Effects of Dietary Blueberry (*Vaccinium ashei* Reade) Leaves on Mildly Postprandial Hypertriglyceridemia

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Abstract: Prevention of postprandial hypertriglyceridemia is an important consideration for reducing the risk of developing cardiovascular disease. While blueberry fruits have been reported to ameliorate lipid metabolism in humans, there are only few research reports on the effects of blueberry leaves (BL). Here, we investigated the efficacy of BL on postprandial hyperlipidemia in subjects with high fasting triacylglycerol (TG) concentrations. Randomized, double-blind, cross-over design study was conducted. The subjects consumed a BL containing beverage or a placebo beverage before a fat-enriched test meal. Blood samples were collected prior to and 1, 2, 3, 4, and 5 hours after consuming the test beverage. The postprandial serum TG and remnant-like particle cholesterol (RLP-C) concentrations were significantly lower in the BL beverage compared with those in the placebo beverage. Additionally, BL was more effective in subjects with high fasting ghrelin with gastric emptying function. In current study, fasting ghrelin correlated with the increase in postprandial serum TG, suggesting that BL ameliorates hypertriglyceridemia through delayed gastric emptying. In conclusion, this pilot study suggests that BL may be useful as an early dietary therapy for treating postprandial hyperlipidemia.

Key words: triacylglycerol, remnant-like particle cholesterol, ghrelin, blueberry leaves

1 Introduction

Fasting and postprandial triacylglycerol (TG) are risk factors for cardiovascular and other chronic diseases⁵, especially postprandial state is clinically important. Several prospective studies have suggested that the postprandial increase in non-fasting serum TG increases the risk of atherosclerosis and ischemic stroke and is an independent risk factor for coronary artery disease²⁻⁵. Additionally, remnant lipoproteins of the metabolites of TG-rich lipoproteins, such as chylomicrons and very-low-density lipoproteins have been recognized as powerful coronary heart disease (CHD) risk factors⁶⁻¹¹. Therefore, therapeutic approaches to reduce not only TG, but also remnant lipoproteins in the postprandial state are believed to be important for the management of people with disorders of lipid metabolism.

Prospective cohort studies strongly suggest that a diet high in fruits and vegetables may protect against cardiovascular disease¹², ¹³. Many studies have suggested natural compounds such as phytochemicals in fruits and vegetables can be important regulators in terms of the risks related to metabolic syndrome¹⁴, ¹⁵. The leaves of blueberry (*Vaccinium ashei* Reade), which belongs to *Ericaceae* plant group, are used as a folk medicine for treating metabolic disorders, such as diabetes, in Europe¹⁶. So far, there have been many studies on the therapeutic efficacy of blueberry fruits¹⁷⁻²⁰, little to none investigations have been done on the leaves. Recently, blueberry leaves (BL) were reported to have lower serum lipids and alleviate hepatic TG accumulation in rats²¹. Moreover, BL reduces visceral fat and ameliorates insulin resistance in diet-induced obese mice²². Although these reports might indicate that BL prevent lifestyle-related diseases, no study has investigated its clinical translation.

In the present study, we investigated the efficacy of BL on postprandial hyperlipidemia in subjects with high fasting TG concentrations.
2 Experimental

2.1 Materials

The sample beverages consisted of a BL beverage containing 300 mg of BL extracts in 100 mL of water and a placebo beverage containing no BL extracts in the same type of bottles and with the same volume and taste as the BL beverage. BL extracts were prepared using a hot water. Briefly, blueberry leaf powder was extracted in 16 parts of hot water at 95-100°C for 30 min twice. Then, the extract was filtered, and heat sterilized. Finally, the extract was dried with a spray dryer, producing a powder. BL extracts contained 127 mg proanthocyanidin/100 mL (MASIS Inc., Food & Drug Nano Analysis, Aomori, Japan). These test beverages were prepared for this study, and both beverages were less than 30 kcal. The sample beverages were prepared by Bizen Chemical Co. Ltd (Okayama, Japan).

2.2 Ethics

This study was carried out in accordance with the Declaration of Helsinki (version 2013) and approved by the ethical Committee of Miyazaki University (Ethical Committee approval no.: I-0025). All participants received a full explanation of the study and provided written informed consent, under the supervision of the physicians in charge. The protocol was registered with the University Hospital Medical Information Network (UMIN) Center (UMIN-CTR, http://www.umin.ac.jp/ctr/index-j.html) in advance (UMIN registration no.: 00030883).

