Cranberry Attenuates Progression of Non-alcoholic Fatty Liver Disease Induced by High-Fat Diet in Mice

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Obesity is characterized by abnormal or excessive fat accumulation, which leads to the development of metabolic syndrome. Because oxidative stress is increased in obesity, antioxidants are regarded as suitable agents for preventing metabolic syndrome. Here, we examined the impact of cranberry, which contains various antioxidants, on metabolic profiles, including that during the progression of non-alcoholic fatty liver disease (NAFLD), in high-fat diet (HFD)-fed C57BL/6 mice. We observed that oxidative stress was diminished in mice that were fed HFD diets supplemented with 1 and 5% cranberry powder as compared with that in HFD-fed control mice. Notably, from 1 week after beginning the diets to the end of the study, the body weight of mice in the cranberry-treatment groups was significantly lower than that of mice in the HFD-fed control group; during the early treatment phase, cranberry suppressed the elevation of serum triglycerides; and adipocytes in the adipose tissues of cranberry-supplemented-HFD-fed mice were smaller than these cells in HFD-fed control mice. Lastly, we examined the effect of cranberry on NAFLD, which is one of the manifestations of metabolic syndrome in the liver. Histological analysis of the liver revealed that lipid-droplet formation and hepatocyte ballooning, which are key NAFLD characteristics, were both drastically decreased in cranberry-supplemented-HFD-fed mice relative to the levels in HFD-fed control mice. Our results suggest that cranberry ameliorates HFD-induced metabolic disturbances, particularly during the early treatment stage, and exhibits considerable potential for preventing the progression of NAFLD.

Key words cranberry; non-alcoholic fatty liver disease (NAFLD); oxidative stress; obesity; metabolic syndrome

INTRODUCTION

In 2015, a total of 107.7 million children and 603.7 million adults worldwide were obese. Obese is a major driver for the increasing prevalence of metabolic syndrome, a cluster of conditions including hyperglycemia, dyslipidemia, and hypertension in the same individuals, and one of the hepatic manifestations of metabolic syndrome is non-alcoholic fatty liver disease (NAFLD). NAFLD is characterized by evidence of hepatic steatosis and lack of secondary causes of hepatic fat accumulation, such as substantial alcohol consumption, long-term use of a steatogenic medication, or monogenic hereditary disorders, and the disease is categorized histologically into non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). Whereas NAFL is defined as the presence of hepatic steatosis in ≥5% of hepatocytes with no evidence of hepatocellular injury in the form of hepatocyte ballooning, NASH is defined as the presence of ≥5% hepatic steatosis and inflammation with hepatocyte injury, including ballooning, with or without any fibrosis, and can lead to cirrhosis, liver failure, and hepatocellular carcinoma. Although NAFLD begins as a simple accumulation of triglycerides (TGs) in the liver, marked hepatic lipid-droplet accumulation represents a risk factor for life-threatening liver-related complications.

The pathological mechanism underlying NAFLD has been described using the “two-hit” and “multiple parallel hits” hypotheses. According to the two-hit hypothesis, the first hit is the development of hepatic lipid accumulation involved in disorders such as obesity, dyslipidemia, and type 2 diabetes mellitus; this is followed by the second hit, which includes oxidative stress, secretion of proinflammatory cytokines, insulin resistance, and gut-derived endotoxin secretion, and this is necessary for the development of NASH. Conversely, the multiple parallel hits hypothesis, which is a new hypothesis, posits that several of the aforementioned hits act in parallel and this ultimately results in liver inflammation.

Oxidative stress is increased in obesity and plays critical roles in the pathogenesis of metabolic syndrome, and oxidative stress has also been implicated in the pathogenesis of NAFLD. Therefore, suppression of oxidative stress is regarded as one of the therapeutic targets in the treatment of metabolic syndrome, including NAFLD. Various antioxidants have been tested in treatments and found to potentially exert beneficial effects in human and animal studies, and, intriguingly, Harrison et al. reported that the combination treatment of vitamins E and C alleviates fibrosis in patients with NAFLD.

Cranberry (Vaccinium oxycoccos L.) (Ericaceae) contains vitamins E and C and a large amount of phenolic polyphenols, including procyanidins and anthocyanins, which exhibit antioxidative activity. Notably, the antioxidative activity of procyanidins has been found to be considerably stronger than that of vitamin E or C, and, among fruits, cranberry contains markedly high levels of procyanidins. Long-term intake of cranberry has previously been shown to...
attenuate hepatic inflammation and steatosis, which results in protection against NAFLD in the high-fat diet (HFD)-fed mice.\textsuperscript{20,21} However, no study to date has examined in detail how metabolic profiles are affected during the early phase of cranberry treatment \textit{in vivo}. Here, we investigated the effect of polyphenol-rich cranberry on obesity-associated metabolic abnormalities over time, including during NAFLD progression, in HFD-fed mice.

