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Inhibitory effects of sweet cherry anthocyanins on the obesity development in C57BL/6 mice

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Abstract

In the present study, purified sweet cherry anthocyanins (CACN) were evaluated to determine their inhibitory effects on adipocyte differentiation of 3T3-L1 cells and their anti-obesity properties in male C57BL/6 mice fed with high-fat diet (HFD). CACN prevented HFD-induced obesity in C57BL/6 mice. In vivo experiment revealed that 40 and 200 mg/kg of CACN in food reduced the body weight by 5.2% and 11.2%, respectively. CACN supplementation could also reduce the size of adipocytes, leptin secretion, serum glucose, triglyceride, total cholesterol, LDL-cholesterol and liver triglycerides. Furthermore, CACN could effectively reduce the expression levels of IL-6 and TNFα genes, markedly increase the SOD and GPx activity. Our results indicated that CACN slowed down the development of HFD-induced obesity in male C57BL/6 mice.

Keywords: sweet cherry; anthocyanin; high-fat diet; obesity
Introduction

Obesity is recognized as a leading global health problem that causes detrimental effects (Haslam & James, 2005; Kopelman, 2000). This condition is known to contribute to the risk of various chronic diseases such as type II diabetes mellitus, coronary heart disease, hypertension, and several types of cancer (Gilbert & Slingerland, 2012; Weyer et al., 2000). Obesity is a complex metabolic disorder induced by a prolonged imbalance between energy intake and energy expenditure levels, in which surplus energy is stored as body lipids mainly in adipose tissues (Arner & Spalding, 2010; Suzuki et al., 2011). Currently available pharmaceuticals developed to treat obesity have a number of limitations such as adverse effects and high rates of secondary failure (Kang & Park, 2012). Therefore, natural products that can be used to treat obesity have been investigated (Costa et al., 2013; Meydani & Hasan, 2010; Yun, 2010).

Anthocyanins are water-soluble pigments commonly found in fruits and vegetables. These substances are the major sub-group of flavonoids and often consumed in a normal diet, including strawberry, blueberry, purple sweet potato, red wine, etc. (McGhie & Walton, 2007; Pojer et al., 2013). Studies have suggested that anthocyanins exhibit anti-obesity properties. For example, anthocyanidins are found in purple corn (Zea mays L.) (Tsuda et al., 2003), black soybean (Glycine max L. Merr.) (Kwon et al., 2007), blood orange (Citrus sinensis L. Osbeck) (Titta et al., 2010), blueberries (Vaccinium angustifolium) (Prior, 2010; Prior et al., 2010b, 2008,
2009), strawberries (*Fragaria ananassa*) (Prior et al., 2009; Prior et al., 2008), and blackberries (*Rubus* sp.) (Kaume et al., 2012). However, limited information about the effect of isolated anthocyanins on obesity is available except blueberry (Prior et al., 2010a), strawberry (Prior et al., 2009) and our recent study on mulberry (Wu et al., 2013). These isolated anthocyanins preventing obesity have focused on monoglycosides, primarily cyanidin-glucoside (Wu et al., 2013), pelargonidin-3-glucoside (Prior et al., 2009), and malvidin-3-glucoside (Prior, 2010). Thus, our knowledge about the mechanism by which isolated anthocyanins with di-glycosides or tri-glycosides inhibit the development of obesity should be enhanced.

Sweet cherry (*Prunus avium* L.) is one of the most common temperate fruits found in Europe, Asia, and Northern Africa because of its good taste as well as health and economical benefits (Damar & Eksi, 2012; McCune et al., 2011). Early studies revealed that sour cherry extracts reduce inflammation and paw edema, alleviate pain caused by gout and arthritis, suppress body weight gain, and prevent skin aging (Einbond et al., 2004; McCune et al., 2011; Sarić et al., 2009; Tall et al., 2004). However, studies on the relationship between sweet cherry anthocyanins (CACN) and anti-obesity effect have not been conducted yet. This study aimed to isolate CACN from sweet cherry and investigate their influence on the development of obesity.
Materials and methods

Chemical reagents

3T3-L1 pre-adipocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). DMEM, insulin, antibiotic mixture, isobutylmethylxanthine (IBMX), dexamethasone (DEX), and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY). Cyanidin-3-glucoside, cyanidin-3-rutinoside, and pelargonidin-3-rutinoside were purchased from Polyphenols Laboratories (Sandnes, Norway). All of the other chemicals were of reagent grade.

