Dietary supplementation with purified mulberry (*Morus australis* Poir) anthocyanins suppresses body weight gain in high-fat diet fed C57BL/6 mice

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**A B S T R A C T**

We present our experiment about adding anthocyanins to the daily food of mice. Three kinds of anthocyanins (cyanidin-3-glucoside, cyanidin-3-rutinoside and pelargonidin-3-glucoside) purified from Chinese mulberry (*Morus australis* Poir) were evaluated for suppressing body weight gain of the male C57BL/6 mice fed with high-fat diet (HFD). The results from a 12-week experiment show that consumption of purified mulberry anthocyanins (MACN) of 40 or 200 mg/kg can significantly inhibit body weight gain, reduce the resistance to insulin, lower the size of adipocytes, attenuate lipid accumulation and decrease the leptin secretion. Thus, dietary supplementation with MACN can protect against body weight gain of the diet-induced obese mice.

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### 1. Introduction

Obesity has become one of the main public health problems in recent decades because it could cause an increased risk of chronic diseases, such as type II diabetes and coronary heart disease (Hassel & James, 2005; Weyer, Foley, Bogardus, Tataranni, & Pratley, 2000). It is a complex metabolic disorder induced by an imbalance between calorie intake and metabolic expenditure, which is expressed as an increase in adipocyte number (hyperplasia) and size (hypertrophy) (Arner & Spalding, 2010; Hirsch & Batchelor, 1976; Suzuki et al., 2011). Currently available therapeutic approaches for treating obesity have a number of limitations, such as adverse effects and high rates of secondary failure (Derosa & Maffioli, 2012; Mayer, Hocht, Puyo, & Taira, 2009), so investigating potential natural products to prevent obesity has increasingly attracted the interest of the academic community (Yun, 2010).

In recent years, many natural pigments from vegetables and fruits have attracted scientists’ interest because of their non-toxicity and beneficial health effects (Hsu & Yen, 2007; Kim & Park, 2011; Pandey & Rizvi, 2009). As a natural pigment, anthocyanin is one of the major sub-group of flavonoids responsible for the red, blue and purple colour of many plant tissues (Castaeda-Ovando et al., 2009; He & Giusti, 2010). It also possesses antineoplastic, anti-inflammatory and lipoxygenase inhibition effects (Kim & Park, 2011). What is more, some anthocyanins have been documented in the prevention of diet-induced obesity (Prior, 2010; Zafra et al., 2007), such as anthocyanidins from purple corn (Zea mays L.) (Tsuda, Horio, Uchida, Aoki, & Osawa, 2003), black soybean (*Glycine max* L. Merr.) (Kwon et al., 2007), blood orange (*Citrus sinensis* L. Osbeck) (Titta et al., 2010), cornelian cherries (*Cornus mas*) (Jayaprasakam, Olson, Schutzki, Tai, & Nair, 2006), blueberries (*Vaccinium angustifolium*) (Prior et al., 2009, 2010), strawberries (*Rubus sp.*) (Kaume, Gilbert, Brownmiller, Howard, & Devred, 2012). These have been shown to be useful to ameliorate obesity in vivo. However, all these studies, with the exception of blueberries and strawberries, have used anthocyanins rich extracts. So increasing knowledge about how purified anthocyanins exert influence on the preventing obesity is necessary and worthwhile.

Chinese mulberry (*Morus australis* Poir) is traditionally used in medicines because of its pharmacological effects, for example, anti-fever diuretics, liver protection, blood pressure reduction and cardiovascular diseases amelioration (Huang, Chang, Wu, Huang, & Wang, 2011; Priya, 2012). Recent research has suggested that mulberry extracts exhibit an anti-obesity effect (Peng, Liu, Chang, Chyau, & Huang, 2011). Although mulberry is rich in anthocyanins, it is still unknown whether its specific anthocyanins...
possess pharmacological effects on anti-obesity. In this study, we purified anthocyanins from mulberry and investigated their effect on the development of obesity.

2. Materials and methods

2.1. Materials

Cyanidin-3-glucoside, cyanidin-3-rutinoside and pelargonidin-3-glucoside were obtained from Polyphenols Laboratories (Sandnes, Norway). All the other chemicals were reagent grade.

