Inhibition of Low-Density Lipoprotein Oxidation by Nagano Purple Grape (Vitis vinifera × Vitis labrusca)

Masumi KAMIMARU1, Yoshimi KISHIMOTO2, Mariko TANI3, Kunihiko ANDOH4, Kazunori UTSONOMIYA4, and Kazuo KONDO2

1Department of Food Technology, Nagano Prefecture General Industrial Technology Center, 205–1 Nishibamba Karita, Nagano 380–0921, Japan
2Institute of Environmental Science for Human Life, Ochanomizu University, 2–1–1 Ohtsuka, Bunkyo-ku, Tokyo 112–8610, Japan
3Andoh Orthopaedic Clinic Matsushiro, 152–2 Matsushiro, Matsushiro-cho, Nagano 381–1231, Japan
4Division of Diabetes, Metabolism and Endocrinology, Department of Internal Medicine, Jikei University School of Medicine, 3–25–8 Nishi-Shinbashi, Minato-ku, Tokyo 105–8461, Japan

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Summary The Nagano Purple grape (Vitis (V.) vinifera × V. labrusca) is a hybrid created by a cross between Kyoho (V. vinifera × V. labrusca) and Rosario Bianco (V. vinifera) grapes. The grape, including its skin, can be eaten and contains no seeds because of gibberellin treatment. The skins of various fruits have been shown to contain antioxidant activity. However, it is unclear whether the Nagano Purple grape contains antioxidant activity. We prepared the skins and dried fruits (including the skins) of the Nagano Purple grape, so as to assay for the presence of an antioxidant activity. We examined the concentration of polyphenols in the grape and further assayed whether components in the grape inhibited the oxidation of low-density lipoprotein (LDL). We detected the presence of cyanidin-3-glucoside (Cy-3-glc), five anthocyanidins and resveratrol in the skins. A trace of resveratrol was detected in the pulp. LDL collected from human subjects 1 h following the consumption of the skins or dried fruits revealed significant inhibition of LDL oxidation compared to that observed in fasting venous blood samples. We further observed the antioxidant activity of Cy-3-glc. Our results suggest that the consumption of the Nagano Purple grape can give rise to resistance to LDL oxidation.

Key Words anthocyanin, antioxidant, low-density lipoprotein, grape

The Nagano Purple grape is a new triploid grape cultivar (Vitis (V.) vinifera × V. labrusca) with large, dark-colored berries. This cultivar resulted from a cross between the tetraploid grape, Kyoho (V. vinifera × V. labrusca) and the diploid grape, Rosario Bianco (V. vinifera) in 2004. It was released by the Nagano Fruit Tree Experiment Station, Suzaka-shi, Nagano, Japan, in 2005. The entire grape can be eaten, including its skin and it contains no seeds because of gibberellin treatment.

Grapes contain a large amount of various phenolic compounds in their skins, pulp, and seeds (1–5). These phenolic compounds, including anthocyanin, proanthocyanidin (catechins) and condensed tannin, may be the source of putative health benefits derived from processed goods such as wine (6–8). A wide range of polyphenolic constituents have been reported to exhibit antitumor and anti-inflammatory effects, as well as the ability to block cellular events predisposing to atherosclerosis and coronary heart disease (CHD) (9–13). In recent years, resveratrol has been shown to exhibit several biological activities, including cardioprotection and antioxidant activities, inhibition of platelet aggregation, and anti-inflammatory activity (14–18). Furthermore, fruits whose skins contain various polyphenols have been shown to exhibit high antioxidant activity in vitro (19, 20). However, the presence of such components in the skin or pulp of the Nagano Purple grape and the antioxidant activity of the grape remained to be empirically demonstrated.

Modifications of low-density lipoprotein (LDL) have been implicated in the pathogenesis of atherosclerosis. LDL oxidation can be mediated by various processes, such as by the action of transition metal ions (copper or iron), nitric oxide/superoxide radicals, or by the action of peroxidase enzymes (21, 22). For example, the free radical-mediated oxidation of LDL leads to lipid peroxidation, which is the autooxidation of the polyunsaturated fatty acid chains of lipids by a radical chain reaction (23). A diet rich in antioxidants might counteract these processes by inhibiting the oxidation of LDL. Therefore, the use of antioxidants to prevent the formation of oxidized LDL might be useful to reduce the atherogenicity associated with modified LDL.