MATERIALS AND METHODS

\textbf{Animals and Treatments} The experimental diets used, normal diet (ND) (CE-2) and HFD (High Fat Diet 32, 56.7\% kcal from fat), were obtained from CLEA Japan (Tokyo, Japan). Cranberry powder (biokia cranberry powder, product number 500452), which was processed 100\% of cranberries and contained neither additives nor added sugar, was manufactured by Kiantama Oy (Suomussalmi, Finland); the anthocyanin and proanthocyanidin contents reported by the manufacturer were 120 and 2600 mg/100 g, respectively.\textsuperscript{22} Cranberry powder was included in HFD.

Male 6-week-old C57BL/6 mice were purchased from Nippon SLC (Hamamatsu, Japan). After acclimation for 1 week, the mice were divided into four treatment groups: (1) ND, (2) HFD, (3) HFD + 1\% cranberry powder (HFD + CB1), and (4) HFD + 5\% cranberry powder (HFD + CB5). The diets of CB-supplemented HFD were prepared in our laboratory. Subsequently, the mice were further divided into four groups and, after 1, 2, 4, or 8 weeks feeding on the different diets, were anesthetized by inhaling isoflurane (3\%) (Pfizer, Tokyo, Japan) and dissected. No relevant adverse effects were observed in the experimental animals. The mice were maintained on a 12/12-h light/dark cycle and provided free access to water and food. Body weight was measured once a week, and the total food intake for each cage (5 mice/cage) was measured. All animal procedures were approved by the Institutional Animal Care and Use Committee of Osaka Ohtani University (approval ID 1701) and performed in accordance with institutional guidelines and regulations for animal experiments at Osaka Ohtani University.

\textbf{Analysis of Oxidative Stress} To examine the level of oxidative stress in the liver, nitroguanosine formation was assessed. Mouse livers were embedded in Tissue-Tek OCT compound (Sakura Finetek Japan, Tokyo, Japan) and sectioned, and the frozen sections were fixed overnight in peridate lysine paraformaldehyde solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Immunohistochemical staining was performed using anti-nitroguanosine antibody (10\,µg/mL, COSMO BIO, Tokyo, Japan), ImmPRESS-Alkaline Phosphatase Reagent (Vector Laboratories, Burlingame, CA, U.S.A.), and Alkaline Phosphatase Substrate Kit I VECTOR RED (Vector), according to manufacturer protocols. For semi-quantitative histopathological comparison, each section was analyzed using NIH Image Analysis Software, ImageJ (NIH, Bethesda, MD, U.S.A.).

\textbf{Fasting Blood Glucose} Blood samples were obtained from the tail vein of fasted (16h) mice at 1, 2, 4, and 8 weeks after beginning the diets. Fasting blood glucose levels were determined using a Gluestar Sensor Neo (Sanwa Kagaku Kenkyusho, Nagoya, Japan).

\textbf{Serum TG Level} Blood samples were collected through retro-orbital bleeding at 1, 2, 4, and 8 weeks after beginning the diets, and then serum was obtained by centrifuging the samples. Serum TG levels were determined using a LabAssay Triglyceride kit (Wako Pure Chemical Industries, Ltd.).

\textbf{Serum Alanine Aminotransferase (ALT)} Blood samples were collected through retro-orbital bleeding at 1, 2, 4, and 8 weeks after beginning the diets, and serum was obtained by centrifuging the samples. Serum ALT levels were analyzed at the Oriental Yeast Corporation (Tokyo, Japan).

\textbf{Liver Histology} For histopathological examination, liver samples were collected from mice, washed with phosphate-buffered saline, and fixed in 10\% buffered formalin. The samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) at Applied Medical Research (Osaka, Japan).

For Oil Red O staining, mouse liver samples were embedded in Tissue-Tek OCT compound, and then frozen sections were prepared; subsequently, the sections were stained with Oil Red O staining solution (Muto Pure Chemicals, Tokyo, Japan), as per the manufacturer’s protocol, and then counterstained with hematoxylin. For semi-quantitative histopathological comparison, each section was analyzed using ImageJ.