2.2. Methods of purifying anthocyanins from mulberry (MACN)

Fresh mulberry was purchased from a local fruit market in Hangzhou. 4 kg of mulberry were weighed out and extracted three times with methanol/formic acid (9:1, v/v). The combined extract was subjected to vacuum evaporation to remove the solvent and subsequently loaded onto an equilibrated Amberlite XAD-7 column. The column was saturated with 1% formic acid, and subsequently the binding anthocyanins were eluted with 1% formic acid in methanol. The methanol eluent was collected and subjected to vacuum evaporation again. Once evaporated, the concentrate was extracted with ethyl acetate until there was no colour change in the organic layer. The aqueous layer was lyophilized and stored at −80 °C until using. The purified anthocyanins were identified by using the Chromatograph HPLC/ESI/MS/MS (from Agilent 1290 Infinity LC) coupled to a 6400 series triple quadrupole mass spectrometer. The concentrations of each anthocyanin were determined by using UltiMate 3000 series HPLC.

2.3. Animals and diets

The male C57BL/6 mice with 4 weeks of age were purchased from the Branch of National Breeder Center of Rodents (Shanghai, China). After purchase the mice were housed at 23 ± 3 °C, and subjected to a 12 h light/dark cycle. The mice had free access to both food and water, and were handled according to the guidelines of the Instituted Animal Care and Use Committee of Zhejiang University for the care and use of laboratory animal (Zju2012-2-01-011Y).

After 7-day adaptation, all the experimental mice \( n = 48 \) were randomly divided into four groups according to the daily food intake of: (1) the control group fed with low-fat diet (LFD, 10% calories from fat); (2) the control group fed with high-fat diet (HFD, 45% calories from fat); (3) the test group fed with HFD added with MACN of 40 mg every kilogramme food; (4) the test group fed with HFD added with MACN of 200 mg of every kilogramme food.

The low-fat diet (LFD) was to contain 3.85 kcal in each gramme of food, of which there were 20% kcal from protein, 70% kcal from carbohydrates and 10% kcal from fat. The high-fat diet (HFD) was to contain 4.73 kcal in each gramme of food, of which there were 20% kcal from protein (from Mediscience Ltd., Yangzhou, China). The diets were replaced once every 2 days to prevent oxidation of the fats in diets. Body weight and the food intake quantity were measured once weekly.

2.4. Biochemical analyses of serum

Serum ALT, AST, glucose, triglycerides and total cholesterol levels were analysed by enzymatic methods using commercially available kits (Elabscience). Serum insulin and leptin levels were measured by immunooassay using a corresponding rat/mouse ELISA kit according to the manufacturer's protocols. The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated from insulin and glucose values by using Matthew’s formula (Matthews et al., 1985).

2.5. Histology analysis of liver and epididymal white adiposes

The liver and epididymal white adipose were fixed in a buffer solution of 10% formalin. Liver samples were stained with Oil Red O and hematoxylin to examine their cellular architecture and lipid accumulation. The fixed epididymal white adipose tissues were stained with haematoxylin and eosin (H&E) to analyse its adipocyte’s size.

2.6. Hepatic lipid analysis

The samples of mice liver were homogenised in phosphate-buffered saline and the total lipids were estimated by Folch's method (Folch, Lees, & Sloane Stanley, 1957). The liver triglycerides and total cholesterol were quantified with the same enzymatic method as used for serum analysis.

2.7. Statistical analysis

Statistical analyses for all sample groups were performed by the SPSS statistical software. A mean and its standard error for each group were calculated. Significant differences between these means were determined by post hoc Duncan’s multiple range tests. Probability values lower than 0.05 were accepted as significant.

3. Results

3.1. Characterization of purified mulberry anthocyanin (MACN)

The Chromatograph HPLC/ESI/MS/MS was used to determine anthocyanin compositions of MACN. Table 1 suggests there are three kinds of anthocyanins in MACN, which are cyanidin-3-glucoside, cyanidin-3-rutinoside and pelargonidin-3-glucoside. Then the identified anthocyanins were characterised by HPLC. As shown in Fig. 1, the retention times of cyanidin-3-glucoside, cyanidin-3-rutinoside and pelargonidin-3-glucoside are 15.9, 17.5 and 20.6 min, respectively. In addition, the concentration of each anthocyanin was measured (see Table 1). The results indicate that the main anthocyanins of mulberry are cyanidin-3-glucoside and cyanidin-3-rutinoside, which is in agreement with the previous studies.

3.2. Influence of diet MACN on food intake and body weight

In order to determine whether MACN affects body weight of the C57BL/6 mice, MACN (at 40 or 200 mg/kg) was administered daily for 12 weeks. There were no abnormal clinical signs throughout the 12-week experiment. After 12 weeks, the mice fed with HFD induced greater weight gain in comparison with the control LFD mice. Weight gain was 44.7% higher for the HFD group as compared to the LFD group (see Fig. 2A). Furthermore, administration of MACN at 40 mg/kg suppressed the body weight gain for the

<table>
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<td><strong>Mass spectrometry data and the content of purified mulberry anthocyanins (MACN).</strong></td>
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Values are expressed as mean ± SEM.
HFD-fed mice by 11.8%, but the higher level of MACN produced a 21.4% decrease instead (see Fig. 2A).

