterine mass, although not as much as estrogen therapy (Diel et al. 2004). Treatment with resveratrol has only a weak estrogenic effect and induces proliferation of uterine cells, but it suppresses the metabolic alteration in OVX rats (Majumdar et al. 2014). Therefore, menopausal women have increased risks of metabolic diseases, but some plant bioactive compounds may reduce the risks without greatly increasing uterine proliferation, and the effects may or may not be related to phytoestrogenic effects of those compounds.

Black carrots (*Daucus carota L.*) are mostly grown in Middle Eastern countries and have recently been grown in South Korea. They are rich in anthocyanins as well as carotenoids that have many health benefits. No studies have yet assessed benefits of black carrots in metabolic diseases, but anthocyanins in other fruits and vegetables such as blueberry have been studied. Foods containing anthocyanins typically have natural food coloring from red to deep violets, and different colors of foods are the result of different compositions of anthocyanins (Tsuda 2012). Since the different compositions of anthocyanins contribute distinct health benefits (Guo et al. 2012a, b), different foods rich in anthocyanins may have different functions. However, anthocyanins have limitations for use as functional foods. Anthocyanins are very sensitive to heat, light, and pH changes and are susceptible to oxidation and loss of functions (Noh et al. 2013). During the initial processing, black carrot anthocyanins are oxidized by native enzyme polyphenol oxidases to make brown pigments (Jing and Giusti 2007). However, heating can denature the native enzymes in the foods and inhibit the production of brown pigments, but heat and light themselves can change the colors (Noh et al. 2013; Jing and Giusti 2007). Furthermore, anthocyanins containing glycosides are less efficiently absorbed in the intestine and biological functions may be altered because the anthocyanins undergo structural changes during digestion (Felgines et al. 2002; Hidalgo et al. 2012). The fermentation of black carrots may eliminate some of the limitations. After fermentation anthocyanins may be stable against oxidation and better absorbed due to the removal of glycosides from the anthocyanins.

We hypothesized that fermentation of black carrot would change the structure of anthocyanins to improve stability, bioavailability and bioactivity and the long-term consumption of black carrot extracts fermented with *Aspergillus oryzae* or *Lactobacillus plantarum* would prevent menopausal symptoms such as impairments of energy, glucose, and lipid metabolisms in estrogen-deficient animals with diet-induced obesity. The present study examined the hypothesis and investigated the mechanisms of the metabolic effects of black carrot extracts in ovariectomized (OVX) rats fed high-fat diets.

Materials and methods

Preparation of fermented black carrots and their extracts

Black carrots were provided by Well-Run B&F (Cheoan, Korea) and extracted with water at 70 °C for 2 h in an

ultrasonic extractor. The extracts were concentrated with a rotary evaporator by 50 % and were centrifuged at 8000×*g* for 30 min. The concentrates were then freeze-dried. The concentrated extracts were fermented with *A. oryzae* SRCM 23 or *L. plantarum* SRCM 9 obtained from Institute of Sunchang Fermented Soybean Products (Sunchang, Korea). *A. oryzae* SRCM 23 and *L. plantarum* SRCM 9 were cultivated in YM broth at 25 °C for 72 h and MRS broth at 37 °C for 24 h in a shaker (160 rpm, Jeio Tech, Daejeon, Korea), respectively, to expand the number of *A. oryzae* and *L. plantarum*. Black carrot extracts were sterilized at 120 °C, 1.5 atm for 15 min and cooled to room temperature. They were inoculated with 1 % (v/v) of either *Aspegillus* or *Lactobacillus* and fermented at 25 °C and 37 °C for 120 h, respectively, and then freeze-dried.

The contents of total anthocyanins and carotenoids

The yields of back carrots without fermentation and with fermentation by *A. oryzae* and *L. plantarum* were 12.8, 13.1, and 13.0 %, respectively. The lyophilized extracts were dissolved in methanol.

Total anthocyanin contents were measured using a pH differential method (Noh et al. 2013; Lin and Chou 2009). Briefly, the extract was diluted in a pH 1.0 solution (0.1 M HCl, 25 mM KCl) and in a pH 4.5 solution (0.4 M CH_3-COONa). The absorbances of the mixtures were then measured at 534 and 700 nm against distilled water. Cyanidin-3-glucoside (ChromaDex, USA) was used as a standard and results were expressed as mg of cyanidin-3-glucoside equivalents in 100 g of dried sample). The total anthocyanin content was calculated using the equation described previously.

Total carotenoid content was measured using a spectrophotometric method. Samples were diluted with distilled water and 80 % methanol (1:1:2, v/v/v). The mixture was mixed with *n*-hexane. The mixture was centrifuged for 5 min at 4 °C, and absorbance of the hexane layer was measured at 350 nm spectrophotometrically. Results were expressed as mg of β-carotene (Sigma, St. Louise, USA) in 100 g of dried sample.

The contents of some anthocyanins in the extracts of black carrots with and without fermentation were analyzed by HPLC (Agilent Technologies, USA) with a Luna C18 column (4.6 × 250 mm, 5 μm; Phenomenex, USA). The mobile phase consisted of the solvents, distilled water (A) and acetonitrile (B). The following gradient was used: 0 min, A:B 100:0 (v/v); 10 min, A:B 88:12; 20 min, A:B 80:20; 35 min, A:B 60:40; 40 min, A:B 10:90; and 42 min, A:B 0:100. The mobile phase flow rate was 1.0 mL/min, the column temperature was 30 °C, the injection volume was 10 μL, and UV detection was at 254 nm. Cyanidin-3-rutinoside, delphine-3-glucoside, malvidin-3,5-diglycosides, and malvidin-3-glucoside (1–600 μg/mL; ChromaDex) and cyanidin chloride, delphinidin chloride, and malvidin chloride (Sigma) were used as standards.

Animals and experimental design

Female Sprague–Dawley rats (weighing 278 ± 20 g, aged 9–10 weeks) were housed individually in stainless steel cages in a controlled environment (23 °C and with a 12-h light/dark cycle). All surgical and experimental procedures were approved by the Animal Care and Use Review Committee of Hoseo University, Korea (2012-03). Experimental animals were freely given water and a high-fat diet containing black carrot extracts for a 12-week experimental period to determine long-term effects of estrogen-deficient metabolism alteration (Ko et al. 2013). The high-fat diet was a semi-purified, modified AIN-93 formulation for experimental animals (Reeves et al. 1993). The diet consisted of 40 percent energy (En %) from carbohydrates, 20 En % from protein and 40 En % from fats. The major carbohydrate, protein and fat sources were starch plus sugar, casein (milk protein) and lard (CJ Co., Seoul, Korea), respectively.

Rats underwent OVX or a sham operation under anesthesia induced by intramuscular injections of a mixture of ketamine and xylazine (100 and 10 mg/kg body weight, respectively). A mid-ventral incision was made, and the ovary was isolated by the ligation of the most proximal portions of the oviduct, and it was removed with scissors. This procedure was repeated on the contralateral side. The same procedure was carried out for the sham groups except for the removal of the ovaries (Ko et al. 2013). Forty OVX rats were randomly assigned to the following four groups of ten rats each: (1) 2 % black carrot extracts (OVX-BC), (2) 2 % black carrot fermented with *A. oryzae* (OVX-BCAO), (3) black carrot fermented with *L. plantarum* (OVX-BCLP) and (4) 2 % dextrose (placebo; OVX-control). Ten sham-operated rats were assigned to a high-fat diet containing 2 % dextrose (Sham-control) as the normal-control group.

Metabolic analysis

Overnight-fasted serum glucose levels, food and water intake, and body weight were measured every Tuesday at 10 a.m. At the 11th week of the experimental period an oral glucose tolerance test (OGTT) was performed in overnight feed-deprived rats by orally administering 2 g/kg body weight of glucose every 10 min for 90 and 120 min, and serum insulin levels were measured at 0, 20, 40, 60, 90 and 120 min (Ko et al. 2013). Serum glucose and insulin levels were measured using a Glucometer (Accuchek, Roche Diagnostics, Indianapolis, IN) and RIA kit (Linco Research,

Billerica, MA), respectively. Homeostasis model assessment for insulin resistance index (HOMA-IR) was calculated as fasting serum insulin (μU) X fasting serum glucose (mmol/L)/22.5.

At the end of the study, rats were anesthetized with ketamine and xylazine (100 and 10 mg/kg body weight, respectively). Blood samples for serum isolation were collected by abdominal cardiac puncture. After blood collection, human insulin (5 U/kg body weight; Lily) was injected through the inferior vena cava of the rats to determine insulin signaling in the liver and after 10 min and their tissues were collected. Peri-uterine and retroperitoneal fat masses and uteruses were then removed and weighed. Uterus index was calculated as uterus weight divided by body weight.