\textbf{Quantitative RT-PCR Analysis of Gene Expression in the Liver} Total RNA was extracted from mouse liver using TRizol (Life Technologies, Carlsbad, CA, U.S.A.), according to the manufacturer’s instructions, and the mRNA levels of interleukin-6 (IL-6), sterol regulatory element binding protein-1c (SREBP-1c), peroxisome proliferator-activated receptor γ (PPARγ), and monocyte chemotactic protein-1 (MCP-1) were determined through quantitative RT-PCR performed using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan); mRNA levels were normalized to the β-actin mRNA level. The following thermal-cycling protocol was used: 60s at 95\(^\circ\)C, followed by 40 cycles of 15s at 95\(^\circ\)C and 60s at 63\(^\circ\)C. The sequences of the primers used in this study are shown in Supplementary Table.

\textbf{Statistical Analysis} Statistical significance of differences was determined using Tukey’s test; \(p<0.05\) was considered significant. All data are presented as means ± standard deviation (S.D.).

\section*{RESULTS}

\textbf{Effects of Cranberry on Body Weight and Food Intake} To investigate the effect of cranberry on body weight, mice in the four diet groups were weighed weekly (Fig. 1A). The body weight of HFD-fed mice was remarkably higher than that of ND-fed mice. In contrast, the body weight of cranberry-supplemented-HFD-fed mice was markedly lower than that of HFD-fed control mice, and, notably, the cranberry-supplemented-HFD-fed mice exhibited drastically diminished weight gain as compared with HFD-fed mice until 2 weeks after beginning the diets. Conversely, total food intake was higher in the two cranberry-supplemented-HFD-fed groups than that in the HFD group (Figs. 1B, C). The exact reason for difference in total food intake was unknown; however, cranberry-supplementation might attenuate the oily smell of the HFD due to the high lipid content of the diet. Therefore, mice might prefer eating cranberry-supplemented HFD than HFD only. These results indicated that cranberry suppressed the HFD-induced increase in body weight and concomitantly...
triggered hyperphagia.

**Cranberry Reduces Oxidative Stress in the Liver** To examine whether cranberry suppresses oxidative stress, frozen liver sections were stained with an antibody against nitroguanosine, a marker of oxidative stress. Nitroguanosine was detected at a higher level in the livers of HFD-fed mice than cranberry-supplemented-HFD-fed mice at almost all time points examined (Figs. 2A, B). Moreover, we used quantitative RT-PCR to measure the mRNA level of IL-6, a proinflammatory cytokine that is expressed under oxidative stress, and our results showed that cranberry treatment trended to suppress IL-6 expression (Fig. 2C). These results indicated that cranberry reduced obesity-induced oxidative stress in the liver.

**Influence of Cranberry on Glucose and Lipid Metabolism** The effect of cranberry on glucose metabolism was examined by measuring fasting blood glucose levels at 1, 2, 4, and 8 weeks after beginning the diets. Fasting blood glucose was higher in HFD-fed mice than in ND-fed mice, but the glucose levels did not differ in a statistically significant manner among the HFD, HFD + CB1, and HFD + CB5 groups (Fig. 3).

Next, we investigated how cranberry affects lipid metabolism. First, serum TG levels were significantly lower in cranberry-supplemented-HFD-fed mice than in HFD-fed mice at 1 and 2 weeks after beginning the diets (Fig. 4A). Second, histological analysis revealed that adipocytes in epididymal adipose tissue in cranberry-supplemented-HFD-fed mice were markedly smaller than those in epididymal adipose tissue in HFD-fed mice at 1 and 2 weeks after starting the diets (Supplementary Fig.). Third, we quantified the mRNA level of sterol regulatory element binding protein-1c (SREBP-1c), a master regulator of lipid metabolism; at 1 week after beginning the diets, hepatic SREBP-1c mRNA level was lower in mice in the HFD + CB5 group than in mice in the HFD group (Fig. 4B), although this suppressive effect produced by cranberry supplementation was lost by 2 weeks after beginning the diets. Fourth, Oil Red O staining of liver sections revealed a higher amount of lipid droplets in HFD-fed mice than in cranberry-supplemented-HFD-fed mice at 1 and 2 weeks after starting the diets (Figs. 4C, D). These results suggested that cranberry reduces lipid accumulation and thus might prevent NAFLD progression.

