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Basic nutritional investigation

Dietary sweet cherry anthocyanins attenuates diet-induced hepatic steatosis by improving hepatic lipid metabolism in mice

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ABSTRACT

Objective: Anthocyanins have been reported to have beneficial effects on obesity and obesity-related metabolic disorders (e.g., insulin resistance and dyslipidemia). The objective of this study was to examine the beneficial effects of sweet cherry anthocyanins (SWCN) on high-fat diet-induced liver steatosis and investigate the underlying molecular mechanism.
Methods: C57 BL/6 J mice were fed low-fat diet, high-fat diet, or high-fat diet supplemented with SWCN of 200 mg/kg for 15 wk. The hepatic gene expression profile was analyzed by DNA microarray analysis.
Results: SWCN supplementation alleviated high-fat diet-induced liver steatosis in mice. Microarray analysis of hepatic gene expression profiles indicated that SWCN treatment significantly changed the expression profiles of 1119 genes which were enriched in 16 pathways, such as PPAR signaling pathway, steroid biosynthesis, fatty acid metabolism, and biosynthesis of unsaturated fatty acids.
Conclusion: These results confirmed the previous findings regarding the occurrence and development of hepatic steatosis under high-fat-diet conditions, elucidated that SWCN protected from diet-induced hepatic steatosis and the beneficial effects might be involved in multiple molecular pathways, especially the PPARγ pathway.

Introduction

Liver steatosis, characterized by excessive accumulation of fat in hepatocytes, is a common chronic liver disease and considered to be an initial stage of non-alcoholic fatty liver disease (NAFLD) [1,2]. Mounting evidence suggests that hepatic steatosis is a mediator of the metabolic syndrome and closely associated with obesity, diabetes, and hyperlipidemia [3,4]. Several studies indicated that high-fat diet (HFD)-induced liver steatosis was largely due to the increased hepatic lipogenesis and decreased fatty acid oxidation [5,6]. Some drugs used for the treatment of hepatic steatosis, are usually accompanied by some adverse effects [7].

Anthocyanins are a major subgroup of pigments, consumed by people through dietary vegetables, fruits, beans, and red wine. Ongoing studies report the beneficial roles of anthocyanins, such as their antioxidative, antiinflammatory, anticarcinogenic, and antihyperlipidemic effects [8–11]. Moreover, cyanidin-3-glucoside (C3 G) and many other kinds of anthocyanins (e.g. blueberry anthocyanins, elderberry anthocyanins, and tart cherry anthocyanins) have been reported to ameliorate hepatic steatosis and adipose inflammation [12,13]. Previously, we demonstrated that dietary purified SWCN, containing three kinds of anthocyanins (cyanidine-3-[2 G-glucosylrutinoside], cyanidine-3-rutinoside, and pelargonidin-3-rutinoside), could significantly decrease high-fat diet-induced body weight gain and insulin-resistance in mice [8], but the influence of SWCN on hepatic steatosis was not fully elucidated. The present study aimed to evaluate the potential effects of SWCN on HFD-induced hepatic steatosis and investigate the underlying molecular

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mechanisms using gene microarray analysis followed by KEGG pathway analysis.

Materials and methods

Preparation of sweet cherry anthocyanins

The method for extraction and identification of SWCN have been described previously [8]. The contents of total polyphenol and total flavonoids were determined using the methods described previously [14,15], and the total polyphenols and total flavonoid contents were expressed as microgram gallic acid (GAE) and rutin equivalents (RE) and per milligram of SWCN respectively. One mg SWCN powder contained 394.79 ± 25.36 μg cyanidine-3-(2 G-glucosylrutinoside), 490.33 ± 13.62 μg cyanidine-3-rutinoside and 23.54 ± 1.95 μg pelargonidin-3-rutinoside [8], and the total polyphenol and total flavonoids contents of 1 mg SWCN were 17.35 ± 0.62 μg GAE and 50.89 ± 3.59 μg RE, respectively.

Animal care and experimental protocol

Thirty male C57 BL/6 J mice (four-week old) were obtained from the National Breeder Center of Rodents (Shanghai, China) and maintained under 12 h/12 h light/dark cycle with free access to food and water. All the protocols in this research were approved by the Committee on the Ethics of Animal Experiments of Zhejiang University (Permission Number: ZJU201550501) and the experimental procedures were performed in accordance with the National Institutes of Health regulations for the care and use of animals in research. All the animals were housed five per cage with room temperature of 23 ± 3°C. After 1 wk for acclimation, the mice were randomly divided into three groups (n = 10): LFD group, mice fed low-fat diet; HFD group, mice fed high-fat diet; HFD + SWCN group, mice fed high-fat diet supplemented with SWCN. The compositions and energy densities of modified AIN-93 G diets [16] were listed in the Supplementary Table S1. In the HFD + SWCN group, mice were given aqueous solution of SWCN at 200 mg/kg by oral gavage at 1400 h each day for 15 wk. The mice in LFD group and HFD group received an equal volume of saline alone. The body weights and food intakes were monitored weekly.

Animal sacrifice and sample collection

At week 15, the mice were sacrificed after a 12-h fast by decapitation. Blood samples were collected and centrifuged at 2000 g for 15 min to separate the serum. The livers were collected, rinsed with cold saline, and then weighed. Approximately, 0.4 × 0.4 × 0.4 cm sized liver samples was dissected and stored in 10% buffered neutral formalin, used for histologic evaluation. The remaining pieces of livers were flash frozen in liquid nitrogen, and then stored at −80°C before use.