Isolation of anthocyanins from sweet cherry

Fresh sweet cherry was obtained from the Agricultural Logistics Center in Hangzhou. Anthocyanins were purified according to Prior (2008) with slight modifications. In brief, cherry pulp was extracted with methanol/water/formic acid (90:9:1, v/v) for 6 h in a shaking water bath at 4 °C. The resulting mixture was centrifuged at 10,000 ×g for 30 min. Afterward, the supernatant was collected and the residue was extracted again as described. The two supernatants were concentrated by vacuum evaporation and subsequently placed in an Amberlite XAD-7 column. The anthocyanins were adsorbed on the column. Sugars, organic acids, and other water-soluble compounds were removed from the column by using 1% formic acid mixed in distilled water. The binding anthocyanins were then eluted with 1% formic acid in methanol. Methanol
eluent was evaporated again to remove methanol. The concentrated eluent was extracted thrice with ethyl acetate. Afterward, the aqueous layer was collected and lyophilized to yield dry purified CACN powder. The powder was stored at –80 °C until use.

**Identification and quantification of anthocyanin by HPLC-ESI-MS/MS Analysis**

The anthocyanin compounds were determined using the Agilent 1290 Infinity liquid chromatograph system with quaternary pump and Infinity auto-sampler, coupled to a 6400 series triple-quadrupole mass spectrometer. Analyses were carried out using full scan, data dependent MS/MS scanning from m/z 100–1000. The capillary voltage ±3.0 kV, gas flow 6L/min, temperature of the source 325 °C and temperature of the desolvation gas 350 °C. The concentrations of each anthocyanin were determined by using UltiMate 3000 series HPLC equipped with a UV detector (UltimateTM 3000 wavelength).

**Cell culture and adipocyte differentiation**

The effect of CACN on lipid accumulation in 3T3-L1 cells was investigated according to the protocol of Suzuki et al (2011) and García-Diaz et al (2011), with slight modification. Briefly, 3T3-L1 pre-adipocytes were incubated in a culture medium containing DMEM, 10% FBS, 1.5 g/L of sodium bicarbonate, penicillin (100 U/mL), and streptomycin (100 µg/mL). The cell culture was incubated at 37 °C in an incubator humidified with 5% CO₂. Adipogenic agents (0.5 mM IBMX, 1 µM DEX,
and 1 µM insulin) were added to the culture medium to induce adipocyte differentiation. Cells were induced for two days with adipogenic factors. The medium was then changed to a normal culture medium and replaced with a fresh medium at an interval of 2 d. From 0 d to 6 d, the cells were exposed to chemical reagents (DMSO and CACN). CACN was dissolved using sterile water. At 7 d, the harvested 3T3-L1 adipocytes were washed with phosphate-buffered saline (PBS), fixed with 10% glutaraldehyde, and stained with 0.5% oil red O. The concentration of stained lipids was determined by extracting the dye with 100% isopropanol and obtaining the absorbance at 500 nm.