Food intake for the LFD group was 3.4 g/day throughout the experiment, which was higher than three HFD group, whereas the caloric intake (13.1 kcal/day) was lower (see Fig. 2B). We noticed that there were no significant differences about daily food intake and caloric intake between two HFD groups with MACN and the group without MACN. These data shows MACN does not affect the mouse’s food intake and caloric intake (see Fig. 3), but it can significantly decrease a HFD-induced body weight gain (see Fig. 2B).

There are no significant differences in the weights of heart, liver and kidney of the mice in these four groups (not shown here). However, as expressed in a percentage of body weight, heart, liver and kidney are smaller for the HFD-fed mice than the LFD-fed mice (see Table 2). The weight of epididymal fat is much higher for the HFD-fed mice compared to the control LFD-fed mice, but in the administration of MACN its weight will be not increased so much.

3.3. Influence of diet MACN on serum parameters

Serum glucose, triglyceride and total cholesterol levels were significantly increased for the HFD-fed mice relative to the control LFD-fed mice (see Table 2). Although MACN decreased HFD-induced serum cholesterol level, it was still higher than the control LFD-fed mice. Compared to the effect on serum cholesterol, MACN affected serum glucose significantly. Furthermore, we found an interesting phenomenon that MACN increased serum triglyceride, which is contrary to the previous experiment by Prior et al. (2010).

The HFD-fed mice showed dramatic elevation of serum parameters of liver injury (ALT and AST) compared to the LFD-fed mice (see Table 2). Intake of MACN did not alter AST level, but effectively attenuated ALT level for the HFD-fed mice.

Fig. 1. HPLC chromatogram profile of mulberry anthocyanins (MACN) at 520 nm. Column: Acclaim® C18 (25 × 4.6 mm, 5 μm) at room temperature; ELSD: drift tube temperature: 40 °C; mobile phase: A (H2O:acetonitrile/formic acid, 87:3:10, 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, 50 min 6% B.

Fig. 2. (A) Time courses of body weight gains for the male C57BL/6 mice. LFD ( ), HFD ( ), HFD with MACN at 40 ( ) or 200 mg/kg ( ) body weight. LFD, low-fat diet control, HFD, high-fat diet. (B) Food intake for the male C57BL/6 mice. LFD ( ), HFD ( ), HFD with MACN at 40 ( ) or 200 mg/kg ( ); (C) Caloric intake for the male C57BL/6 mice. LFD ( ), HFD ( ), HFD with MACN at 40 ( ) or 200 mg/kg ( ). Values are expressed as mean ± SEM. The means marked with superscript letters are significantly different relative to others.
3.4. Influence of diet MACN on adipose and liver morphology

The effects of MACN on lipid accumulation in liver were determined by Oil Red O staining. Mice fed with HFD stained for intense lipid accumulation in the liver (see Fig. 3A). In contrast, MACN alleviated the lipid accumulation, even higher level of MACN stained similar to the control LFD mice. Furthermore, morphological analysis indicated that the lipid droplet size was the largest in the HFD-fed mice, followed successively by the HFD with MACN at 40 mg/kg, the HFD with MACN at 200 mg/kg and the LFD-fed mice (see Fig. 3A). These suggested that MACN were responsible for suppression of liver lipid accumulation.

Fig. 3B exhibits the histology of epididymal white adipose tissue of mice by HE staining. The mice fed with HFD show hypertrophy of the adipocytes in the adipose tissue. MACN whether administrated at low or high levels, lowered the size of adipocytes in the adipose tissue.

3.5. Influence of diet MACN on hepatic lipids

Fig. 4A shows that hepatic content of total lipid, triacylglycerol and cholesterol are elevated for all mice fed with HFD relative to the control LFD, but it is obvious that MACN can reduce lipid, triacylglycerol and cholesterol of liver.