Energy expenditure analysis by indirect calorimetry

After 11 weeks of the assigned treatment, energy expenditure was assessed at the beginning of the dark phase of the light/dark cycle after 6 h of fasting. The rats were placed into metabolic chambers (airflow = 800 mL/min) with a computer-controlled O_2 and CO_2 measurement system (BIOPAC Systems, Inc., Goleta, CA) to determine their calorimetric parameters. The respiratory quotient (RQ) and resting energy expenditure were calculated using previously reported equations (Ko et al. 2013). Average oxygen consumption (VO_2) and average carbon dioxide production (VCO_2) were integrated over periods of 30 min. After the experiment, data were averaged over 1-min intervals and VO_2 and VCO_2 values were corrected for metabolic body size ($kg^{0.75}$). Carbohydrate and fat oxidation were calculated from non-protein oxygen consumption, as were their relative oxidative proportions and the amount of oxygen consumed per gram of substrate oxidized (Ko et al. 2013).

Body composition

After anesthetization with ketamine and xylazine (100 and 10 mg/kg body weight, respectively), the rats were laid in a prone position with their hind legs maintained in external rotation with tape, prior to the euthanizing the rats after 11 weeks of experimental periods. Hip, knee and ankle articulations were in 90° flexion. Upon completion of scanning, lean and fat mass was determined in the abdomen and leg by dual-energy X-ray absorptiometry (DEXA) using an absorptiometer (pDEXA Sabre; Norland Medical Systems Inc., Fort Atkinson, WI, USA), which was equipped with the appropriate software for assessment in small animals (Ko et al. 2013).

Isolation of liver total RNA and real-time PCR

Livers were randomly selected and collected from five rats of each group at the end of each treatment. Each liver was powdered with a cold steel mortar and pestle, and then mixed with a monophasic solution of phenol and guanidine isothiocyanate (TRIzol reagent; Life Science Technology, Rockville, MD) for total RNA extraction, according to the manufacturer's instructions. RNA was quantified by Lamda 850 spectrophotometry (Perkin Elmer, Waltham, MA, USA). The cDNA was synthesized from 1 μg RNA extracted from individual rats using a superscript III reverse transcriptase kit (Life Science Technology). Five different cDNAs from each rat were made from each group, and each cDNA was used for real-time PCR. Equal amounts of cDNA and primers for specific genes were mixed with SYBR Green mix (Bio-Rad, Richmond, CA) in duplicate and amplified using a real-time PCR instrument (Bio-Rad). Thermal cycling conditions were 55 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 94 °C for 20 s, 65 °C for 30 s and 72 °C for 20 s. To assess changes in the expression of genes related to fatty acid synthesis and oxidation in the liver, the expressions of sterol regulatory element-binding protein-1c (SREBP-1c), fatty acid synthase (FAS), peroxisome proliferator-activated receptor-α (PPAR-α) and carnitine palmitoyltransferase-1 (CPT-1), were measured with corresponding primers. Cycle of threshold (CT) for each sample was determined. The gene expression level in unknown samples was quantitated using the comparative CT method (Livak and Schmittgen 2001). ΔCT was calculated via formula: ΔCT = CT (target gene) − CT (endogenous reference gene, β-actin). Relative fold-change in expression was calculated by the equation of $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$. Results were presented as $2^{-\Delta\Delta CT}$.

Immunoblot analysis

The livers were dissected after 10 min of insulin stimulation from five rats of each group was lysed with a 20 mM Tris buffer (pH 7.4) containing 2 mM EDTA, 137 mM NaCl, 1 % NP40, 10 % glycerol, and 12 mM a-glycerol phosphate and protease inhibitors. After 30 min on ice, the lysates were centrifuged for 10 min at 12,000 rpm at 4 °C and protein concentrations determined using a kit (Bio-Rad). Each lysate from randomly selected five rats of each group was used for immunoblotting assay. Lysate samples with equivalent protein level (30–50 μg) were directly resolved by SDS-PAGE (Park et al. 2008). One or two samples from each group were running in the same gel, and this was repeated three times. Immunoblotting with specific antibodies against protein kinase B (PKB or Akt), forkhead box protein O-1 (FOXO-1), acetyl CoA carboxylase (ACC), β-actin and phosphorylated PKB^{Ser473},

FOXO-1^{Ser253}, AMPK^{thr172} and ACC^{Ser79} (Cell Signaling Technology, Beverly, MA) as previously described. The intensity of protein expression was determined using Imagequant TL (Amersham Biosciences, Piscataway, NJ).

3T3-L1 fibroblast differentiation into adipocytes

3T3-L1 fibroblasts were grown and differentiated into adipocytes, as previously described. They were differentiated into adipocytes in high-glucose DMEM media with differentiation inducers containing 1 mg/mL insulin, 50 μM dexamethasone, and 0.8 mM isobutylmethyl xanthine (Sigma, St. Louis, MO) and left for 4 days, followed by an incubation period lasting 6–8 days in high-glucose DMEM without differentiation inducers. During the entire period of incubation, including the period with differentiation inducers, the cells were incubated with 5 or 20 μM cyanidin, malvidin, cyanidin-3-rutinoside, and malvidin-3,5-diglucosides, or 2.5 μM rosiglitazone (Sigma). At the end of the incubation period with compounds, the cells were harvested with a lysis buffer and triglyceride contents were measured in the cells harvested with a lysis buffer without glycerol using a Trinder kit (Young Dong Pharmaceutical Co., Seoul). In addition, these cells were harvested with a monophasic solution of phenol and guanidine isothiocyanate (Trizol reagent, Invitrogen), and total RNA was isolated from the lysate by extraction and precipitation was made with isopropyl alcohol. Reverse transcription was performed with superscript III reverse transcriptase, and PCR was performed with high fidelity Taq DNA polymerase (Invitrogen) to generate cDNA. The mRNA levels of SREBP-1c, FAS and CPT-1 were determined with the proper primers using real-time PCR (Bio-Rad) as described.

Statistical analysis

Statistical analysis was conducted using SAS version 7.0. Results are presented as means ± standard deviations. The effects of black carrot with and without fermentation were determined by one-way analysis of variance. Significant differences between OVX groups were identified by Tukey's tests. The OVX-control and normal-control groups were compared using two-sample t tests. $P < 0.05$ was considered statistically significant.

Results

Total phenolic compounds and flavonoids

All BC extracts, with and without fermentation, were rich in anthocyanins and carotenoids (Table 1). BCAO contained more anthocyanins than BC without fermentation and BCLP. BC also contained carotenoids, but the contents were lower than those of yellow carrots, and they were further decreased by fermentation (Table 1). The contents of cyanidin-3-rutinosides in BC, an indicator compound, were much greater in BC than BCAO and BCLP (Fig. 1; Table 1). Malvidin-3,5-diglycosides and delphine-3-glucoside were detected mainly in unfermented black carrots (Fig. 1). In BCAO and BCLP, cyanidin-3-rutinoside, BCAO malvidin-3,5-diglycosides and delphine-3-glucoside were markedly lowered but their aglycones, cyanidin and malvidin, were markedly increased after fermentation (Table 1). The anthocyanin consumption in the rats when adjusted to a human equivalent dose based on body surface area was 500 mg anthocyanins/60 kg person using the conversion coefficient of 6.2 suggested by the US FDA (Center for Drug Evaluation and Research 2005). This dosage was equivalent to a human consumption of 5 g of black carrot extract which might be unrealistic for a human dosage. However, it may be realistic for an individual consumption of total anthocyanins from a diet that emphasizes anthocyanin-rich foods.

Body weight and energy balance

OVX rats had greater weight gain during the 12-week experimental period than Sham rats and the relative ratios of peri-uterine fat and retroperitoneal fat mass to body weight were higher in OVX rats than Sham rats. In OVX rats, the weight gain and the visceral fat masses were lowered in the descending order of the OVX-control > OVX-BC = OVX-BCLP > OVX-BCAO (Table 2). Uterine weight did not increase after OVX and the treatments with BC, BCLP and BCAO did not prevent its decrease in OVX rats. Serum 17β-estradiol levels were higher by 3.8-fold in Sham-control rats than OVX-control rats, but they tended to increase in OVX-BCAO rats, but not significantly ($P = 0.08$). None of the treatments modulated serum 17β-estradiol levels (Table 2) significantly.

DEXA body composition measurements revealed that OVX lowered lean mass in the abdomen and leg compared to Sham-control rats, whereas fat mass was higher in OVX-control rats (Table 3). BC, BCLP and BCAO treatments completely protected against the loss of lean mass in the abdomen but only BCLP prevented the decrease in lean mass of the legs. Increases in abdominal and leg fat masses were prevented in the descending order of the OVX-control > OVX-BC = OVX-BCLP > OVX-BCAO (Table 3).