In the present study, we report the first identification of polyphenols in the Nagano Purple grape and the effect of the grape on LDL oxidation both in human subjects and in vitro. We demonstrate an effect of the Nagano Purple grape upon the induction of LDL oxidation, which is implicated in the pathogenesis of atherosclerosis.
MATERIALS AND METHODS

Materials. Nagano Purple, Kyoho, and Rosario Bianco grapes were purchased from the market. The dried fruits of Nagano Purple (the berry including its skin) were prepared using a freeze-drying process (65°C, 40 h), which was performed by Asuzacu Foods Inc., Nagano, Japan. The anthocyanin standard, cyanidin-3-glucoside chloride (Cy-3-glc), and the anthocyanin standards, delphinidin chloride (Dp), cyanidin chloride (Cy), petunidin chloride (Pt), pelargonidin chloride (Pg), peonidin chloride (Pn), and malvidin chloride (Mv) were purchased from SSX, Lyon, France. Resveratrol (3,5,4-trihydroxystilbene) and protocatechuic acid were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. The catechin standards, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin gallate (EGCg), (−)-catechin (C), (−)-gallocatechin (GC), (−)-catechin gallate (Cg), and (−)-gallocatechin gallate (GCg) were purchased from Wako Pure Chemical Industries, Ltd. Hypoxanthine (6-hydroxypurine) was purchased from Sigma, St. Louis, MO, USA. 5,5-Dimethyl-1-pyrole-N-oxide (DMPO) was purchased from Labo- tec, Tokyo, Japan. Xanthine oxidase was purchased from Nippon Boehringer Ingelheim, Tokyo, Japan. Diethylentriamine-N,N,N',N''-pentaacetic acid (DTTPA) was purchased from Dojindo, Kumamoto, Japan.

Detection of polyphenols. Because the Nagano Purple grape resulted from a cross between Kyoho and Rosario Bianco grapes, we prepared the skins and pulp from each type of grape (Nagano Purple, Kyoho, and Rosario Bianco grapes) for separate evaluation. We examined the concentration of Cy-3-glc, anthocyanidins, resveratrol, and catechins in grapes by liquid chromatography/mass spectrometry (LC/MS; 1100 Series LC/MSD Trap; Agilent Technologies, Tokyo, Japan) (24–26). The analytical column was an Inertsil ODS-3 (4 μm, 150×4.6 mm I.D.; GL Science, Tokyo, Japan) operated at 40°C. Data was expressed as the weight of each polyphenol versus the base weight of the skin or pulp. Conditions of LC/MS are indicated in Table 2.

For analysis of polyphenols, 5 g of each sample (skin and pulp) was extracted with 30 mL of 80% ethanol, and was evaporated under vacuum to remove the ethanol. The residue was reconstituted in 50 mL of distilled water. Cy-3-glc and catechins were identified by LC/MS.

Anthocyanidins and resveratrol were identified in each sample (skin and pulp) as detailed below. Acid hydrolysis of the anthocyanins was achieved by dissolving 1 mL of the above extracts in 100 μL of 6 N HCl. The solution was then sealed under nitrogen gas and heated to 150°C for 0.5 h. The samples were then cooled immediately in an ice bath, and were quantified by LC/MS.

The analysis of resveratrol was carried out on the above extracts by chromatography on an Amberlite XAD-1180 column (Organo, Tokyo, Japan). By systematic variation of the conditions for adsorption and desorption from the resin with ethyl acetate, a simple solvent extraction was evaporated under vacuum to remove ethyl acetate, and was quantified by LC/MS.

Study subjects and design. Eight healthy male volunteers (aged 42.5±6.6 y) were asked to maintain their habitual diet and lifestyle, and to stop taking vitamin supplements prior to the initiation of the study. The clinical characteristics of the study subjects are indicated in Table 1. This study was approved by the Ethics Committee of Ochanomizu University and carried out in conformity with the Declaration of Helsinki (established in 1964 and revised in 2004). All subjects provided informed consent to participate in our study.

The skins or dried fruits (including the skins) were prepared for a single administration to study participants. After fasting for 12 h, fasting venous blood samples were drawn. Additional venous blood samples were drawn 1 h following consumption of the skins (200 g) or dried fruits (50 g). The design of this study, indicating each dose of consumption (corresponding to 1,000 mg of polyphenol concentration) and blood samples (venous blood samples were drawn 1 h following consumption) was based on our previous examination of LDL oxidation (9, 10, 27, 28).

Sample assay. For analysis of L-ascorbic acid in the samples, we used a Vitamin C Assay Kit (Shima Laboratories, Tokyo, Japan).

To verify whether the Nagano Purple grape exhibit beneficial effects on LDL oxidation, we examined its antioxidant activity by two methods: the lag time assay and measurement of LDL mobility by electrophoresis.