**Cranberry Attenuates NAFLD Progression** Lastly, to investigate the effect of cranberry on NAFLD, we performed a histopathological examination of liver sections obtained at 1, 2, 4, and 8 weeks after beginning the diets. Starting from 1 week after beginning the diets, higher amounts of lipid droplets were detected in the liver in HFD-fed mice than in the liver in ND-fed mice (Figs. 4C, 5A), and at 8 weeks after beginning the diets, not only increased amounts of lipid droplets but also enlarged hepatocytes (relative to control) were observed in the liver in HFD-fed mice (Fig. 5A). Furthermore, ALT levels were higher HFD-fed mice than in ND-fed mice (Fig. 5B). This increase in lipid droplets, enlargement of hepatocytes, and elevation of ALT levels are key features of NAFLD, and thus the findings indicated that the HFD-fed...
mice displayed NAFLD. Notably, cranberry-supplemented-HFD-fed mice exhibited a decrease in lipid droplets and hepatocyte ballooning as compared with HFD-fed mice (Figs. 4C, 5A). The serum ALT levels in cranberry-supplemented-HFD-fed mice and HFD-fed mice did not show statistically significant differences, but the ALT level was lower in HFD + CB1 than in HFD mice (Fig. 5B). Here, we also examined the hepatic mRNA levels of PPARγ and MCP-1, which are detected in patients with NAFLD, at 2 weeks after starting the diets, the levels of both mRNAs were significantly lower in the mice in HFD + CB5 group than in the mice in HFD group (Figs. 5C, D). These results indicated that dietary supplementation with cranberry reduced HFD-induced NAFLD.

DISCUSSION

NAFLD is a major health problem associated with obesity and oxidative stress. In this study, we selected cranberry as
an agent for reducing oxidative stress, and we examined how cranberry affected the metabolic profile of HFD-fed mice, including during NAFLD progression. Our results showed that cranberry treatment caused a reduction in oxidative stress, serum TG concentration, and weight gain. Moreover, histological examination of adipose tissues revealed that adipocytes were enlarged in HFD-fed mice, but that the size of these cells was considerably decreased during the early phase

Fig. 3. Fasting Blood Glucose Levels in Mice
Mice were fed the four diets for 1, 2, 4, or 8 weeks, and before collecting organs, the fasting (16 h) blood glucose levels were determined. Data are expressed as means ± S.D. (n = 5). * p < 0.05, compared with HFD.

Fig. 4. Effect of Cranberry on Lipid Metabolism
Mice were fed the four diets for 1, 2, 4, or 8 weeks. (A) Before dissecting organs, blood samples were collected, and serum TG levels in fasted mice were measured. (B) Quantitative RT-PCR was used to quantify the mRNA expression of SREBP-1c. (C) Liver sections were stained with Oil Red O. Bar = 50 µm. (D) Semi-quantitative analyses of oxidative stress were performed with (C). Data were normalized relative to the corresponding value for HFD and are expressed as means ± S.D. (n = 4–5); * p < 0.05, compared with HFD. (Color figure can be accessed in the online version.)
of cranberry treatment. Notably, cranberry treatment potently reduced lipid droplets and hepatocyte ballooning in the liver, thus suppressing the progress of obesity-induced NAFLD.

Most TG in the plasma is a component of circulating very-low-density lipoprotein, which is produced by the liver. The transcription factor, SREBP-1c, activates the expression of genes related to fatty acid and TG metabolism, and SREBP-1c plays a significant role in accumulation of hepatic TG. Here, hepatic SREBP-1c expression was lower in the mice in HFD + CB5 group than in the mice in HFD group at 1 week after the beginning of the diets, but this inhibitory effect of cranberry was lost by 2 weeks. The conversion of excess carbohydrates to fatty acids is mediated by an increase in SREBP-1c. The amount of food intake was higher in the HFD + CB group than that in the HFD group; therefore, the effect of cranberry on suppression of SREBP-1c was considered to be canceled by 2 weeks after beginning the diets. In addition, serum TG levels of cranberry-supplemented-HFD-fed mice were lower than those of HFD-fed mice at 1 and 2 weeks. Cranberry might reduce serum TG levels by suppressing SREBP-1c expression, but other SREBP-1c-independent mechanisms may also be involved in the regulation of TG synthesis, transport and catabolism.