Animal experiments

All the experimental procedures were approved by the Committee on the Ethics of Animal Experiments of Zhejiang University (Permit Number: Zju201200116) and according to the National Institutes of Health Guide for Care and Use of Laboratory Animals. Forty-eight male C57BL/6 mice (four weeks old) were purchased from the National Breeder Center of Rodents (Shanghai, China) and kept in a specific pathogen-free facility. The mice were provided free access to water and food, housed at 23 ± 3 °C, and subjected to a 12 h/12 h light/dark cycle. The mice were acclimatized for 7 d and then randomly divided in four groups fed with specific diets for 12 weeks: (1) low-fat diet (LFD; 3.85 kcal/g, 20% kcal protein, 70% kcal carbohydrates, 10% kcal fat, supplemental Table 1.); (2) high-fat diet (HFD; 4.73 kcal/g, 20% kcal protein, 30% kcal carbohydrates, 50% kcal fat, supplemental Table 1.).
kcal/g, 20% kcal protein, 35% kcal carbohydrates, 45% kcal fat; Medicience Ltd, Yangzhou, China); (3) HFD with added CACN (40 mg/kg of food); and (4) HFD with added CACN (200 mg/kg of food). The human-equivalent in term of CACN doses based on body surface area is about 0.4 or 2 mg/kg of body weight, respectively (Reagan-Shaw et al., 2008). These groups were referred to as LFD, HFD, HFD40CACN, and HFD200CACN groups, respectively. Body weight and food intake were recorded weekly. At the end of the experimental period, the mice were anesthetized with ketamine-HCl following a 12 fast, and then sacrificed by decapitation. Blood samples, heart, liver, kidney, and adipose tissues were collected, weighed, and stored at –80 °C.

**Serum parameters analysis**

The concentrations of serum glucose, triglycerides, total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL)-cholesterol were determined by enzymatic methods using commercially available kits (Elabscience, Wuhan, China).

Serum insulin and leptin levels were analyzed by immunoassay using a rat/mouse ELISA kit (R&D, Minneapolis, MN, USA) according to the manufacturer’s protocols. A homeostatic model of insulin resistance (HOMA-IR) was assessed based on insulin and glucose levels obtained according to the formula (Matthews et al., 1985).

HOMA-IR = [serum glucose (mmol/L) × serum insulin (mU/L)]/22.5

β-cell function was evaluated based on a β-cell homeostasis assessment score.
(HOMA-BCF):

$$\text{HOMA-BCF} = [20 \times \text{serum insulin (mU/L)} \times \text{plasma glucose (mmol/L)} - 3.5].$$

**Histological analysis of the liver and epididymal white adipose tissue**

Liver and epididymal white adipose tissue samples were fixed with 10% formalin and then stained with oil red O and hematoxylin and eosin (H&E), respectively. Images were captured using a CCD Camera (Olympus Optical, Tokyo, Japan) at a magnification of $\times 100$. The analyses of adipocyte diameter were performed with Adiposoft (Boqué et al., 2009).

**Hepatic lipid and antioxidant enzymes activities analysis**

The liver samples from each mouse were homogenized in PBS, and the total lipids were determined according to a previously described method (Folch et al., 1957). The concentrations of liver triglycerides and total cholesterol were estimated using the same enzymatic kit for serum analysis.

The activity of SOD in the liver was determined using a Total Superoxide Dismutase Assay Kit (Beyotime, Haimen, China) and SOD activity was expressed as U/mg protein. The glutathione peroxidase activity (GPx) was detected using a Cellular Glutathione Peroxidase Assay Kit (Beyotime, Haimen, China).

**Quantitative real-time PCR**

Total RNA from white adipose tissue were extracted with Trizol (Invitrogen
Technologies, USA) according to the manufacturer’s protocol. The single-stranded cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) following the manufacturer’s protocol. Quantitative PCR was performed using LightCycler 480 system (Roche). The 20 µl reaction mixture was prepared as follows: 0.4 µl of forward primer (10 µM) (supplemental Table 2), 10 µl SYBR Green Quantitative PCR SuperMix-UDG (Invitrogen Technologies, USA), 2 µl of cDNA and 0.4 µl of reverse primer (10 µM). The real-time PCR conditions were as follows: 95 for 10 min followed by forty five cycles at 95 °C for 15 s, 60 °C for 5 s; 72 °C for 15 s.

The sequences primer used in the experiments were shown in Table 1. All the results were obtained from at least three independent experiments. The white adipose tissue expression of interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) were examined and normalized using β-actin as an internal control, and the relative fold induction was calculated according to the formula \( 2^{(-\Delta\Delta C)} \) (Rutledge, 2004).