LDL was isolated by ultracentrifugation (100,000 rpm (41,700 ×g), 40 min, 4°C) using a TLA-100.4 fixed-angle rotor (Beckman, Palo Alto, CA), as reported previously (29). The protein content of LDL was determined with a commercial test kit (Micro BCA Protein assay; Pierce Biotechnology, Rockford, IL) using bovine albumin as a standard. Oxidation of LDL (70 μg/mL protein) was initiated by the addition of V-70 (2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile)); Wako Pure Chemical Industries, Ltd.) solution (final concentration 400 μM/mL) at 37°C and we detected the antioxidant activity of LDL by the two methods described below.

We used spectrophotometry (Beckman DU-650; absorbance at 234 nm) to monitor changes in the

Table 1. Clinical characteristics of the subjects.

<table>
<thead>
<tr>
<th>n</th>
<th>8</th>
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<tbody>
<tr>
<td>Age</td>
<td>42.5±5.6</td>
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<tr>
<td>Height (cm)</td>
<td>172.4±5.8</td>
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<tr>
<td>Body weight (kg)</td>
<td>68.2±9.4</td>
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<tr>
<td>BMI</td>
<td>22.9±2.4</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>201.9±19.1</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>87.5±24.7</td>
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<tr>
<td>SBP (mmHg)</td>
<td>115.1±10.4</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>74.1±12.9</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.1±0.1</td>
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Data are means±SD.
quantity of conjugated diene every 4 min for 5 h. The determination of lag time was reported previously (28).

The change in surface charge of the LDL protein was evaluated by an increase in its electrophoretic mobility, which was determined using the TITAN GEL Lipoprotein Kit (Helena Laboratories, Saitama, Japan). As LDL undergoes modification (V70 incubation time: 2 h), it becomes more negatively charged and migrates more efficiently towards the anode due to the increased negative charge. Therefore, alterations of LDL can be measured by comparing the relative mobility of modified LDL.

Antioxidant activity of polyphenol. We analyzed the antioxidant activity of Cy-3-glc and protocatechuic acid using the lag time assay as described above. Protocatechuic acid is a metabolite of Cy-3-glc. Oxidation of LDL (70 µg/mL protein) was initiated by the addition of V-70 solution (final concentration 200 µM) at 37°C. The lag time assay was analyzed three times.

We further examined the scavenging of superoxide...
radicals using electron Spin Resonance (ESR) spectroscopy (30). A mixture of 50 μL of 5 mM hypoxanthine and 50 μL of 0.4 U/mL xanthine oxidase in 0.1 M phosphate buffer, pH 7.8 (total; 200 μL) was used to generate superoxide radicals. We added 35 μL of 5.5 mM DTPA to this solution for chemical stability of the superoxide radicals and then added the spin-trapping reagent, 15 μL of DMPO. Superoxide dismutase (SOD) was used as a standard. The superoxide radical was detected as the spin adduct, DMPO-OOH (DMPO-OH). The spectra was quantified by double-integration using a standard SOD curve and expressed in terms of the SOD activity. Measurements were performed on a JES-FR30EX SERIES SPECTROMETER (Jeol, Tokyo, Japan) under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 100 μT; scanning field, 335 mT; response time, 0.1 s; sweep time, 1 min; microwave power, 4 mW; microwave frequency, 9.42 GHz; temperature, room temperature.

Statistical analysis. Measurements of polyphenols and ESR represent the average of three independent experiments. Measurements of lag time assay in vitro represent the average factor (ratio against control as 1) of three independent experiments. Data for fasting and post consumption of lag time assay are presented as the lag time (min) and the modified LDL are indicted by the relative mobility, and statistical analyses were performed using Student’s paired t-test. Probability values

![Graph](image-url)
were given for the test and \( p<0.05 \) was considered statistically significant.

**RESULTS**

**Detection of polyphenols in grapes**

The results of the polyphenol analysis are presented in Table 2. The indicated values represent the average of three independent analyses. Values of anthocyanidins present in the skins or pulp represent the concentration determined following acid hydrolysis of the samples. Cy-3-glc, anthocyanidins other than for Pg, and resveratrol were detected in the skin of the Nagano Purple grape, whereas only trace resveratrol was detected in the pulp. In the Kyoho grape, we detected Cy-3-glc, anthocyanidins other than for Pg, and resveratrol in the skins. In the Rosario Bianco grape, we detected resveratrol in the grapes and catechins in the grapes were present at levels below the limit of our detection method.