Body weight gain and visceral fat mass were determined by the net of energy balance. Daily energy intakes tended to be higher in Sham-control than OVX-control rats but not significantly ($P = 0.07$) and none of the treatments modulated energy intake (Table 4). Among the anthocyanin

Table 1 Anthocyanin contents of unfermented and fermented black carrots

	Black carrot extracts	Black carrot fermented with *Lactibacillus plantarum*	Black carrot fermented with *Aspergillus oryzae*
Total anthocyanins (mg/g)	46.6 ± 3.9[b]	43.8 ± 4.7[b]	59.8 ± 6.7[a]
Total carotenoids (mg/g)	14.6 ± 2.1[a]	9.5 ± 1.1[b]	10.7 ± 1.3[b]
Cyanidin-3-rutinosides (μg/g)	376.4 ± 0.2[a]	45.0 ± 0.3[b]	61.6 ± 0.2[c]
Malvidin-3,5-diglycosides (μg/g)	10.3 ± 1.1[a]	2.4 ± 0.1[c]	4.5 ± 0.1[b]
Delphinidin-3-glucoside (μg/g)	54.3 ± 1.8	–	–
Cyanidin (μg/g)	1.2 ± 0.1[c]	175.3 ± 1.3[a]	96.5 ± 1.3[b]
Malvidin (μg/g)	6.7 ± 0.2[c]	103.5 ± 1.4[a]	45.3 ± 1.1[b]

Mean ± standard deviation ($n = 3$)

[a,b] Values in the same column with different superscripts were significantly different by Tukey's test at $P < 0.05$

treated groups, OVX-BCAO consumed the most anthocyanins (Table 4). Unlike energy intake, daily energy expenditure was lower in OVX-control rats than in Sham-control rats. OVX-BCAO prevented the decrease in energy expenditure and was similar to the Sham-control rats (Table 4). RQs were not significantly different among any of the groups. Carbohydrate oxidation tended to be higher in OVX-control rats than in Sham-control rats, whereas fat oxidation showing the opposite pattern. BCLP and BCAO treatments prevented the increase in carbohydrate oxidation in OVX rats and decreased fat oxidation in OVX rats (Table 4). Thus, the OVX-BCLP and OVX-BCAO increased fat oxidation thereby suppressing fat accumulation in comparison with the OVX-control.

Glucose metabolism at week 11 of treatment

Overnight-fasted serum glucose and insulin levels were higher in the OVX-controls than the Sham-controls (Table 5). OVX-BCLP and OVX-BCAO tended to decrease serum glucose levels, but they were not significantly different. All treatments with BC, BCLP, and BCAO lowered serum insulin levels in OVX rats in comparison with the OVX-control rats. HOMA-IR, an index of insulin resistance, was markedly increased in OVX-control rats, compared to Sham-control rats, but the increase was prevented in descending order of OVX-control > OVX-BC = OVX-BCLP > OVX-BCAO treatments (Table 5).

Peak serum glucose levels at 30–50 min were higher and remained higher levels during the remaining periods in OVX-control rats than Sham-control rats during OGTT (Fig. 2a). This result suggested that OVX induced glucose intolerance in female rats. The peak of serum glucose levels were lower in descending order of the OVX-control > OVX-BC = OVX-BCLP > OVX-BCAO during OGTT (Fig. 2a). During OGTT, area under the curve (AUC) of glucose at both the first phase (0–50 min) and second phase (50–120 min) was much higher in OVX-control rats than Sham-control rats (Fig. 2b). The first and second phase AUC of serum glucose was lowered in the descending order of the OVX-control > OVX-BC = OVX-BCLP > OVX-BCAO. The AUC of insulin during the first phase was higher in OVX-control rats than Sham-control rats and that of the second phase exhibited the opposite trend of the first phase. The first phase AUC of serum insulin was increased in ascending order of the OVX-control < OVX-BC = OVX-BCLP < OVX-BCAO, whereas the second phase was not significantly different among treatments (Fig. 2c).

Serum lipid profiles after 12 week of supplementation

OVX-control rats exhibited dyslipidemia in the circulation in comparison with the normal-control rats. Serum total and LDL cholesterol and triacylglycerol concentrations were higher in OVX-control rats than the normal-control rats, whereas HDL cholesterol levels were lower in OVX rats (Table 5). BC, BCLP and BCAO treatments similarly lowered serum total and LDL cholesterol levels (Table 5). However, none of the treatments altered serum HDL cholesterol levels in OVX rats. Serum triglyceride levels were decreased in order of OVX-control > OVX-BC = OVX-BCLP > OVX-BCAO (Table 5).

Expressions of hepatic genes involved in fatty acid utilization and hepatic insulin signaling

Hepatic triglyceride contents in the liver were higher in OVX rats than in Sham rats and were lowered by BCLP and BCAO in OVX rats and BCAO decreased it the most (Table 6). Triglyceride storage in the gastrocnemius and quadriceps muscles showed the same trends as that in the liver (Table 6). The expressions of genes related to fatty acid oxidation and fatty acid synthesis were measured to investigate the mechanism of lipid metabolism in the liver.

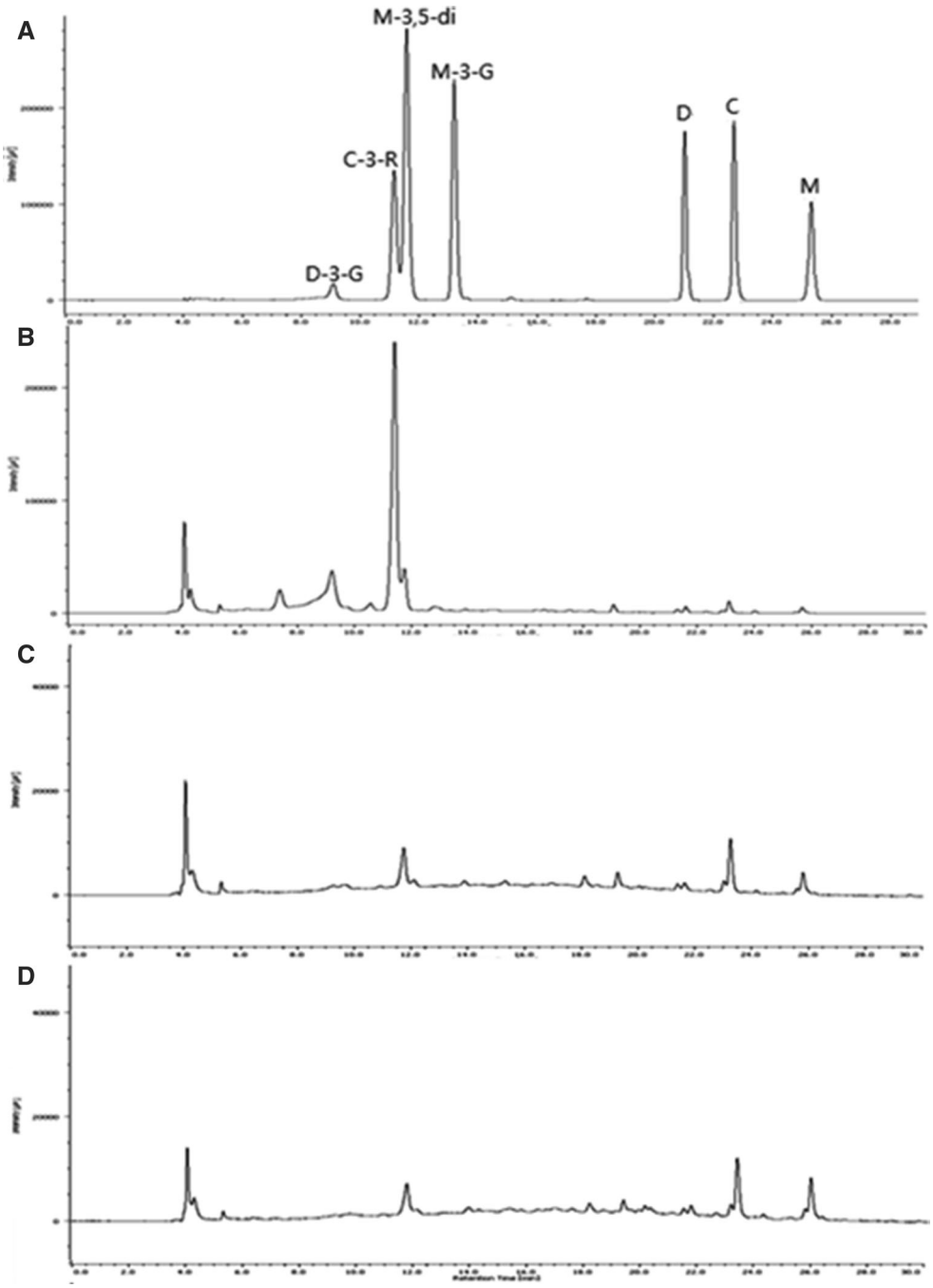

Fig. 1 The chromatograms of anthocyanins in unfermented and fermented black carrots. **A** Standards. **B** unfermented black carrots. **C** black carrots fermented with *Lactobacillus plantarum.* **D** black carrots fermented with *Asperillus oryzae*

The mRNA expression of CPT-1 and PPAR-α was lower in OVX rats than in Sham rats, whereas the expression decreased in the descending order of OVX-control > BC = BCLP > BCAO in OVX rats (Fig. 3a). FAS expression, which is involved in the regulation of fatty acid synthesis, was increased in OVX rats in comparison with Sham rats, but BCLP and BCAO decreased FAS expression. In addition, SREBP-1, a regulator of cholesterol synthesis, was higher in OVX rats compared to Sham rats, whereas BC, BCLP and BCAO treatments suppressed the increase in OVX rats (Fig. 3a).