**Statistical analysis**

The sample groups were statistically analyzed using SPSS 19.0 statistical software. The mean ± standard error of each group was calculated. Significant differences among groups were analyzed with ANOVA and post-hoc Duncan’s multiple range tests. An unpaired Student’s t-test was applied to detect differences of body weight compared to HFD group. P < 0.05 was considered significant.
Results

Characterization of purified CACN

An HPLC-ESI-MS/MS chromatograph was used to identify the anthocyanin composition of CACN. Table 1 shows the three kinds of anthocyanins present in CACN: cyanidin-3-(2\(^G\)-glucosylrutinoside), cyanidin-3-rutinoside, and pelargonidin-3-rutinoside. The identified anthocyanins were further analyzed by HPLC (UltiMate 3000 series). As shown in Fig. 1, the retention times of cyanidin-3-(2\(^G\)-glucosylrutinoside) or peak I, cyanidin-3-rutinoside (peak II), and pelargonidin-3-rutinoside (peak III) were 20.5, 22.9, and 25.6 min, respectively. The concentration of each anthocyanin was also calculated (Table 2). The results suggested that the predominant anthocyanins of sweet cherry are cyanidin-3-rutinoside and cyanidin-3-(2\(^G\)-glucosylrutinoside). This result is consistent with that in previous studies (Ballistreri et al., 2012; Bonerz et al., 2007; Pappas et al., 2011).

Influence of CACN on lipid accumulation in 3T3-L1 cells

We examined the effects of CACN on the differentiation of 3T3-L1 pre-adipocyte to adipocytes to investigate whether or not CACN exhibits inhibitory effects on lipid accumulation in 3T3-L1 cells. At 7 d, 200 µg/mL of CACN reduced lipid accumulation by 30.7% in 3T3-L1 cells (Fig. 2) (p < 0.05).
Influence of CACN on food intake and body weight

The mice were fed with or without CACN for 12 weeks to investigate the anti-obesity effects of CACN. No abnormal clinical signs were observed during this experimental period. The initial average body weight of the mice was 18.33 g, but this average was not significantly different among the four groups (Fig. 3A) (p < 0.05). During the fourth week after HFD was introduced, body weight increased slightly at a higher extent in the HFD-fed mice than in the LFD-fed mice. The weight gain continued progressively at a higher extent in the HFD group. After 12 weeks, CACN intake of 40 and 200 mg/kg reduced the body weight of the HFD-fed mice by 5.2% and 11.2%, respectively, but their body weights remained higher than those of the mice in the LFD group (Fig. 3A) (p < 0.05).

Food intake of the mice in the LFD group was higher than that of the mice in the HFD group (Fig. 3B) (p < 0.05). However, the caloric intake of mice in LFD group was lower compared to HFD group (Fig. 3B) (p < 0.05). We also found no significant differences in daily food intake and caloric intake among HFD-fed mice (Fig. 3B) (p < 0.05), respectively. These data suggest that a decrease in weight gain induced by CACN, is relevant with decreasing food efficiency (Chang et al., 2012; Jayaprakasam et al., 2006) (p<0.05).

The weights of the heart, liver, and kidney remained constant after CACN treatment. However, when expressed as a percentage of body weight, the heart and liver were smaller in HFD-fed mice compared to LFD-fed mice (Table 2) (p < 0.05). The epididymal and abdominal fat weight of mice in HFD group were much higher
than mice in LFD group, but CACN significantly decreased the weight of epididymal and retroperitoneal fat compared to HFD control (Table 2) (p < 0.05).

**Influence of CACN on serum parameters**

The mice in the HFD group showed significantly increased serum glucose, triglyceride, total cholesterol, HDL and LDL-cholesterol levels compared with those in the LFD group (Fig. 4) (p < 0.05). CACN at both doses decreased the serum total cholesterol and LDL-cholesterol level compared to HFD group, but their levels are still higher than those of the mice in the LFD group (Fig. 3C, Fig. 3D) (p < 0.05).

Compared to the effect on serum total and HDL-cholesterol, CACN affected serum glucose and triglyceride significantly (Fig. 3A, Fig. 3B) (p< 0.05). However, treat mice with CACN did not alter HDL-cholesterol (Fig. 3D).