3.6. Influence of diet MACN on insulin and leptin

The insulin and leptin levels in mice serum were examined (Fig. 4B). The results revealed that insulin and leptin secretions into serum increased by comparison with the LFD-fed mice. Although MACN administrated at low and high levels were unable to alter the insulin secretion, the HOMA-IR values (Fig. 4B) and leptin secretion were significantly reduced.
4. Discussion

Anthocyanins are important plant pigments, which have been considered to be associated with a reduced risk of developing chronic diseases (He & Giusti, 2010; Kim & Park, 2011; Prior et al., 2010; Zafra et al., 2007). In this study we focused on the effects of purified mulberry anthocyanins on the development of obesity. The purified mulberry anthocyanins (MACN) containing cyanidin-3-glucoside, cyanidin-3-rutinoside and pelargonidin-3-glucoside have been used in this experiment. The predominant compositions of MACN are cyanidin-3-glucoside and cyanidin-3-rutinoside (see Table 1), which is consistent with previous studies (Dugo et al., 2001; Huang et al., 2011; Liu et al., 2008, 2009; Peng et al., 2011).

As expected, the present investigation has confirmed that HFD can induce a great gain of body weight, an increase of serum and liver lipids, as well as an elevation of insulin and leptin levels. If purified, MACN can be administrated into HFD, body weight gain could be attenuated, serum cholesterol and leptin levels brought down, and hepatic lipids (total lipid, triacylglycerol and cholesterol) decreased. Noticeably, all of the effects exerted by MACN did not cause abnormal clinical signs throughout our 12-week experiment. Some researchers have shown consumption of isolated strawberry anthocyanins (primarily elargonidin-3-glucoside) and purified blueberry anthocyanins (mainly composed of malvidin-3-glucoside and delphinidin-3-glucoside) had lowered body weight gain and body fat. Furthermore, blueberry anthocyanins, composed of two major anthocyanins (malvidin-3-glucoside and malvidin-3-galactoside) decreased serum leptin levels and improved β cell function (Prior et al., 2009, 2010). In the present study, the predominant anthocyanins of MACN are cyanidin-3-glucoside and cyanidin-3-rutinoside, and both of these also exhibit the similar functions. Based upon the data available, cyanidin-3-glucoside and cyanidin-3-rutinoside may contribute to suppress body weight gain, but the specific impact of anthocyanin on the development of obesity is still unknown and further investigation is needed.

The control mice fed with LFD consumed more food than the mice fed with HFD. However, intake of MACN did not affect the food intake (see Fig. 2B). There were no significant differences in daily food intake among three HFD-fed groups. Daily calorie intake was not different between the HFD-fed mice and the HFD with MACN (at 40 or 200 mg/kg, respectively) fed mice (see Fig. 2B). These data suggest that a decrease in weight gain induced by MACN, is irrelevant with decreasing food or calorie intake, and decreasing food efficiency may account for this (Jayaprakasam et al., 2006; Min et al., 2012).

The C57BL/6 mice are susceptible to diet induced obesity, which displayed as massive liver lipid accumulation (see Fig. 3A). Feeding mice HFD increases liver lipids, triglycerides and cholesterol significantly (see Fig. 4A), but MACN dramatically countered this. The action of MACN to suppress hepatic cholesterol and triglycerides in HFD mice, should result in a reduction of cholesterol and triglycerides in body tissues, but its effect on serum triglyceride contrasts to this. It that may be the consumption of MACN might regulate lipid metabolism by affecting hepatic lipid oxidation lipogenesis (Jayaprakasam et al., 2006).

The increase of serum insulin level and HOMA-IR values indicate that insulin resistance appeared in the HFD fed mice (Prior et al., 2010; Weyer et al., 2000). In the present study, insulin resistance (assessed by HOMA-IR) slightly appeared in the HFD-fed mice, and the intake of MACN reduced resistance to insulin (Fig. 4B).

Leptin is a product of the obese gene, secreted in white adipose tissue, which plays an important role in regulating energy homeostasis and lipid metabolism. Most obese animals exhibit high ser-
um leptin concentrations (Haslam & James, 2005). In agreement with previous studies (Prior et al., 2009, 2010), the leptin level of HFD-fed mice has been elevated (Fig. 4B). In addition, MACN treatments have slowed down the development of obesity, and a similar trend present in the leptin levels. In the current investigation, feeding mice with HFD could increase weight of their epididymal fat and induced hypertrophy adipocytes (Fig. 3B). The intake of MACN could reduce weight of their epididymal fat and lower the size of adipocytes. This phenomenon may suggest that MACN directly affect both the number and the size of adipocytes in adipose tissue, which is likely associated with production of leptin (Prior, 2010).

In summary, consumption of MACN suppresses body weight gain of the high-fat fed C57BL/6 mice. Intake of MACN at 40 mg/kg inhibits body weight gain by 11.8%, and the high level of MACN intake in the Public Interest (200903044).

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