OVX attenuated the phosphorylation of Akt and FOXO-1, whereas the treatment prevented the suppression of their phosphorylation in the order of OVX-control, BC, BCLP, and BCAO (Fig. 3b). In addition, the phosphorylation of AMPK and ACC was lower in OVX and it was reversed by the treatments with BC, BCLP and BCAO. BCAO increased the phosphorylation of AMPK and ACC the most

Table 2 Metabolic parameters at the end of the 12-week treatment

	OVX-CON ($n = 10$)	OVX-BC ($n = 10$)	OVX-BCLP ($n = 10$)	OVX-BCAO ($n = 10$)	Sham-CON ($n = 10$)
Body weight (g)	420.1 ± 33.3^{a}	412.4 ± 32.2^{a}	409.7 ± 28.5^{a}	380.3 ± 30.3^{b}	$386.2 \pm 40.5^{\dagger}$
Body weight gain (g)	107.8 ± 13.8^{a}	94.7 ± 10.7^{b}	91.4 ± 12.5^{b}	72.4 ± 11.5^{c}	$85.7 \pm 10.3^{\dagger}$
Peri-uterine fat/body weight (g/kg bw)	0.046 ± 0.005^{a}	$0.042 \pm 0.007^{a,b}$	0.04 ± 0.005^{b}	0.035 ± 0.004^{c}	$0.037 \pm 0.004^{\dagger}$
Retroperitoneal fat/body weight (g/kg bw)	0.033 ± 0.004^{a}	0.035 ± 0.005^{a}	0.028 ± 0.003^{b}	0.023 ± 0.003^{c}	$0.026 \pm 0.003^{\dagger}$
Uterus weight (g)	0.22 ± 0.05	0.25 ± 0.05	0.26 ± 0.04	0.27 ± 0.06	$0.73 \pm 0.10^{\dagger}$
Serum 17β-estradiol levels (pg/ml)	1.4 ± 0.4	1.7 ± 0.6	1.8 ± 0.6	2.0 ± 0.7	$5.3 \pm 1.0^{\dagger}$

OVX-CON, OVX rats fed a high-fat diet (HFD) with 2 % dextrin; BC, OVX rats fed HFD with 2 % lyophilized water extracts of black carrot; BCAO, 2 % lyophilized water extract of black carrot fermented with *Asperillus oryzae*; BCLP, OVX rats fed HFD with 2 % lyophilized water extract of black carrot fermented with *Lactobacillus plantarum*; Sham-CON, Sham rats fed a high-fat diet (HFD) with 2 % dextrin

Mean ± standard deviation ($n = 10$)

† Significantly different from the OVX-CON by two-sampled t test at $P < 0.05$

^{a,b,c} Values in the same column with different superscripts were significantly different by Tukey's test at $P < 0.05$

Table 3 Measurement of lean body and fat mass by DEXA

	OVX-CON ($n = 10$)	OVX-BC ($n = 10$)	OVX-BCLP ($n = 10$)	OVX-BCAO ($n = 10$)	Sham-CON ($n = 10$)
Lean mass in the abdomen (g/cm^3)	20.8 ± 2.8^{b}	24.6 ± 3.6^{a}	25.5 ± 3.7^{a}	$24.9 \pm 3.2^{a,*}$	$26.3 \pm 3.4^{\dagger}$
Fat mass in the abdomen (g/cm^3)	17.5 ± 2.9^{a}	$16.6 \pm 3.25^{a,b}$	15.0 ± 2.4^{b}	$12.4 \pm 2.8^{c,*}$	$11.4 \pm 2.7^{\dagger}$
Lean mass in the leg (g/cm^3)	5.33 ± 0.71^{b}	5.74 ± 0.89^{b}	6.76 ± 1.13^{a}	$6.05 \pm 0.87^{a,b,*}$	$6.89 \pm 1.08^{\dagger}$
Fat mass in the leg (g/cm^3)	5.12 ± 0.72^{a}	4.48 ± 0.65^{b}	4.39 ± 0.69^{b}	3.78 ± 0.57^{c}	$3.27 \pm 0.58^{\dagger}$

OVX-CON, OVX rats fed a high-fat diet (HFD) with 2 % dextrin; BC, OVX rats fed HFD with 2 % lyophilized water extracts of black carrot; BCAO, 2 % lyophilized water extract of black carrot fermented with *Asperillus oryzae*; BCLP, OVX rats fed HFD with 2 % lyophilized water extract of black carrot fermented with *Lactobacillus plantarum*; Sham-CON, Sham rats fed a high-fat diet (HFD) with 2 % dextrin

Mean ± standard deviation ($n = 10$)

† Significantly different from the OVX-CON by two-sampled t test at $P < 0.05$

^{a,b} Values in the same column with different superscripts were significantly different by Tukey's test at $P < 0.05$

Table 4 The parameters of indirect calorimetry at the end of experiment

	OVX-CON ($n = 10$)	OVX-BC ($n = 10$)	OVX-BCLP ($n = 10$)	OVX-BCAO ($n = 10$)	Sham-CON ($n = 10$)
Energy intake (kcal/day)	100.9 ± 12.6	100.4 ± 9.2	98.4 ± 9.2	93.7 ± 12.0	94.7 ± 9.4
Anthocyanin intake (mg/kg bw/day)	–	50.4 ± 4.6^{b}	46.8 ± 4.4^{b}	65.5 ± 8.4^{a}	–
Energy expenditure ($kcal/kg^{0.75}$/day)	114.1 ± 16.7^{b}	120.5 ± 14.7^{b}	$126.6 \pm 15.8^{a,b}$	$136.8 \pm 15.6^{a,*}$	$139.0 \pm 17.4^{\dagger}$
Respiratory quotient	0.84 ± 0.03	0.83 ± 0.03	0.81 ± 0.04	0.80 ± 0.04	0.79 ± 0.04
VO_2 ($mL/kg^{0.75}$/min)	16.3 ± 2.4^{b}	17.2 ± 1.9^{b}	$18.1 \pm 2.3^{a,b}$	$19.5 \pm 2.0^{a,*}$	$19.8 \pm 2.2^{\dagger}$
VCO_2 ($mL/kg^{0.75}$/min)	13.7 ± 2.0^{b}	$14.2 \pm 1.9^{a,b}$	$14.6 \pm 1.8^{a,b}$	$15.6 \pm 1.7^{a,*}$	$15.7 \pm 2.0^{\dagger}$
Carbohydrate oxidation ($mL/kg^{0.75}$/min)	5.5 ± 0.7^{a}	$5.3 \pm 0.7^{a,b}$	4.7 ± 0.7^{b}	$4.6 \pm 0.6^{b,*}$	$4.2 \pm 0.6^{\dagger}$
Fat oxidation ($mL/kg^{0.75}$/min)	6.6 ± 0.9^{c}	7.4 ± 0.9^{b}	$8.7 \pm 1.2^{a,b}$	$9.9 \pm 1.3^{a,*}$	$10.6 \pm 1.4^{\dagger}$

OVX-CON, OVX rats fed a high-fat diet (HFD) with 2 % dextrin; BC, OVX rats fed HFD with 2 % lyophilized water extracts of black carrot; BCAO, 2 % lyophilized water extract of black carrot fermented with *Asperillus oryzae*; BCLP, OVX rats fed HFD with 2 % lyophilized water extract of black carrot fermented with *Lactobacillus plantarum*; Sham-CON, Sham rats fed a high-fat diet (HFD) with 2 % dextrin

Mean ± standard deviation ($n = 10$)

† Significantly different from the OVX-CON by two-sampled t test at $P < 0.05$

^{a,b} Values in the same column with different superscripts were significantly different by Tukey's test at $P < 0.05$

Table 5 Serum glucose and lipid profiles at overnight fasting state

	OVX-CON	OVX-BC	OVX-BCLP	OVX-BCAO	Sham-CON
Glucose (mg/dL)	122 ± 10	121 ± 12	117 ± 9	114 ± 10	101 ± 10‡
Insulin (mg/dL)	1.44 ± 0.21[a]	1.25 ± 0.19[b]	1.21 ± 0.21[b]	1.16 ± 0.16[b,*]	1.15 ± 0.25‡
HOMA-IR	8.6 ± 1.0[a]	7.5 ± 0.9[b]	7.1 ± 1.0[b]	6.6 ± 0.7[c,*]	5.8 ± 0.7‡
Total cholesterol (mg/dL)	106.1 ± 7.2[a]	94.1 ± 10.9[b]	92.9 ± 10.8[b]	90.2 ± 10.2[b]	91.7 ± 8.2‡
LDL cholesterol (mg/dL)	48.0 ± 4.7[a]	36.5 ± 4.8[b]	36.3 ± 4.7[b]	33.8 ± 3.6[b]	31.1 ± 3.4‡
HDL cholesterol (mg/dL)	44.2 ± 5.4	45.8 ± 5.1	45.3 ± 4.4	46.2 ± 5.5	49.3 ± 4.7‡
Triglyceride (mg/dL)	69.2 ± 7.4[a]	58.8 ± 6.5[b]	56.6 ± 6.8[b,c]	52.3 ± 6.3[c]	56.3 ± 7.2‡

OVX-CON, OVX rats fed a high-fat diet (HFD) with 2 % dextrin; BC, OVX rats fed HFD with 2 % lyophilized water extracts of black carrot; BCAO, 2 % lyophilized water extract of black carrot fermented with *Asperillus oryzae*; BCLP, OVX rats fed HFD with 2 % lyophilized water extract of black carrot fermented with *Lactobacillus plantarum*; Sham-CON, Sham rats fed a high-fat diet (HFD) with 2 % dextrin

Mean ± standard deviation ($n = 10$)

‡ Significantly different from the OVX-CON by two-sampled t test at $P < 0.05$

[a,b] Values in the same column with different superscripts were significantly different by Tukey's test at $P < 0.05$

(Fig. 3b). Thus, BCAO had the greatest potential to prevent hepatic steatosis in OVX rats.