Serum leptin and insulin levels in mice were examined (Table 3). Serum leptin was increased in HFD-fed mice. CACN (at 40 or 200 mg/kg of food) lowered the serum leptin levels compared with the HFD control (Table 3). The content of serum insulin was not altered by the amount of fat or CACN in the diet, but high HOMA-IR values in HFD-fed mice returned to normal levels by CACN supplementation. By contrast, the HOMA-BCF score was reduced in HFD-fed mice but improved in HFD plus CACN-fed mice (Table 3) (p < 0.05).

**Influence of CACN on the morphologies of the liver and the adipose tissue**

Morphological analysis of liver exhibits that mice fed with HFD stained for intense
lipid accumulation (Fig. 5A) (p<0.05) compared to LFD-fed mice. CACN whether administrated at low or high dose, significantly reduced the lipid accumulation in liver tissue.

Fig. 5B indicates the histology of epididymal white adipose tissue of the mice. The HFD induces hypertrophy of the adipocytes after 12 weeks. The adipocytes cell size was markedly attenuated when HFD-fed mice were treated with CACN at high dose (Fig. 5B) (p<0.05).

**Influence of CACN diet hepatic lipids and antioxidant enzymes activities**

Table 3 shows the increased hepatic content of total lipid, triacylglycerol, and cholesterol of the mice fed with the control HFD compared with the control LFD, but high dose of CACN could evidently decrease HFD induced liver triacylglycerol and cholesterol (Table 3) (p < 0.05).

The markedly reduction of total SOD and GPx activity in the liver of mice in HFD group was observed compared to LFD group (Fig. 6A) (P < 0.05). CACN at both dosed significantly increased the SOD and GPx activity compared to the HFD control (Fig. 6A) (p < 0.05).

**Influence of CACN on mRNA expression level of TNF-α and IL-6**

The mRNA expression levels of IL-6 and TNFα were determined in white adipose tissue (Fig. 6B). As compared to the LFD group, mice fed with HFD caused an up-regulation of IL-6 and TNFα genes. CACN at both dose markedly reduced the
expression levels of IL-6 and TNFα compared to the HFD control (Fig. 6B) (p < 0.05).

Discussion

Anthocyanins are the major sub-group of flavonoids responsible for the purple, blue and red color of many plant tissues. Recently, these substances have attracted scientists’ interest because of their non-toxicity and healthy benefits, including beneficial effects on obesity and related metabolic complications (Del Rio et al., 2013). In this study, we explored the effect of isolated anthocyanins with di-glycosides and tri-glycosides on HFD-induced obesity, and the effect of anthocyanin on oxidative stress and inflammation were also investigated.

Dietary fat can increase body weight and adiposity in humans and animals (Speakman et al., 2007). As expected, the present study confirmed that HFD indeed induced a great obesity, a markedly increase of serum and liver lipid profiles, as well as an elevation of leptin and the expression of IL-6 and TNFα levels, and a decrease of liver total SOD and GPx activity. If appropriate CACN administrated into HFD weight gain could be prevented, serum parameters (glucose, triglyceride, total cholesterol, LDL-cholesterol, leptin) brought down, hepatic lipids (total cholesterol, triglyceride, and lipid) attenuated, the expression levels of IL-6 and TNFα markedly reduced, the SOD and GPx activity increased. Researchers have also demonstrated that the consumption of isolated strawberry anthocyanins (mainly pelargonidin-3-glucoside) (Prior et al., 2009), blueberry anthocyanins (primarily
malvidin-3-glucoside and delphinidin-3-glucoside) (Prior et al., 2010a), mulberry anthocyanins (mainly cyanidin-3-glucoside) (Wu et al., 2013) (our recent work) decreases body weight gain. Based upon the data available, CACN may contribute to inhibit the body weight gain. Analytically, it is difficult to determine a particular anthocyanin in CACN singly responsible for the anti-obesity effect and further investigation is needed and worthwhile.