2.3 Subjects

Women and men, 20-60 years old, with a fasting serum TG level of 1.13 mmol/L (100 mg/dL) to 2.25 mmol/L (199 mg/dL) were recruited from the Miyazaki region of Japan. The inclusion criteria were based on the onset point of increased risk of coronary heart disease according to the results of a retrospective cohort study

Subjects were excluded if they had underlying disorders that affect lipid metabolism, including cardiorespiratory dysfunction, renal damage, hepatic disease, gastrointestinal disease or endocrine disease, as judged by blood and urine data collected at the time of a screening visit or from medical histories. Subjects taking anti-hyperlipidemic or anti-hyperglycemic agent or using functional food products that affect lipid or glucose metabolism were also excluded. The subjects were randomly assigned to each sequence (ingestion order) with stratified randomization for gender, age, and fasting TG using computer-generated random numbers under blind conditions.

2.4 Study protocol

The study had a randomized, double-blind, crossover design. The following measurements were conducted at the screening visit, after overnight fasting for at least 12 hours: anthropometric parameters (height, weight, waist circumference); visceral fat area using an impedance instrument (EW-FA90; Panasonic Corporation, Osaka, Japan), which has results highly correlated with the computed tomography method; blood pressure; serum lipids (TG, low-density lipoprotein [LDL]-cholesterol, high-density lipoprotein [HDL]-cholesterol); plasma glucose; hemoglobin A1c; hepatic function (serum alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transpeptidase, alkaline phosphatase, lactate dehydrogenase and total protein); serum albumin; serum uric acid; serum creatinine; serum total bilirubin; serum urea nitrogen; and urine analysis by urine test strip.

Subjects consumed the fat-enriched test meal after consuming the test beverage (BL or placebo) after overnight fasting for at least 12 hours. After a washout period of two weeks, the second experiment was conducted using the other test beverage and the same test meal. The subjects were instructed to maintain their habitual diet and physical activity during the study period and to record their meal contents for 3 days before each visit. The test meal consisted of corn potage soup (200 g) (Nagoya Seiraku Co. Ltd, Nagoya, Japan), butter (19 g) (Megmilk Snow Brand Co. Ltd, Sapporo, Japan) and lard (15 g) (Megmilk Snow Brand Co. Ltd, Sapporo, Japan), the total nutrient composition of which was calculated as 40.3 g fat, 4.1 g protein and 17.2 g carbohydrate (total calorie 1816 kJ).

Blood samples were collected before consuming the test beverage to provide baseline values, and at 1, 2, 3, 4, and 5 hours after consuming the test beverage. Plasma samples were collected in sodium fluoride tubes. Blood was centrifuged at 1,500 × g for 15 min at 4°C to separate the serum and plasma. The concentrations of serum TG, remnant-like particle cholesterol (RLP-C), apolipoprotein B48 (ApoB48), insulin, leptin, and plasma glucose and ghrelin were analyzed. Serum TG was measured using a standard enzymatic colorimetric assay (Serotec Co., Ltd., Sapporo, Japan). Serum RLP-C was measured using a homogeneous assay (Hitachi Chemical Diagnostics Systems Co., Ltd., Tokyo, Japan). Serum insulin (Abbott Japan Co., Ltd., Tokyo, Japan) and ApoB48 (Fujirebio Inc., Tokyo, Japan) were measured by the CLEIA assay. Serum leptin was measured by radioimmunoassay (Merck Millipore Co., Ltd., Danvers, MA). Plasma glucose was measured by a glucose oxidase (GOD) immobilized electrode method (A&T Corporation, Yokohama, Japan). Serum RLP-C, ApoB48 and leptin were analyzed by SRL Inc (Tokyo, Japan) and the other serum and plasma samples were analyzed by Clinical Laboratory, University of Miyazaki Hospital.

To measure plasma ghrelin, blood was collected directly into tubes containing EDTA-2K and aprotinin; the tube was immediately centrifuged for plasma isolation at 4°C. The isolated plasma was treated with one-tenth of its volume of 1 N HCl and the tube was rocked gently. This sample was then used for plasma ghrelin measurement using an auto-
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3 Results

3.1 Subjects

Sixteen people who met the inclusion criteria were registered as subjects. Twelve subjects completed the study and four dropped out of the study: one who stopped the test due to feeling ill after the fasting blood draw (before ingesting the test meal) during the first test and did not complete all two tests, two who could not participate in all two tests due to personal reasons, and the other who could not eat test meal. Of 12 subjects that completed the study, two people were excluded from the analysis according to the study protocol: one who regularly ate functional foods in test periods and the other who had decreased serum insulin from the fasting state to postprandial state, leaving a subject group comprising 10 people who met the study protocol requirements. The characteristics of subjects are shown in Table 1.