**Grape consumption in healthy subjects**

The concentration of Cy-3-glc and resveratrol contained in 200 g of skins was determined to be 19 mg and 0.3 mg, respectively. The concentration of L-ascorbic acid was below the detection limit. The corresponding concentrations contained in 50 g of dried fruit were 5.4 mg (Cy-3-glc), 0.1 mg (resveratrol), and 2.9 mg (L-ascorbic acid). For the lag time assay and measurement of LDL mobility, the oxidation of LDL (70 \( \mu \)g/mL protein) isolated from venous blood samples was initiated by the addition of V-70 solution at 37°C.

Lag time is defined as the lag phase of conjugated diene formation following incubation of LDL with V70. The lag time is indicated in real time (min). LDL isolated from blood samples collected from human subjects 1 h following consumption of the dried fruits revealed significant inhibition of LDL oxidation compared to LDL isolated from fasting venous blood samples \( (p<0.05) \). LDL isolated from blood samples collected from human subjects 1 h following consumption of the dried fruits exhibited significantly suppressed LDL modification compared to LDL isolated from fasting venous blood samples \( (p<0.05) \).

**Lag time assay and scavenging of superoxide**

We next employed the lag time assay to evaluate the antioxidant activity of Cy-3-glc and protocatechuric acid, and we further examined the scavenging of superoxide. LDL \( (70 \mu \text{g/mL protein}) \) was incubated with Cy-3-glc \( (0, 1, 5 \mu \text{M}) \) or protocatechuric acid \( (0, 1, 5 \mu \text{M}) \) prior to the addition of V-70 (final concentration 200 \( \mu \text{M} \)) at 37°C. The effects of these reagents on the lag phase of conjugated diene formation in LDL incubated in vitro are shown in Fig. 3 (Representative data is shown). Both reagents prolonged the lag time compared to the control (no reagent). The lag time assay was analyzed three times. The lag time was prolonged by an average factor (ratio against control as 1) of 1.10 in the presence of 1 \( \mu \text{M} \) Cy-3-glc and by a factor of 1.50 in the presence of 5 \( \mu \text{M} \) Cy-3-glc (Fig. 3a). Compared to the control (no reagent), the lag time was prolonged by a factor of 1.67 in the presence of 1 \( \mu \text{M} \) protocatechuric acid, and by a factor of 1.84 in the presence of 5 \( \mu \text{M} \) protocatechuric acid (Fig. 3b).

Since the lag time assay indicated the presence of antioxidant activity in Cy-3-glc and protocatechuric acid, we next analyzed the ability of Cy-3-glc and protocatechuric acid to scavenge superoxide radicals. The ESR spectrum is indicated in Fig. 4a. The level of adduct
(DMPO-OOH, DMPO-OH) formation was decreased by increasing the concentration of SOD. Therefore, a standard curve using SOD was generated (Fig. 4b) and scavenging activity was measured in terms of SOD activity (Fig. 4c). Cy-3-glc and protocatechuic acid were both shown to be able to scavenge superoxide by this assay (Cy-3-glc: 2,000 units/g (SOD activity; unit/g), protocatechuic acid: 14,600 units/g (SOD activity; unit/g)).

**DISCUSSION**

We have shown that the consumption of the fresh skins and dried fruits of Nagano Purple grapes led to increased resistance of LDL to oxidation in human subjects. We have also shown that a compound (Cy-3-glc) identified in the Nagano Purple grape and one of the metabolites of Cy-3-glc, protocatechuic acid, exhibit antioxidant activity.

The skins of various fruits have been shown to contain high antioxidant activity. However, an antioxidant activity or useful components in the Nagano Purple grape remained to be identified. The effect of consumption of grape skins in human subjects is particularly complicated by the use of pesticide. In the present study, we have identified an antioxidant activity within the Nagano Purple grape, as assessed both by the inhibition of LDL oxidation in human subjects and by in vitro analysis.