In our recent study, purified mulberry anthocyanins have been studied relative to effects on obesity. The mulberry anthocyanins reduce the resistance to insulin, markedly lower the size of adipocytes, attenuate lipid accumulation, and decrease the secretion of leptin (Wu et al., 2013). Our present results are in agreement with these aspects. But mulberry anthocyanin at both dose affected serum glucose (markedly lower the LFD control) more than sweet cherry anthocyanin relatively (Fig. 4A). Furthermore, we found an interesting phenomenon that CACN significantly decreased serum triglyceride, which is contrary to our recent result. The reasons for such differences remain unclear, but these differences are probably attributed to the structural variations in aglycone and sugar moieties. There might be dose dependent effects of anthocyanin consumption on body weight gain (Prior et al., 2010a). High dose of CACN (200 mg/kg of food) is more efficacious in preventing obesity, reduction the serum triglyceride, total cholesterol, LDL-cholesterol levels, hepatic lipids, and the expression of IL-6, while increasing the SOD activity.

C57BL/6 mice are susceptible to diet-induced obesity (Speakman et al., 2008; White, 2007). In this study, the mice fed with HFD showed high lipid accumulation in
the liver. Furthermore, HFD significantly increased serum glucose, triglycerides, total, HDL and LDL-cholesterol, but CACN countered this effect except for HDL-cholesterol. In previous study, purple corn (Tsuda et al., 2003), strawberry (Prior et al., 2009), blueberry (Prior et al., 2010a) and blackberry (Kaume et al., 2012) anthocyanin inhibited elevation of total liver lipids concentrations induced by HFD. CACN consumption might affect lipid metabolism by affecting hepatic lipid oxidation and lipogenesis (Jayaprakasam et al., 2006; Prior et al., 2009; Rolo et al., 2012; Tsuda et al., 2003).

Animal studies have shown that C57BL/6 mice fed with a HFD supplemented with anthocyanins exhibit improved insulin resistance and β-cell function (Lenquiste et al., 2012; Prior et al., 2010a; Sancho & Pastore, 2012; Tsuda et al., 2003). In the present study, insulin resistance (assessed by HOMA-IR) was observed in the HFD-fed mice, and CACN intake improved insulin resistance. β-cell function (assessed by HOMA-BCF score) weakened in the HFD-fed mice, and CACN improved this situation.

Leptin is a product of a gene encoding obesity traits and is secreted by adipose tissues; leptin has an important function in lipid metabolism (Maratos-Flier, 2008; Prieur et al., 2008). Studies have suggested that obesity models exhibit high serum leptin concentrations that decrease when animals are treated with anthocyanins (Prior, 2009, 2010b). A similar observation was found in our study. In addition, CACN treatment reduced the leptin levels. In the present study, HFD could increase the weight of the abdominal fat and induce hypertrophy in adipocytes. CACN
supplementation could decrease the weight of the epididymal fat and the size of adipocytes. In addition, CACN exhibited inhibitory effects of lipid accumulation in 3T3-L1 cells. CACN may directly affect both the number and the size of adipocytes, and this observation is probably likely associated with inhibition of adipose cell differentiation and proliferation (Boque et al., 2013; Suzuki et al., 2011).

Accumulated evidence indicates that obesity and associated hepatic fat accumulation induced by HFD can cause oxidative stress by decreasing the antioxidant enzymes activities (Lugogo et al., 2011). Several studies have demonstrated the beneficial effects of polyphenols against oxidative stress (Nabavi et al., 2012; Sarić et al., 2009). In this study we found that antioxidant properties in liver were affected by CACN. Furthermore, CACN at both dose significantly increased the antioxidant defenses compared to the HFD control. Therefore, CACN was shown to partially prevent oxidative stress.

Obesity is associated with a state of chronic or low grade systemic inflammation which increases production of obesity-related inflammatory cytokines, such as IL-1β, IL-6, TNFα, leptin and decrease anti-inflammatory cytokine levels, such as adiponectin (Bondia-Pons et al., 2012; Fernandez-Sanchez et al., 2011). We found HFD fed mice were under the pathophysiologic condition of inflammation associated with obesity, which evidenced by high levels of IL-6, TNFα and leptin. CACN exerted potentially anti-inflammatory effect (Table 3 and Fig. 6B). The mechanisms whereby anthocyanins exert anti-obesity effects are still not clear. Possible mechanisms suggested have included: suppression of the mRNA levels of the fatty
acid and triacylglycerol synthesis related gene (Tsuda et al., 2003), decreased the
production of inflammatory cytokines (DeFuria et al., 2009), reduced the food
efficiency (Chang et al., 2012; Wu et al., 2013), reduced the expression of
neuropeptide Y (NPY) and increased the γ-amino butyric acid receptor (GABA\textsubscript{B1}R) in
hypothalamus (Badshah et al., 2013).

