Purple Grape Juice Improves Endothelial Function and Reduces the Susceptibility of LDL Cholesterol to Oxidation in Patients With Coronary Artery Disease

James H. Stein, MD; Jon G. Keevil, MD; Donald A. Wiebe, MD; Susan Aeschlimann, RDMS, RVT; John D. Folts, PhD

Background—In vitro, the flavonoid components of red wine and purple grape juice are powerful antioxidants that induce endothelium-dependent vasodilation of vascular rings derived from rat aortas and human coronary arteries. Although improved endothelial function and inhibition of LDL oxidation may be potential mechanisms by which red wine and flavonoids reduce cardiovascular risk, the in vivo effects of grape products on endothelial function and LDL oxidation have not been investigated. This study assessed the effects of ingesting purple grape juice on endothelial function and LDL susceptibility to oxidation in patients with coronary artery disease (CAD).

Methods and Results—Fifteen adults with angiographically documented CAD ingested 7.7 ± 1.2 mL · kg⁻¹ · d⁻¹ of purple grape juice for 14 days. Flow-mediated vasodilation (FMD) was measured using high-resolution brachial artery ultrasonography. Susceptibility of LDL particles to oxidation was determined from the rate of conjugated diene formation after exposure to copper chloride. At baseline, FMD was impaired (2.2 ± 2.9%). After ingestion of grape juice, FMD increased to 6.4 ± 4.7% (P = 0.003). In a linear regression model that included age, artery diameter, lipid values, and use of lipid-lowering and antioxidant therapies, the effect of grape juice on FMD remained significant (mean change 4.2 ± 4.4%, P < 0.001). After ingestion of grape juice, lag time increased by 34.5% (P = 0.015).

Conclusions—Short-term ingestion of purple grape juice improves FMD and reduces LDL susceptibility to oxidation in CAD patients. Improved endothelium-dependent vasodilation and prevention of LDL oxidation are potential mechanisms by which flavonoids in purple grape products may prevent cardiovascular events, independent of alcohol content. (Circulation. 1999;100:1050-1055.)

Key Words: antioxidants ■ arteries ■ coronary disease ■ endothelium

Regular consumption of alcohol-containing beverages is associated with a decreased risk of cardiovascular events, such as myocardial infarction, stroke, and cardiac death.1-6 Although the benefits of moderate alcohol consumption seem to be related to its ability to raise high-density lipoprotein cholesterol (HDL-C) levels and inhibit platelet aggregation,7-10 some epidemiological studies suggest that the greatest degree of cardioprotection is related to ingestion of red wine, rather than beer or spirits.5,11,12 This casts doubt on the hypothesis that alcohol, per se, mediates all of the cardioprotective benefits of alcohol-containing beverages and suggests that other components of these products, such as polyphenols, have cardiac benefits.5,11,12

Flavonoids are polyphenol derivatives of 2-phenyl-1-benzopyran-4-1 that are present in fruits, vegetables, nuts, and seeds.13 Flavonoid intake is associated with a reduced risk of coronary events.14-18 The flavonoid components of grape products, including red wine and purple grape juice (GJ), inhibit collagen-mediated platelet aggregation.5,19-21 Although flavonoids in red wine and purple GJ reduce the susceptibility of low-density lipoprotein cholesterol (LDL-C) to oxidative stress in vitro, antioxidant effects in humans have been demonstrated only after ingestion of red wine, not purple GJ.22-24 In vitro, flavonoid components of red wine and purple GJ induce endothelium-dependent vasodilation of arterial rings, a phenomenon mediated by the nitric oxide–guanosine 3’,5’ cyclic monophosphate (NO-cGMP) pathway.25,26 Endothelial dysfunction accelerates the development of atherosclerosis and may be one of the earliest manifestations of this disease.27-30 Improved endothelial function may explain the rapid clinical benefits observed in trials of lipid-lowering therapy.29-31

Although improved endothelial function and prevention of LDL oxidation are potential mechanisms by which ingestion of red wine and flavonoids may reduce cardiovascular risk, the in vivo effects of grape products on these parameters are not known. The purpose of this study was to assess the effects of purple GJ on endothelial function.
and LDL susceptibility to oxidation in patients with coronary artery disease (CAD).

Methods

Experimental Protocol
Fifteen subjects with angiographically documented CAD were recruited for this study. Subjects with unstable angina, uncontrolled diabetes mellitus, or recent medication changes were excluded. Subjects were prohibited from consuming fruit products, tea, or alcoholic beverages during the study. They were encouraged not to change their diet otherwise and daily dietary logs of all food and beverages consumed during the study were reviewed to assure dietary compliance. Tobacco users were prohibited from smoking on the morning of endothelial function studies.