The Nagano Purple grape contains no seeds. Therefore, we prepared either the fresh skins alone or the dried fruits including the skins. Because this grape was released recently in Nagano, we first established a polyphenol assay, so as to examine the components of the grape, in particular the polyphenol content. We detected abundant anthocyanidins (hydrolysis products) in the skin of the Nagano Purple grape relative to the levels detected in either Kyoho or Rosario Bianco grapes (Table 2). Using liquid chromatography (LC) in combination with tandem mass spectrometry (MS/MS), anthocyanidin glucoside(s) (Mv-glc, Dp-glc, Mv-diglc, Cy-glc, Pt-glc, Pn-glc) and acylated anthocyanidin glucoside(s) (Dp-glc-ace (acetic acid), Dp-diglc-cou (p-coumaric acid), Pt-glc-ace, Pt-glc-cou, Dp-glc-cou, Mv-diglc-cou, Cy-gly-cou, Mv-glc-cou) were identified in the skin of the Nagano Purple grape (data not shown). A wide variety of Mv glucoside(s) was recognized in the skins, but unfortunately we could not obtain the standard of Mv glucoside(s). Therefore, we focused on Cy-3-glc, accounting for nearly 90% of total Cy glucoside. Anthocyanins are water-soluble pigments that confer a blue, purple, or red color to plants. The fraction of total anthocyanins absorbed and excreted in the urine (in the native form or as metabolites) has been reported to be far below 1% of intake, which is lower than that found for other flavonoids (31). Because of the low level of absorption, there have been few studies of the biological benefits of anthocyanin consumption in human studies. Vitaglione et al. have reported that protocatechuic acid is a major metabolite of Cy-3-glc in humans (31). In addition, protocatechuic acid and p-hydroxybenzoic acid were identified in rat plasma after administration of a high dose of Cy-3-glc and Pg, respectively (32, 33). However, few studies have evaluated the absorption or beneficial effects of anthocyanidins other than Cy in humans.

We also detected a greater resveratrol content in Nagano Purple and Rosario Bianco grapes than in Kyoho grapes (Table 2). Resveratrol (trans-resveratrol) is a nonflavonoid polyphenol that can be isolated from grape skin. Resveratrol has been reported to scavenge free radicals and to protect LDL from peroxidative degradation (34). Furthermore, the consumption of wine containing anthocyanins and resveratrol has been
shown to lead to reduced concentrations of glucose and lipid in the plasma of streptozotocin-induced diabetic rats (35) and resveratrol consumption has been shown to improve survival of mice fed a high-calorie diet (9). However, there are few reports describing the antioxidant activity of native grapes, as opposed to processed products such as wine, in humans.

We have evaluated the effects of a single consumption of Nagano Purple grapes so as to determine the effects of beneficial components in the grapes upon LDL oxidation. We observed an extension of the conjugated diene formation of LDL and the suppression of LDL modification. The lag time of LDL samples collected from human subjects 1 h following the consumption of the skins or dried fruits was significantly increased compared to that observed in LDL samples from fasting subjects (Figs. 1a and 2a). We further observed that oxidative modification of LDL samples obtained from subjects 1 h following the consumption of the skins or dried fruits was reduced relative to the modification of LDL obtained from fasting subjects (Figs. 1b and 2b). Our results suggest that these effects are due to the absorption of beneficial compounds from the grapes into the blood. Although we tried to detect polyphenols in plasma and urine samples by LC/MS, we were unable to detect the presence of anthocyanins or resveratrol. This may be due to the low level of absorption of these components, as described above. To address this issue, more sensitive methods for the evaluation of these components are needed.

We next examined the antioxidant activity of grape components and metabolites using the lag time assay and a radical scavenging assay. A typical human diet and lifestyle is associated with a plasma concentration of Cy of roughly several 100 nM. The antioxidant activity of anthocyanins (in the nM to μM range) present in various fruits and vegetables has been established by determining the ORAC (Oxygen Radical Absorbance Capacity) value or the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity (1–4, 36, 37). We examined the antioxidant activity of Cy-3-glc and protocatechuic acid based on their effects upon the lag time assay. Both Cy-3-glc and protocatechuic acid (1 μM and 5 μM) prolonged the lag time (Fig. 3). We further showed that both Cy-3-glc and protocatechuic acid have superoxide scavenging activity. Previous reports have shown that neither Cy-3-glc nor protocatechuic acid inhibit xanthine oxidase (38), indicating that we can use this method to determine scavenging activity. We also confirmed the antioxidant activity of resveratrol and L-ascorbid acid using the lag time assay (data not shown). These results confirm data that has been reported previously (28, 39–42). Taken together, the results suggest in addition to resveratrol and L-ascorbid acid, anthocyanins including Cy-3-glu and its metabolite may also suppress LDL oxidation in humans.

In conclusion, we document here the presence of polyphenols in the Nagano Purple grape and we show the effect of the fresh skins of the grape and dried fruits on LDL oxidation. We demonstrate the effect of the Nagano Purple grape upon the induction of LDL oxidation implicated in the pathogenesis of atherosclerosis. These results suggest that the consumption of not only processed products but also fresh grapes confers LDL resistance to oxidation.

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