In summary, purified CACN attenuated the body weight gain of the HFD-fed
C57BL/6 mice. Furthermore, CACN supplementation could attenuate the size of
epidydimal adipocytes, bring down serum glucose, lipids and leptin levels
significantly, improve the hepatic lipid profiles, effectively reduce the expression
levels of inflammatory gene and markedly increase the activity of antioxidant
enzymes. Therefore, CACN might be used as a potential agent against obesity.

Acknowledgments

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(Grant No. 31000775), and Zhejiang Innovation Program for Graduates (Grant No.
YK2011008).
References


**Figure Captions**

Fig. 1 HPLC chromatogram profile of sweet cherry anthocyanins at 520 nm. Column: Promosil C18 (4.6 mm × 250 mm; 5 µm) at room temperature; mobile phase: A (H₂O/acetonitrile/formic acid, 90:5:5, v/v/v) and B (water/acetonitrile/formic acid, 40:50:10, v/v/v); flow rate: 0.5 mL/min; elution gradient: 0 min 5% B, 20 min 20% B, 30 min 40% B, 40 min 60% B, 45 min 90% B, and 50 min 6% B.

Fig. 2 Sweet cherry anthocyanins (CACN) inhibited 3T3-L1 adipocyte differentiation. 3T3-L1 pre-adipocyte differentiation was induced in the presence of DMSO or 25, 50, 100, 200, and 400 µg/mL of CACN for 6 d. At 7 d, the cells were stained with oil red O. Data are expressed as the ratio of control levels (n = 3, p < 0.05 compared with no CACN).

Fig. 3 (A) Time courses of the body weight of male C57BL/6 mice; Data were presented as the mean ± S.E.M. (n=12 per group, p<0.05 compared with HFD) (B) Food intake for the male C57BL/6 mice. LFD (□), HFD (■), HFD 40CACN (○), HFD200CACN (▲). Data were presented as the mean ± S.E.M. and analyzed with ANOVA and post-hoc Duncan’s multiple range tests (p<0.05). The means marked with superscript letters are significantly different relative to others.

Fig. 4 Serum serum glucose, triglyceride, total cholesterol, HDL and LDL-cholesterol in mice. LFD (□), HFD (■), HFD 40CACN (○), HFD200CACN (▲). Data were presented as the mean ± S.E.M. (n=12 per group) and analyzed with ANOVA and post-hoc Duncan’s multiple range tests (p<0.05). The means marked with superscript letters are significantly different relative to others.
Fig. 5 Morphological changes in the liver and the epididymal adipose tissue of the male C57BL/6 mice. (A) Oil red O was used to stain the liver sections of mice; (B) H&E staining of epididymal adipose tissue and adipocyte size (% distribution). Original magnification ×100. Representative sections are obtained from three mice of the dietary group. Data were presented as the mean ± S.E.M. (n=3)

Fig. 6 (A), Hepatic antioxidant enzymes activities, (B) Gene expression determined by quantitative real-time PCR. Expression of IL-6, TNFα in the epididymal adipose tissue. LFD (□), HFD (■), HFD 40CACN (▓), HFD200CACN (░). Data were presented as the mean ± S.E.M. (n=12 per group) and analyzed with ANOVA and post-hoc Duncan’s multiple range tests (p<0.05). The means marked with superscript letters are significantly different relative to others.
Table 1 Mass spectrometry data and purified sweet cherry anthocyanins

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<td>I</td>
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<td>757/611/287</td>
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<td>II</td>
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<td>III</td>
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Data are expressed as mean ± S.E.M.
Table 2 Heart, kidneys, heart, and adipose tissue weights of C57BL/6 mice