The suppression of lipid synthesis by cyanidin and malvidin

From the animal study, BCLP and BCAO had better lipid lowering activity than BC. The lipid lowering activity of cyanidin and malvidin, the major components of BCLP and BCAO, was measured in 3T3-L1 adipocytes. Triglyceride accumulation was decreased in a dose-dependent manner, and it was much lower in the descending order of DMSO (control) > cyanidin-3-rutinoside, malvidin-3,5-diglycosides > malvidin > cyanidin (Fig. 4a). The decrease in triglyceride was related to increased mRNA expression of CPT-1 and PPAR-α and attenuated the mRNA expression of FAS and SREBP-1c. Cyanidin and malvidin decreased mRNA expression of FAS and SREBP-1c and increased that of PPAR-α and CPT-1 (Fig. 4b).

Discussion

Many forms of anthocyanins occur in fruits and vegetables and the different combinations of anthocyanins may have different colors and functions (Tsuda 2012). Fermentation of anthocyanins may enhance their intestinal absorption and bioactivities by removing glycosides. No previous studies have investigated the alleviation of postmenopausal symptoms by anthocyanin-rich foods fermented with *L. plantarum* and *A. oryzae*, although resveratrol, a bioactive compound of wine (the alcohol fermentation of anthocyanin-rich foods) has been studied (Mobasheri and Shakibaei 2013; Nguyen et al. 2012). The present study revealed that fermenting the extracts of black carrots with *L. plantarum* and *A. oryzae* changed the anthocyanin composition by increasing anthocyanin aglycones (-cyanidin and malvidin) and the changes in composition improved their effectiveness for improving energy homeostasis with BCAO being the most effective for increasing daily energy expenditure and fat oxidation. BC, BCLP and BCAO all prevented the exacerbation of glucose intolerance and dyslipidemia in OVX rats, but BCAO was most effective for menopausal symptoms in OVX rats. Thus, the fermentation of black carrots prevented the exacerbation of metabolic disturbances induced by estrogen deficiency.

Bioavailability studies indicate that after taking anthocyanins, their concentrations in plasma and urine often remain low since anthocyanins are degraded by intestinal bacteria (Fang 2014; de Ferrars et al. 2014). After the consumption of 500 mg of radioactively labeled cyanidin-3-glucoside, 35 metabolites (17 in serum, 31 in urine, and 28 in feces) were identified (de Ferrars et al. 2014; Fang 2014). Hidalgo et al. (2012) reported that bacterial fermentation of a mixture of anthocyanins resulted in the formation of gallic, syringic and *p*-coumaric acids. Fermentation changes the forms of anthocyanins, degrading them to smaller byproducts that are more easily absorbed and with altered bioactivity (Vergara-Salinas et al. 2013; Wu et al. 2012) although some anthocyanin glycosides were also detected in serum and urine. Anthocyanin aglycones are reported to have greater bioavailability glycosylated anthocyanins. Among the glycosylated anthocyanins with the same aglycone structure, varying the sugar moiety of the glycosides and the position of the same glycoside on the same anthocyanin rings affects bioavailability (Kamonpatana et al. 2014; Jin et al. 2011; Ichiyanagi et al. 2006). The present study showed that fermentation of black carrot with either of *L. plantarum* or *A. oryzae* changed the composition of anthocyanins, such

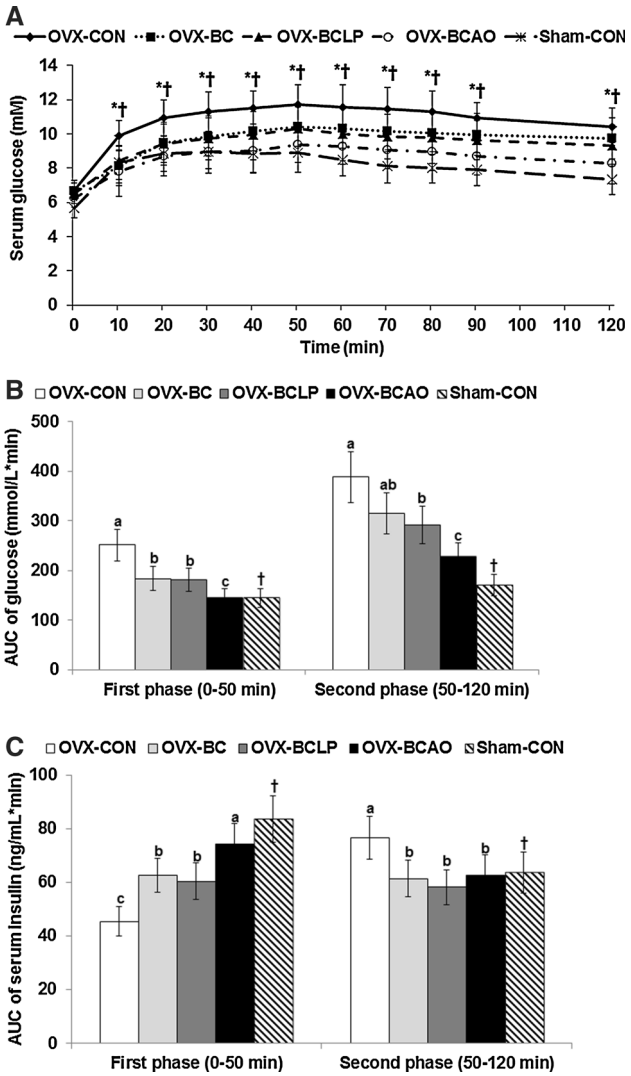

Fig. 2 Serum glucose levels and area under the *curve* of glucose and insulin during oral glucose tolerance test (OGTT) at week 11 of experimental periods. OVX-control, OVX rats fed a high-fat diet (HFD) with 2 % dextrin; BC, OVX rats fed HFD with 2 % lyophilized water extracts of black carrot; BCAO, 2 % lyophilized water extract of black carrot fermented with *Asperillus oryzae*; BCLP, OVX rats fed HFD with 2 % lyophilized water extract of black carrot fermented with *Lactobacillus plantarum*; Normal-control, Sham rats fed a HFD with 2 % dextrin changes in serum glucose levels were measured during OGTT (**a**). The average of the area under the *curve* (AUC) of glucose (**b**) and insulin (**c**) during the first part (0–40 min) and second part (40–120 min) of OGTT. Each *dot* and *bar* represents the mean ± SD ($n = 10$). *Significantly different among the treatment groups of OVX rats at $P < 0.05$. [a,b,c]Different *superscript letters* indicate significant differences among OVX rats by Tukey's test at $P < 0.05$. †Significantly different between the OVX-CON and Sham-CON groups by two-sampled *t* test at $P < 0.05$

that anthocyanin glycosides such as cyanidin-3-rutinosides, malvidin-3,5-diglycosides, delphine-3-glucoside decreased and anthocyanin aglycones such as cyanidin and malvidin increased. Fermentation of milk with Chingshey purple sweet potato by lactic acid bacteria strains such as *Lactobacillus acidophilus* increases antioxidant activities as measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals and enhances its scavenging effects against superoxide anion radicals (Wu et al. 2012). Fermentation of black bean extracts with *Aspergillus* increases DPPH free radical-scavenging activity, Fe^{2+}-chelating activity, and reducing power in comparison with the non-fermented black beans although heating decreases the antioxidant capacity (Lin and Chou 2009). These results suggest that fermentation with *Lactobacillus* or *Aspergillus* may improve antioxidant capacity of foods containing anthocyanins. However, few studies have evaluated the benefits anthocyanin aglycones for postmenopausal symptoms (Jia et al. 2013; Pompei et al. 2012).