Biochemical analyses

Serum levels of total cholesterol (TC), triacylglycerol (TG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured by an automatic biochemistry analyzer (ACCUTE TBA-40 FR, Japan) following the manufacturer's instructions. For the determination of hepatic TG and TC levels, liver tissues were homogenized, and the total lipids were extracted with 2:1 (v/v) mixture of chloroform:methanol using the method previously described [17], then the concentrations of hepatic TG and TC were determined using commercially available kits (Elabscience, Wuhan, People's Republic of China).

Histopathological analysis

The sections of liver were fixed in 10% buffered formalin, dehydrated, embedded in paraffin, sliced at 4 μm thickness, and then stained with hematoxylin and eosin (H&E). For oil red O staining, the sections of liver were embedded in optimal cutting temperature gel, then air-dried tissue sections of 6 μm were dipped in buffered formalin and washed with oil red O solution. Image capture was performed using an optical microscope (Olympus CX41, Shinjuku, Japan). The degree of hepatic steatosis was scored using the previously described method [18].

RNA preparation

Approximately 80 mg of frozen liver tissue was dissected and immediately homogenized in liquid nitrogen. Total RNA was extracted using Trizol (Invitrogen Technologies, Waltham, MA, USA) according to the manufacturer's instructions. The contaminating genomic DNA was removed by DNase-I digestion in solution, using Takara's RNeasy mini kit and DNase reagents. The quantity and purity of isolated RNA were determined using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Gene microarray profiling

The gene expression profiling by Illumina Mouse WG-6_v2.0 (Illumina, Inc, San Diego, CA, USA) platform was carried out by YiMeiTongDe (Beijing Emei Tongde Technology Development Co. Ltd., Beijing, China). Equal amounts of total RNA from five mice in each dietary group were pooled and treated as one sample before analysis to minimize the individual variability. Triplicate assays for each RNA sample were performed. Briefly, the total RNA (300 ng) was reverse-transcribed into cDNA, using T7 Oligo(dT) primers according to an Illumina TotalPrep RNA Amplification Kit (Ambion Inc., Naugatuck, CT, USA). After the cDNA purification, the cDNA was transcripted into Biotin-labeled complementary RNA (cRNA) in vitro. The hybridization was performed with biotin-labeled complementary RNA (cRNA) in BeadChip WG-6 array. After incubation at 58°C for 16 h, the BeadChips WG-6 were washed with fresh wash tray according to Illumina Whole-Genome Gene Expression Direct Hybridization Assay, stained with streptavidin-Cy3 and then the Beadchips were scanned with an Illumina Beadchip Reader as described in the Illumina manual. The array intensity data were first analyzed using the Illumina Genome Studio for visualization and normalization. The average normalization method was applied in all analyses and average background correction was performed using Beadstudio software. *P* values for differential expression were calculated using the Illumina Custom Error Model algorithm. For advanced data analysis, the three technical replicates were pooled and calculated by the averaging method to identify differentially expressed genes with a fold change in direction of ≥1.6 and *P* values less than 0.05. Gene Ontology-biological process (GO-BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed based on the Database for Annotation, Visualization, and Integrated Discovery (DAVID).

Real-time quantitative polymerase chain reaction (PCR)

The expression pattern of the selected differentially expressed genes in the microarray data analyses were validated by real-time quantitative PCR. The selected genes should meet the following criteria: Present in the differentially expressed gene list and involved in the significantly changed pathways identified using DAVID database. One μg total RNA was converted to cDNA in a 20 μL reaction mixture using a reverse transcription kit (Takara, Shiga, Japan) according to the manufacture's instruction. And real-time quantitative PCR was performed on Applied Biosystems (Waltham MA, USA) 7500 Real-Time PCR system. The reactions were conducted in a 20 μL mixture, each containing 1 μL cDNA, 10 μL SYBR Green QPCR Master Mix (Roche, Basel, Switzerland), and optimized concentrations of paired-primers (300 nM). The amplifications were performed according to the following thermoscycling program: 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C and 60 s at 60°C followed by melting curve for 60 s at 95°C, then gradual decrease to 50°C, 25 s at 50°C, then gradual increase to 95°C, and 25 s at 95°C. The primer sequences are presented in the Supplementary Table S4. All the samples were analyzed as triplicates, and the relative expression of mRNA was normalized using the geometric mean of 5 housekeeping genes, including β-actin, RPS18, B2 M, TBP, and ARBP.

Statistical analysis

All the statistical analyses were performed using SPSS 19.0 statistical software. Data are expressed as mean ± SD. The statistical significance was analyzed using Kruskal-Wallis Test for steatosis grading and one-way ANOVA and post-hoc Duncan's multiple range test for others. $P < 0.05$ was considered to be statistically significant.

Results

SWCN alleviated HFD-induced hepatic steatosis

At week 15, the mice in HFD + SWCN group displayed a significant reduction in body weight, liver weight, and liver index (percentage of liver/body weight) compared with HFD mice. In addition, the serum levels of TG, TCH, HDL-C, and LDL-C in HFD mice were substantially higher than those in LFD mice, but the SWCN supplementation resulted in a significant reduction in these serum parameters compared with HFD mice (Table 1). H&E staining of liver tissues showed that mice fed high-fat diet developed a high degree of hepatic steatosis with severe