3.2 Postprandial lipid parameters

As a primary outcome, the IAUC of serum TG concentration were significantly lower in the BL beverage compared with the placebo beverage (Fig. 1C). Moreover, the IAUC of serum RLP-C concentration were significantly lower in the BL beverage compared with the placebo beverage (Fig. 1F). In the exploratory analysis, changes in postprandial serum TG and RLP-C from the initial values (Δ) were significantly lower in the BL beverage compared with the placebo beverage when assessed by the linear mixed-effects model with fixed effects for group (Fig. 1B, 1E). On the other hand, changes in postprandial serum ApoB48 concentration from the initial values (Δ) were tended to be lower in the BL beverage compared with the placebo beverage (Fig. 1H), but the IAUC was not significantly different (Fig. 1I).

A stepwise analysis as a multiple regression analysis on the relationship between the postprandial TG response in the index of IAUC and fasting blood parameters (TG, RLP-C, ApoB48, glucose, insulin, leptin, ghrelin concentrations) indicated that fasting ghrelin concentration was the only explanatory variable for the postprandial TG response (Table 2). Therefore, when the relationship between the BL effect (IAUC for serum TG concentration after ingestion of placebo beverage minus IAUC for serum TG concentration after ingestion of BL beverage) and fasting ghrelin concentration was examined, they had a significant positive correlation (Fig. 2).

3.3 Postprandial other blood parameters

None of the other parameters measured in this study (glucose, insulin, leptin, ghrelin concentrations) were significantly different between two test beverages (Table 3).

4 Discussion

In the present study, we investigated the effects of BL on postprandial serum lipid parameters. Compared with the placebo beverage, the consumption of the BL beverage significantly decreased not only serum TG but also serum

Table 1  Characteristics of subjects.

|                         | All  |
|-------------------------|------|
| Male/female             | 5/5  |
| Age (y)                 | 46.3 ± 2.9 |
| Height (cm)             | 161.8 ± 3.1 |
| Weight (kg)             | 69.8 ± 3.9 |
| Body mass index (kg/m²) | 26.8 ± 1.6 |
| Waist circumference (cm)| 91.4 ± 3.6 |
| Visceral fat area (cm²) | 109.2 ± 17.6 |
| Systolic blood pressure (mmHg) | 127.8 ± 3.4 |
| Diastolic blood pressure (mmHg) | 79.7 ± 2.2 |

Values are means ± SE
RLP-C. RLP-C was developed to evaluate the cholesterol concentration of remnant lipoproteins\(^1\). Kugiyama et al. demonstrated that RLP-C and RLP-TG measurements appear to provide a more precise assessment of CHD risk than plasma TG\(^2\). McNamara also reported that RLP-C was an independent CHD risk factor in women participating in the Framingham Offspring study and was superior to TG in this regard\(^3\). These data suggested that the suppression of the postprandial increase in RLP-C, achieved in our preliminary study by ingestion of BL, may be helpful to construct a nutritional therapeutic strategy for reducing CHD risk.

As shown in Fig. 1H, the increase in serum ApoB48 concentration was slightly lower in the BL beverage compared with the placebo beverage. ApoB48 is one of the apolipoproteins secreted from the small intestine and is present in chylomicrons. Therefore, ApoB48 is an indicator of chylomicron quantity, but our observation did not reveal an obvious effect of BL on ApoB48. On the other hand, Hara et al. reported that oolong tea polymerized polyphenols decreased postprandial serum TG as well as TG concentration in chylomicrons\(^4\). Further studies may be required to...
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Table 2  Multiple regression analysis (stepwise method) between the postprandial TG response (TG-IAUC) and fasting blood parameters (TG, RLP-C, ApoB48, glucose, insulin, leptin, ghrelin).

|        | r    | β    | R    | adjR² | F     |
|--------|------|------|------|-------|-------|
| Ghrelin| 0.755| 0.755| 0.755| 0.517 | 10.635|
| TG     | 0.091|      |      |       |       |
| Glucose| –0.554|     |      | 0.517 |       |
| Insulin| –0.344|     |      | 0.344 |       |
| Leptin | –0.215|     |      | 0.215 |       |
| RLP-C  | –0.135|     |      | 0.135 |       |
| ApoB48 | –0.106|     |      | 0.106 |       |

Multiple regression equation: TG-IAUC = 2.503 + 0.125 ghrelin, p = 0.012
Fasting TG, RLP-C, ApoB48, glucose, insulin, and leptin were excluded in this analysis.
r: simple correlation coefficient, β: standard partial regression coefficient, R: multiple correlation coefficient, adjR²: adjusted coefficient of determination, TG: triacylglycerol, RLP-C: remnant-like particle cholesterol, ApoB48: apolipoprotein B48, IAUC: incremental area under the curve.