Estrogen deficiency induces a marked increase in body fat storage in humans and in OVX rats and other experimental animals (Gourlay et al. 2014; Lizcano and Guzmán 2014; Butera 2010) and a high-fat diet accelerates the increases in body fat mass (Ko et al. 2013). Several studies have reported that the consumption of anthocyanins limits body weight gain and fat mass both in diet-induced and genetic animal models of obesity (Wu et al. 2013, 2014), although 4 week consumption of dried purple carrots did not change body weight gain or serum lipid concentrations in men (Wright et al. 2013). The consumption of anthocyanins (20–200 mg/kg body weight) from different foods, such as sweet cherry, blueberry, mulberry, and honeysuckle suppresses body weight gain, decreases body fat and prevents dyslipidemia in male C57BL/6 mice and rats fed a high-fat diet (Wu et al. 2013, 2014). The mechanism of preventing body weight gain has not been well-studied. Badshah et al. (2013) found that the intake of anthocyanins from black bean seed coat decreases body weight gain by lowering daily food intake by decreasing the expression of neuropeptide Y and increasing γ-amino butyric acid receptor in the hypothalamus of male rats. However, in the present study, BC, BCLP and BCAO decreased body weight gain and fat mass in OVX rats without changing daily food intake. There was a non-significant tendency for BCAO tended to decrease food intake in OVX rats ($P = 0.11$), whereas BCAO did markedly increase daily energy expenditure. Thus, the present study indicates that BCAO decreases body fat mainly by increasing daily energy expenditure.

Anthocyanins are reported to improve glucose metabolism by enhancing insulin sensitivity and insulin secretion (Pinent et al. 2005; Jayaprakasam et al. 2005). Male C57BL/6 mice fed with a high-fat diet supplemented with anthocyanins exhibited improved insulin resistance (Seymour et al. 2011; Guo et al. 2012a, b). The consumption of anthocyanins from tart cherry decreased serum glucose levels and hyperinsulinemia while increasing the expression of PPAR-α in Dahl salt-sensitive rats (Guo et al.

Table 6 Triglyceride levels in the liver and skeletal muscles

	OVX-CON	OVX-BC	OVX-BCLP	OVX-BCAO	Sham-CON
Hepatic triglycerides (mg/g tissue)	1.05 ± 0.14^{a}	0.88 ± 0.10^{b}	0.77 ± 0.10^{b}	0.63 ± 0.09^{c}	$0.72 \pm 0.10^{\ddagger}$
Triglyceride in the gas muscle (mg/g tissue)	1.31 ± 0.19^{a}	$1.12 \pm 0.17^{a,b}$	1.07 ± 0.14^{b}	$0.95 \pm 0.13^{b,*}$	$0.78 \pm 0.22^{\ddagger}$
Triglyceride in the quadriceps muscle (mg/g tissue)	2.87 ± 0.32^{a}	$2.62 \pm 0.35^{a,b}$	2.27 ± 0.36^{b}	$2.07 \pm 0.27^{b,*}$	$1.85 \pm 0.24^{\ddagger}$

OVX-CON, OVX rats fed a high-fat diet (HFD) with 2 % dextrin; BC, OVX rats fed HFD with 2 % lyophilized water extracts of black carrot; BCAO, 2 % lyophilized water extract of black carrot fermented with *Asperillus oryzae*; BCLP, OVX rats fed HFD with 2 % lyophilized water extract of black carrot fermented with *Lactobacillus plantarum*; Sham-CON, Sham rats fed a high-fat diet (HFD) with 2 % dextrin

Mean ± standard deviation ($n = 10$)

‡ Significantly different from the OVX-CON by two-sampled t test at $P < 0.05$

^{a,b} Values in the same column with different superscripts were significantly different by Tukey's test at $P < 0.05$

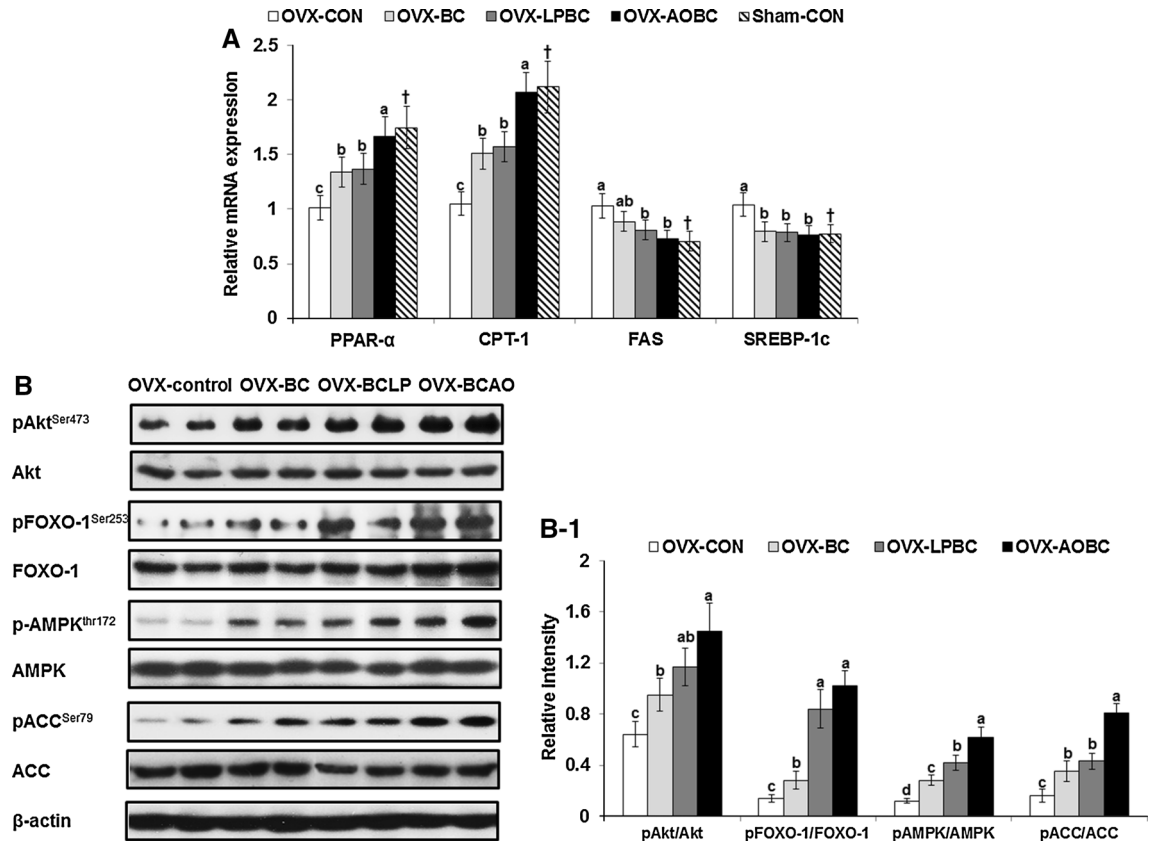

Fig. 3 Hepatic expression of genes involved in fatty acid utilization and hepatic insulin signaling at the end of the experimental periods. OVX-control, OVX rats fed a high-fat diet with 2 % dextrin; BC, OVX rats fed a high-fat diet with 2 % lyophilized water extracts of black carrot; BCAO, 2 % lyophilized water extract of black carrot fermented with *Asperillus oryzae*; BCLP, OVX rats fed a high-fat diet with 2 % lyophilized water extract of black carrot fermented with *Lactobacillus plantarum*; normal-control, Sham rats fed a high-fat diet with 2 % dextrin. The mRNA levels of hepatic genes involved in fatty acid synthesis and oxidation were measured by real-time PCR (**a**). Hepatic insulin signaling was determined by immunoblotting assays (**b**). Each *bar* represents the mean ± SD ($n = 5$). ^{a,b,c}Different *superscript letters* indicate significant differences among OVX rats by Tukey's test at $P < 0.05$. †Significantly different between the OVX-CON and Sham-CON groups by two-sampled t test at $P < 0.05$

2012a, b), whereas consuming a high-fat diet containing blueberry extracts also increased insulin sensitivity in Zucker rats (Seymour et al. 2011). The present study showed somewhat different results from the previous studies: BC and fermented BC (BCLP and BCAO) lowered overnight-fasted serum insulin levels without changing serum glucose levels, and HOMA-IR, an indicator of insulin resistance, was reduced in the descending order of OVX-control > OVX-BCLP = OVX-BC > OVX-BCAO. Glucose-stimulated insulin secretion at the first part of OGTT was increased in ascending order of OVX-control < OVX-BCLP = OVX-BC < OVX-BCAO. Thus,