In NAFLD development, oxidative stress represents the main mechanism underpinning the “second hit” that leads to reactive oxygen species (ROS) formation. Lipid accumulation in the liver impairs the oxidative capacity of mitochondria, and the consequent increase in the generation of ROS and reactive aldehydic derivatives cause oxidative stress, leading to induction of inflammatory cytokines and insulin resistance. In this study, cranberry treatment was found to suppress HFD-induced oxidative stress, analyzed by means of anti-nitroguanosine staining, and reduce hepatic lipid droplets, detected through Oil Red O staining. Cranberry contains numerous antioxidants (e.g., vitamins E and C, anthocyanins, and proanthocyanidins); proanthocyanidins especially have been found to have strong antioxidant activity and are present in a high content in cranberries. These antioxidants, including proanthocyanidin, might interact to suppress the progression of NAFLD.

Okuno et al. reported that adipocyte-specific inhibition of oxidative stress using overexpression of catalase (Cat) and superoxide dismutase 1 (SOD1), Cat/SOD1 double-transgenic mice, decreased ectopic lipid accumulation, particularly in the liver, and enhanced adipose expansion. Our results are consistent with this as we observed suppression of hepatic lipid accumulation in cranberry-supplemented-HFD-fed mice; however, adipocytes in the adipose tissues of cranberry-supplemented-HFD-fed mice were smaller than those in HFD-fed mice during the early treatment phase in our study. Exact
reasons for this discrepancy are currently unknown. The study by Okuno et al. eliminated adipocyte-specific oxidative stress and they showed similar profiles of plasma TG between Cat/SOD1 double-transgenic mice and wild-type mice. In our study, in contrast, systemic suppression of oxidative stress by cranberry would attenuate whole-body expression of inflammatory cytokines and thereby reduce insulin resistance, resulting in reduced whole-body lipids, such as serum TG levels and lipid droplets in the adipocytes and the liver.

Although total food consumption was higher in cranberry-supplemented-HFD-fed mice than in HFD-fed mice, weight gain was suppressed, adipose-tissue cells were smaller, and serum TG concentrations were lower. We hypothesized that besides exhibiting antioxidant activity, cranberry might potentially activate fatty acid oxidation. However, the expression of fatty acid oxidation-related genes (such as the PPARα gene) did not differ markedly between mice treated with and without cranberry (data not shown). We next hypothesized that cranberry might suppress fatty acid synthesis. However, the expression of hepatic SREBP-1c, which induces fatty acid synthesis-related gene expression, was not suppressed in HFD + CB mice compared with that in HFD mice except for 1 week after the diet. Cranberry might have effects on other factors such as lipid absorption and transport. A recent study demonstrated that cranberry extract reversed high-fat/high-sucrose diet-induced metabolic disturbances by altering the gut microbiota. However, further investigation is necessary to confirm these findings.

Cranberry lowered serum TG concentration and adipocyte size during the early treatment phase (first 2 weeks), but the effects were not detected at 8 weeks (Figs. 4, 5). Thus, the ability of cranberry to counteract HFD-induced metabolic disturbances might be limited to a few weeks. However, at all the time points examined (up to 8 weeks), body weights of cranberry-supplemented-HFD-fed mice were lower than those of the mice that were fed HFD without cranberry supplementation. Therefore, the effect exerted by cranberry during the early treatment phase might contribute to the attenuation of weight gain, as well as to the suppression of NAFLD progression, even in the late phase.

Our findings agree with those of a previous study indicating that cranberry extract reduces hepatic inflammation and NAFLD in HFD-fed obese C57BL/6 mice. Glisan et al. and Anhê et al. demonstrated that body weight and adiposity in cranberry-supplemented-HFD-fed mice showed no change relative to that in HFD-fed mice, although our results showed that the body weight of cranberry-supplemented-HFD-fed mice was lower than that of HFD-fed control mice. This discrepancy between the findings could be attributed to experimental design. The mice used in the study by Glisan et al. were pre-fed HFD (60% kcal fat, 20% kcal protein, and 20% kcal carbohydrate) for 11 weeks and those of Anhê et al. were pre-fed high-fat/high-sucrose diet (65% kcal lipid, 15% kcal protein, and 20% kcal carbohydrates); thus, these studies were intervention. In contrast, the mice in our study were not pre-fed HFD (56.7% kcal fat, 20.1% kcal protein, and 23.2% kcal nitrogen free extract), that means prevention study. Therefore, cranberry ameliorated HFD-induced obesity, particularly during the early treatment stage.

In summary, our findings suggest that in HFD-fed mice, cranberry supplementation can attenuate NAFLD progression in conjunction with reducing oxidative stress, as well as markedly lower serum TG concentration and weight gain during the early treatment phase. Thus, cranberry might represent an efficient candidate agent for preventing the development of metabolic syndrome, including NAFLD.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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