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<th>LFD Control</th>
<th>HFD Control</th>
<th>HFD40CACN</th>
<th>HFD200CACN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart (g 100 g⁻¹)</td>
<td>0.50±0.04ᵃ</td>
<td>0.38±0.03ᶜ</td>
<td>0.45±0.06ᵇ</td>
<td>0.44±0.06ᵇ</td>
</tr>
<tr>
<td>Liver (g 100 g⁻¹)</td>
<td>4.05±0.03ᵃ</td>
<td>2.92±0.02ᵇ</td>
<td>2.79±0.15ᵇ</td>
<td>3.93±0.15ᵃ</td>
</tr>
<tr>
<td>Kidney (g 100 g⁻¹)</td>
<td>1.32±0.08ᵃ</td>
<td>0.97±0.22ᶜ</td>
<td>1.22±0.07ᵃᵇ</td>
<td>1.25±0.08ᵃ</td>
</tr>
<tr>
<td>Epididymal (g 100 g⁻¹)</td>
<td>1.92±0.11ᵃ</td>
<td>5.67±0.26ᵈ</td>
<td>4.61±0.34ᶜ</td>
<td>3.68±0.25ᵇ</td>
</tr>
<tr>
<td>Retroperitoneal (g 100 g⁻¹)</td>
<td>1.01±0.21ᵃ</td>
<td>1.87±0.17ᶜ</td>
<td>1.52±0.13ᵇ</td>
<td>1.43±0.19ᵃᵇ</td>
</tr>
</tbody>
</table>

Data were presented as the mean ± S.E.M. (n=12 per group) and analyzed with ANOVA and post-hoc Duncan’s multiple range tests. The means marked with superscript letters are significantly different compared with the other groups (p<0.05).
Table 3 Hepatic lipids, leptin and insulin levels of the male C57bl/6 mice

<table>
<thead>
<tr>
<th>Item</th>
<th>LFD Control</th>
<th>HFD Control</th>
<th>HFD40CACN</th>
<th>HFD200CACN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver lipids (mg/g)</td>
<td>43.55 ± 3.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68.15 ± 5.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>54.4 ± 4.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.48 ± 5.42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triglycerides (mg/g)</td>
<td>21.18 ± 1.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.65 ± 4.87&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.02 ± 4.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.16 ± 2.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cholesterol (mg/g)</td>
<td>1.89 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.72 ± 0.56&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.42 ± 0.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.47 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>2.46 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.50 ± 0.58&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.60 ± 0.57&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.42 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>2.09 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.29 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.17 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.07 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>13.30 ± 1.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.79 ± 2.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.90 ± 1.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.20 ± 2.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HOMA-BCF</td>
<td>30.69 ± 3.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.95 ± 2.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.52 ± 5.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.28 ± 4.53&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data were presented as the mean ± S.E.M. (n=12 per group) and analyzed with ANOVA and post-hoc Duncan's multiple range tests. The means marked with superscript letters are significantly different compared with the other groups (p<0.05). HOMA-IR, Insulin resistance was assessed by homeostasis assessment model; HOMA-BCF, β cell function was estimated by homeostasis assessment score.
Figure 1

Absorbance at 520 nm (mAu)

Retention time (min)
Figure 2

A

Control

Sweet cherry anthocyanins (200 µg/mL)

B

Relative Oil Red O Abs. (%)

Sweet cherry concentration(µg/mL)
Figure 3

A

![Body Weight Graph](image)

- LFD Control
- HFD Control
- HFD40CACN
- HFD200CACN

Weeks of Experiment

B

![Food Intake Graph](image)

Food intake (g/d/mouse)

Food intake (kcal/d/mouse)
Figure 4

A

Serum glucose (mmol/L)

B

Serum triglyceride (mmol/L)

C

Serum total cholesterol (mmol/L)

D

Serum lipoprotein cholesterol (mmol/L)

HDL

LDL
Figure 5

A  Liver

B  Adipose

LFD Control

HFD Control

HFD40CACN

HFD200CACN

5 µm

5 µm

5 µm

5 µm

µm <30 30-60 60-100 >100

µm <30 30-60 60-100 >100

µm <30 30-60 60-100 >100

µm <30 30-60 60-100 >100
Figure 6

A. Hepatic antioxidant enzymes activity (U/mg protein)

B. Relative gene expression

- IL-6
- TNFα

Letters indicate statistical significance.