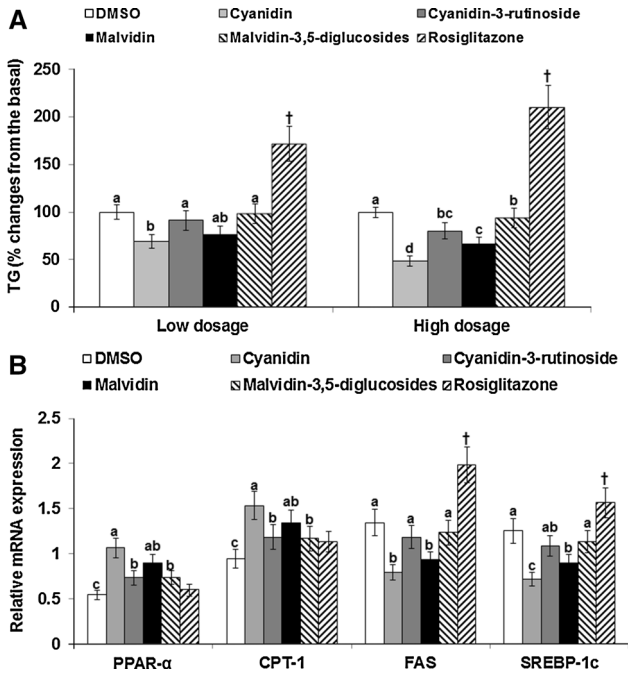

Fig. 4 Triglyceride accumulation and mRNA expression of adipogenic markers in 3T3-L1 mature adipocytes. **a** Triglyceride contents in 3T3-L1 adipocytes after the vehicle (DMSO), 5 or 20 μM cyanidin, malvidin, cyanidin-3-rutinoside, or malvidin-3,5-diglycoside or 5 or 2 μM rosiglitazone were incubated with high-glucose DMEM media in 3T3-L1 fibroblasts during the entire period of incubation, including the period with differentiation inducers ($n = 3$). **b** The mRNA expression of CPT-1, FAS, and SREBP-1c in fully differentiated 3T3-L1 adipocytes after the treatment with vehicle, 20 μM cyanidin, malvidin, cyanidin-3-rutinoside, or malvidin-3,5-diglycoside or 2 μM rosiglitazone in high-glucose DMEM media during the entire period of incubation, including the period with differentiation inducers. The results represented the ratio of mRNA levels of the gene of interest (CPT-1, FAS, and SREBP-1c) and those of the housekeeping gene (β-actin) in arbitrary units (AU) ($n = 3$). ^{a,b,c}*Superscript letters* indicate a significant difference at $P < 0.05$ according to the Tukey's test. †Significantly different from the control (DMSO) by two-sampled t test at $P < 0.05$

BCAO supplementation improved glucose metabolism by potentiating glucose-stimulated insulin secretion and reducing insulin resistance in OVX rats fed a high-fat diet. Therefore, the fermentation-induced changes in anthocyanin composition appear to change how the anthocyanins alter glucose metabolism in estrogen-deficient animals.

Hepatic steatosis may be associated with oxidative hepatocellular damage, inflammation, activation of fibrogenesis, and systemic insulin resistance (Fruci et al. 2013). The present study found that BCAO decreased hepatic fat storage mainly by increasing fatty acid oxidation and decreasing lipogenesis. The increase in fatty acid oxidation was associated with potentiating pAkt → pFOXO-1 and pAMPK → pACC. In the in vitro pat of this study, cyanidin and malvidin lowered lipid accumulation in 3T3-L1 adipocytes in comparison with the cyanidin-3-rutinoside and malvidin-3,5-diglycosides by increasing mRNA expressions of PPAR-α and CPT-1 and decreasing FAS and SREBP-1c. Thus, BCAO has the potential to prevent hepatic steatosis in OVX rats. Consistent with the present study, anthocyanin supplementation was shown to decrease hepatic lipid accumulation, hepatic insulin resistance and oxidative stress in male mice and rats (Valenti et al. 2013). The alleviation of hepatic steatosis is associated with the activations of PPAR-α and AMPK, which induce lipolysis (Hwang et al. 2011; Guo et al. 2011; Seymour et al. 2011). Postmenopausal women and estrogen-deficient female animals easily develop hepatic steatosis with diets in high fat and cholesterol (Kamada et al. 2011; Florentino et al. 2013). OVX was shown to be associated with decreased hepatic expression of catabolic gene expression including CPT-1 by 53 % and β-hydroxyacyl-CoA dehydrogenase by 27 %, and increased hepatic anabolic gene expression of sterol regulatory element-binding protein-1c by 106 %, ACC by 72 % and stearoyl CoA desaturase-1 by 109 % (Kamada et al. 2011). Furthermore, in an OVX Sprague–Dawley rat model, 17β-estradiol treatment decreased fatty acid synthesis in a hepatic zone 3-specific manner through increasing the phosphorylation of acetyl coenzyme-A carboxylase via an estrogen receptor-α-mediated pathway (Zhang et al. 2013). Menopausal women have been shown to exhibit hepatic steatosis which is ameliorated by estrogen replacement therapy, although the results are inconsistent (Florentino et al. 2013; McKenzie et al. 2006). Thus, the activation of estrogen receptors in the liver may prevent hepatic steatosis by increasing β-oxidation of fatty acids and decreasing lipogenesis. A few studies have evaluated the association of phytoestrogens and hepatic steatosis in estrogen-deficient animals. Genistein increases the rate of β-oxidation through increasing the activity of succinate dehydrogenase and CPT-1 in OVX rats (Choi et al. 2012). However, in postmenopausal women, 12-week consumption of elderberry extracts rich in anthocyanins (500 mg/day) did not change biomarkers of liver function, although the plasma concentrations of anthocyanin metabolites of the elderberry treatment group were greater ($P = 0.02$) than that of the control at week 12 of the treatment (Curtis et al. 2009).

In conclusion, fermentation of BC with *L. plantarum* or *A. oryzae* changed the composition of anthocyanins. AO fermentation of BC was especially effective for potentiating its ability to improve energy, glucose and lipid metabolism in OVX rats. It was associated with increased aglycones, especially cyanidin and malvidin. BCAO activated the phosphorylation of AMPK to increase PPAR-α and CPT-1 gene expression which increased fatty acid oxidation and potentiated hepatic insulin signaling which suppressed fatty acid synthesis. BCAO has potential therapeutic efficacy for treating postmenopausal metabolic

disturbances in estrogen-deficient animals, and deserves further study in postmenopausal women.

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Conflict of interest The authors declare no competing financial interest.

Ethical standard All institutional and national guidelines for the care and use of laboratory animals were followed.

References

Ainy E, Mirmiran P, Zahedi Asl S, Azizi F (2007) Prevalence of metabolic syndrome during menopausal transition Tehranian women: Tehran Lipid and Glucose Study (TLGS). Maturitas 58:150–155

Badshah H, Ullah I, Kim SE, Kim TH, Lee HY, Kim MO (2013) Anthocyanins attenuate body weight gain via modulating neuropeptide Y and GABAB1 receptor in rats hypothalamus. Neuropeptides 47:347–353

Baños G, Guarner V, Pérez-Torres I (2011) Sex steroid hormones, cardiovascular diseases and the metabolic syndrome. Cardiovasc Hematol Agents Med Chem 9:137–146

Björntorp P (1996) The regulation of adipose tissue distribution in humans. Int J Obes Relat Metab Disord 20:291–302

Butera PC (2010) Estradiol and the control of food intake. Physiol Behav 99:175–180

Center for Drug Evaluation and Research (2005) Guidance for industry: Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. US Department of Health and Human Services Food and Drug Administration, Washington

Chedraui P, San Miguel G, Vintimilla-Sigüenza I, Villacreses D, Romero-Huete L, Domínguez A, Jaramillo W, Escobar GS, Pérez-López FR, Genazzani AR, Simoncini T, Research Group for Omega Women's Health Project (2013) The metabolic syndrome and its components in postmenopausal women. Gynecol Endocrinol 29:563–568

Choi JS, Koh IU, Song J (2012) Genistein reduced insulin resistance index through modulating lipid metabolism in ovariectomized rats. Nutr Res 32:844–855

Curtis PJ, Kroon PA, Hollands WJ, Walls R, Jenkins G, Kay CD, Cassidy A (2009) Cardiovascular disease risk biomarkers and liver and kidney function are not altered in postmenopausal women after ingesting an elderberry extract rich in anthocyanins for 12 weeks. J Nutr 139:2266–2271

de Ferrars RM, Czank C, Zhang Q, Botting NP, Kroon PA, Cassidy A, Kay CD (2014) The pharmacokinetics of anthocyanins and their metabolites in humans. Br J Pharmacol 171:3268–3282

Diel P, Geis RB, Caldarelli A, Schmidt S, Leschowsky UL, Voss A, Vollmer G (2004) The differential ability of the phytoestrogen genistein and of estradiol to induce uterine weight and proliferation in the rat is associated with a substance specific modulation of uterine gene expression. Mol Cell Endocrinol 221:21–32

Fang J (2014) Bioavailability of anthocyanins. Drug Metab Rev 46:508–520

Felgines C, Texier O, Besson C, Fraisse D, Lamaison JL, Rémésy C (2002) Blackberry anthocyanins are slightly bioavailable in rats. J Nutr 132:1249–1253

Florentino GS, Cotrim HP, Vilar CP, Florentino AV, Guimarães GM, Barreto VS (2013) Nonalcoholic fatty liver disease in menopausal women. Arch Gastroenterol 50:180–185

Fruci B, Giuliano S, Mazza A, Malaguarnera R, Belfiore A (2013) Nonalcoholic Fatty liver: a possible new target for type 2 diabetes prevention and treatment. Int J Mol Sci 14:22933–229366

Gourlay ML, Hammett-Stabler CA, Renner JB, Rubin JE (2014) Associations between body composition, hormonal and lifestyle factors, bone turnover, and BMD. J Bone Miner Metab 21:61–68