Fig. 2  BL was more effective in the subjects with fasting high ghrelin concentration. There were significantly correlations between fasting ghrelin concentration and BL effect (N = 10). Please refer Fig. 1 for abbreviations.

A potential mechanism underlying the suppressive effect of BL on the postprandial hyperlipidemia is suggested by a previous study on apple polyphenols, in which proanthocyanidins in BL inhibited pancreatic lipase. Proanthocyanidins are a group of polyphenols widely distributed in nature (in fruits, vegetables and their beverage products such as red wine and tea). They have been shown to have protective effects against several diseases. Horigome et al. reported that proanthocyanidins from various plants had inhibitory effects on digestive enzymes such as trypsin, alpha-amylase, and lipase. Among various plants, proanthocyanidins in apple polyphenols have been shown to be highly effective in pancreatic lipase inhibition, thereby preventing postprandial elevation of plasma TG in rodent and human. Furthermore, the inhibitory effects of the proanthocyanidins increased according to the degree of polymerization from dimer to pentamer. Similarly, proanthocyanidins with DPs of pentamer or greater showed maximal inhibitory effects on pancreatic lipase. Other studies have confirmed a clear relationship between the proanthocyanidins DP and their inhibitory effects on pancreatic lipase. For example, the inhibition of lipase activity by fractions of grape seed procyanidins and of cocoa extracts also increased with increasing DP, wherein an inverse correlation between log IC₅₀ and DP was observed. Thus, higher levels of DP were associated with more potent inhibitory activities of the proanthocyanidins. On the other hand, Kimura et al., in addition to analyzing the effect of the DP, studied the effect of the proanthocyanidin-linkage type in the inhibition exerted on pancreatic lipase activity, and so the inhibition of pancreatic lipase activity demonstrated to be related not only to higher molecular sizes but also the proportions of A-type linkages. BL is rich in polyphenols such as chlorogenic acid, rutin, and proanthocyanidins. Takeshita et al. reported that the mean degree of polymerization of proanthocyanidins was 7.7 in a study on suppression of expression of Subgenomic Hepatitis C Virus RNA by proanthocyanidins in BL. Furthermore, Matsuo et al. elucidated the presence of A-type in proanthocyanidins in BL by mass spectral analysis. In this study, we considered that the TG-lowering remarkable effect of BL was due to

Investigate TG concentrations in chylomicrons rather than on the number of chylomicron particles. The TG concentrations in chylomicrons rather than on the number of chylomicron particles.
According to the data presented herein, the TG-lowering effect of BL correlated significantly with fasting ghrelin. Ghrelin is a 28 amino acid peptide produced predominantly in the stomach by the enteroendocrine cellular system. Ghrelin has been shown to modify energy homeostasis by stimulating appetite, food intake and inducing adiposity. Ghrelin also mediates several gastrointestinal functions. It has been shown to accelerate gastric emptying in healthy subjects and in patients with gastroparesis. Despite reports from previous studies showing that postprandial glucose decreased due to delayed gastric emptying, the relationship between delayed gastric emptying and decreased postprandial TG was unknown until recently. Sato et al. reported that the TG-lowering effect of metformin was mediated by delayed gastric emptying for the first time. In the present study, fasting ghrelin level was extremely correlated with the postprandial TG response, such that the TG-lowering effect of BL was remarkable in subjects with high fasting ghrelin. On the other hand, Serrano et al. reported that proanthocyanidins led to a reduction in gastric emptying. Our results suggest that the TG-lowering effect of BL may be related lipase inhibition as well as to delayed gastric emptying by proanthocyanidins in BL. However, further studies are needed to elucidate the detailed mechanism of this effect.

5 Conclusion

BL was effective at decreasing postprandial hyperlipidemia, especially in people with high fasting ghrelin. This pilot study indicates that BL may be useful as an early dietary therapy for treating postprandial hyperlipidemia.

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Conflicts of Interest

The authors declare no conflict of interest.

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