Guo H, Li D, Ling W, Feng X, Xia M (2011) Anthocyanin inhibits high glucose-induced hepatic mtGPAT1 activation and prevents fatty acid synthesis through PKCζ. J Lipid Res 52:908–922

Guo H, Liu G, Zhong R, Wang Y, Wang D, Xia M (2012a) Cyanidin-3-O-β-glucoside regulates fatty acid metabolism via an AMP-activated protein kinase-dependent signaling pathway in human HepG2 cells. Lipids Health Dis 11:10

Guo H, Xia M, Zou T, Ling W, Zhong R, Zhang W (2012b) Cyanidin 3-glucoside attenuates obesity-associated insulin resistance and hepatic steatosis in high-fat diet-fed and db/db mice via the transcription factor FoxO1. J Nutr Biochem 23:349–360

Hidalgo M, Oruna-Concha MJ, Kolida S, Walton GE, Kallithraka S, Spencer JP, de Pascual-Teresa S (2012) Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial growth. J Agric Food Chem 60:3882–3890

Hwang YP, Choi JH, Han EH, Kim HG, Wee JH, Jung KO, Jung KH, Kwon KI, Jeong TC, Chung YC, Jeong HG (2011) Purple sweet potato anthocyanins attenuate hepatic lipid accumulation through activating adenosine monophosphate-activated protein kinase in human HepG2 cells and obese mice. Nutr Res 31:896–906

Ichiyanagi T, Shida Y, Rahman MM, Hatano Y, Konishi T (2006) Bioavailability and tissue distribution of anthocyanins in bilberry (Vaccinium myrtillus L) extract in rats. J Agric Food Chem 54:6578–6587

Jayaprakasam B, Vareed SK, Olson LK, Nair NG (2005) Insulin secretion by bioactive anthocyanins and anthocyanidins present in fruits. J Agric Food Chem 53:28–31

Jia Y, Kim JY, Jun HJ, Kim SJ, Lee JH, Hoang MH, Kim HS, Chang HI, Hwang KY, Um SJ, Lee SJ (2013) Cyanidin is an agonistic ligand for peroxisome proliferator-activated receptor-alpha reducing hepatic lipid. Biochim Biophys Acta 1831:698–708

Jin Y, Alimbetov D, George T, Gordon MH, Lovegrove JA (2011) A randomized trial to investigate the effects of acute consumption of a blackcurrant juice drink on markers of vascular reactivity and bioavailability of anthocyanins in human subjects. Eur J Clin Nutr 65:849–856

Jing P, Giusti MM (2007) Effects of extraction conditions on improving the yield and quality of an anthocyanin-rich purple corn (*Zea mays L*) color extract. J Food Sci 72:C363–C368

Kamada Y, Kiso S, Yoshida Y, Chatani N, Kizu T, Hamano M, Tsubakio M, Takemura T, Ezaki H, Hayashi N, Takehara T (2011) Estrogen deficiency worsens steatohepatitis in mice fed high-fat and high-cholesterol diet. Am J Physiol Gastrointest Liver Physiol 301:G1031–G1043

Kamonpatana K, Failla ML, Kumar PS, Giusti MM (2014) Anthocyanin structure determines susceptibility to microbial degradation and bioavailability to the buccal mucosa. J Agric Food Chem 62:6903–6910

Kim HK, Nelson-Dooley C, Della-Fera MA, Yang JY, Zhang W, Duan J, Hartzell DL, Hamrick MW, Baile CA (2006) Genistein decreases food intake, body weight, and fat pad weight and causes adipose tissue apoptosis in ovariectomized female mice. J Nutr 136:409–414

Ko BS, Kim DS, Kang S, Ryuk JA, Park S (2013) *Prunus mume* and *Lithospermum erythrorhizon* extracts synergistically prevent visceral adiposity by improving energy metabolism through potentiating hypothalamic leptin and insulin signaling in ovariectomized rats. Evid Based Complement Alternat Med 2013:750986

Lin YC, Chou CC (2009) Effect of heat treatment on total phenolic and anthocyanin contents as well as antioxidant activity of the extract from *Aspergillus awamori*-fermented black soybeans, a healthy food ingredient. Int J Food Sci Nutr 60:627–636

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408

Lizcano F, Guzmán G (2014) Estrogen deficiency and the origin of obesity during menopause. BioMed Res Int 2014:757461

Majumdar AS, Giri PR, Pai SA (2014) Resveratrol- and melatonin-abated ovariectomy and fructose diet-induced obesity and metabolic alterations in female rats. Menopause 21:876–885

McKenzie J, Fisher BM, Jaap AJ, Stanley A, Paterson K, Sattar N (2006) Effects of HRT on liver enzyme levels in women with type 2 diabetes: a randomized placebo-controlled trial. Clin Endocrinol 65:40–44

Mobasheri A, Shakibaei M (2013) Osteogenic effects of resveratrol in vitro: potential for the prevention and treatment of osteoporosis. Ann N Y Acad Sci 1290:59–66

Nguyen BT, Kararigas G, Wuttke W, Jarry H (2012) Long-term treatment of ovariectomized mice with estradiol or phytoestrogens as a new model to study the role of estrogenic substances in the heart. Planta Med 78:6–11

Noh HJ, Jang SY, Park JJ, Yun HS, Park S (2013) Browning prevention of black carrot extract and the quality characteristics of jelly supplemented with black carrot extract. Korean J Food Cult 28:293–302

Park S, Hong SM, Sung SR, Lee JE, Kwon DY (2008) Extracts of Rehmanniae radix, Ginseng radix and Scutellariae radix improve glucose-stimulated insulin secretion and β-cell proliferation through IRS2 induction. Genes Nutr 2:347–351

Pinent M, Bladëa MC, Salvadöa MJ, Arola L, Ardëavol A (2005) Metabolic fate of glucose on 3T3-L1 adipocytes treated with grape seed derived procyanidin extract (GSPE): comparison with the effects of insulin. J Agric Food Chem 53:5932–5935

Pompei A, Toniato E, Innocenti P, D Alimonte I, Cellini C, Mattoscio D, Cotellese R, Bosco D, Ciccarelli R, Dadorante V, D Orazio N, Martinotti S, Robuffo I (2012) Cyanidin reduces preadipocyte differentiation and relative ChREBP expression. J Biol Regul Homeost Agents 26:253–264

Reeves PG, Nielsen FH, Fahey GC Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123:1939–1951

Seymour EM, Tanone II, Urcuyo-Llanes DE, Lewis SK, Kirakosyan A, Kondoleon MG, Kaufman PB, Bolling SF (2011) Blueberry intake alters skeletal muscle and adipose tissue peroxisome proliferator-activated receptor activity and reduces insulin resistance in obese rats. J Med Food 14:1511–1518

Tchernof A, Després JP (2013) Pathophysiology of human visceral obesity: an update. Physiol Rev 93:359–404

Tsuda T (2012) Dietary anthocyanin-rich plants: biochemical basis and recent progress in health benefits studies. Mol Nutr Food Res 56:159–170

Valenti L, Riso P, Mazzocchi A, Porrini M, Fargion S, Agostoni C (2013) Dietary anthocyanins as nutritional therapy for nonalcoholic fatty liver disease. Oxid Med Cell Longev 2013:145421

Vergara-Salinas JR, Bulnes P, Zúñiga MC, Pérez-Jiménez J, Torres JL, Mateos-Martín ML, Agosin E, Pérez-Correa JR (2013) Effect of pressurized hot water extraction on antioxidants from grape pomace before and after enological fermentation. J Agric Food Chem 61:6929–6936

Wright OR, Netzel GA, Sakzewski AR (2013) A randomized, double-blind, placebo-controlled trial of the effect of dried purple carrot on body mass, lipids, blood pressure, body composition, and inflammatory markers in overweight and obese adults: the QUENCH trial. Can J Physiol Pharmacol 91:480–488

Wu TY, Tsai CC, Hwang YT, Chiu TH (2012) Effect of antioxidant activity and functional properties of Chingshey purple sweet potato fermented milk by *Lactobacillus acidophilus*, *L delbrueckii subsp lactis*, and *L gasseri* strains. J Food Sci 77:M2–M8

Wu T, Tang Q, Gao Z, Yu Z, Song H, Zheng X, Chen W (2013) Blueberry and mulberry juice prevent obesity development in C57BL/6 mice. PLoS ONE 8:e77585

Wu T, Tang Q, Yu Z, Gao Z, Hu H, Chen W, Zheng X, Yu T (2014) Inhibitory effects of sweet cherry anthocyanins on the obesity development in C57BL/6 mice. Int J Food Sci Nutr 65:351–359

Zhang H, Liu Y, Wang L, Li Z, Zhang H, Wu J, Rahman N, Guo Y, Li D, Li N, Huhtaniemi I, Tsang SY, Gao GF, Li X (2013) Differential effects of estrogen/androgen on the prevention of nonalcoholic fatty liver disease in the male rat. J Lipid Res 54:345–357