Subjects were provided with purple GJ (Welch’s 100% Concord Grape) and instructed to drink approximately 4 mL/kg twice daily for 14 days. For an average 80 kg patient, this amounted to approximately 640 mL (~21 ounces) per day of GJ. This quantity of GJ contains ~112 g of carbohydrates.

Brachial Artery Reactivity Protocol
Brachial artery (BA) reactivity studies were performed in the morning on the day of phlebotomy in the fasting state. Subjects were allowed to take their morning medications and on day 14 consumed their morning dose of GJ. After a 10-minute rest, in a temperature-controlled room (68 to 70°F), the diameter of the right BA and baseline forearm blood flow were measured with a 7.5 MHz, trapezoidal, linear array vascular ultrasound transducer and a Hewlett-Packard 2500 or 5500 Sonos ultrasound system. Increased forearm blood flow was induced by inflation of a pneumatic blood pressure tourniquet placed around the widest part of the forearm to a systolic pressure of 200 mm Hg, or 50 mm Hg greater than the systolic blood pressure, whichever was lower. This was followed by deflation after 4.5 minutes. Repeat blood flow scans were obtained immediately thereafter, and repeat BA diameters were measured after 1 minute. Fifteen minutes were allowed for vessel recovery, and repeat resting BA diameter and blood flow scans were obtained.

Sublingual nitroglycerin (400 μg) was administered, and final scans were performed after 3 minutes. A single lead ECG was monitored throughout the study. Blood pressure was measured in the left upper arm before the first scan, before administration of sublingual nitroglycerin, and every 5 minutes thereafter until it returned to baseline.

Ultrasound images were recorded on magneto-optical disks, using the digital storage and retrieval software of the ultrasound system. The BA was imaged 2 to 15 cm above the elbow and scanned in the midsagittal section with the focus zone set to the depth of the near wall. Depth and gain settings were used to optimize the images of the lumen/arterial wall interface. Images were magnified as necessary. Vessel diameters were measured in triplicate by a single observer on 2 occasions using proprietary software (MedArchive Viewer 1.5a, Secure Archive LLC). All measurements were performed blinded to subject information, GJ ingestion, and study date. The BA diameter after reactive hyperemia to the baseline diameter, expressed as a percent change. Nitroglycerin-mediated vasodilation (NTGMD) was calculated in an analogous fashion. Volumetric flow rates were calculated by multiplying the time velocity integral of the angle-corrected Doppler flow signal by the heart rate and the mean cross-sectional vessel area. Changes in blood flow were expressed as percentages of the resting flow measurements.

Measurement of Lipid and Insulin Levels
Baseline serum lipid levels were obtained after a 12-hour fast; however, subjects consumed their morning GJ dose on day 14, ~2 hours before phlebotomy. Total serum cholesterol levels were measured using a cholesterol ester/oxidase enzymatic procedure. HDL-C levels were measured directly using an enzymatic colorimetric method that incorporated polyethylene glycol-modified cholesterol ester oxidase. Serum triglycerides (TG) levels were measured using a glycerol kinase-based enzymatic procedure. LDL-C was calculated by the Friedewald formula. Insulin levels were measured by radioimmunoassay.

Isolation of LDL Particles and Determination of Susceptibility to Oxidation
LDL particles were isolated from serum by sequential density ultracentrifugation between densities of 1.006 and 1.063 g/mL using a Beckman Optima ultracentrifuge at 100 000 rpm (~400 000 g). The LDL containing fraction was desalted with a 2-mL column of preswollen 12% cellulose and 0.1 mol/L phosphate buffered saline (PBS) with 10 mmol EDTA. The protein concentration of LDL was measured by a Lowry protein method using a Cobas FARA centrifugal analyzer (Roche) and adjusted to a concentration of 0.5 g/mL using EDTA-containing PBS. A 100-μL aliquot of LDL was mixed with 900 μL of PBS without EDTA. LDL oxidation was initiated by adding 10 μL of freshly prepared CuCl2 (final concentration, 5 μmol/L). The rate of conjugated diene formation was measured at 234 nm at 30°C for 5 hours. Absorbance measurements were obtained every 3 minutes using a Beckman DU 7500 spectrophotometer (Beckman Instruments). From the kinetic profile of the LDL preparations, the lag time was defined as the time (in minutes) between initiation of conjugated diene production after addition of CuCl2 and the intercept of the maximum slope of the absorbance curve at the time of maximum conjugated diene production.32 These studies also were performed and interpreted blinded to subject information, GJ ingestion, and study date.

Statistical Analysis
Continuous variables were described by means±SD, except TG levels, which were described by median and range values. Changes in FMD and NTGMD were described as means with 95% CI. Initially, variables were compared using repeated measures t tests, except for TG levels; these were compared using the Wilcoxon signed rank test. Correlations between normally distributed parameters were described using Pearson’s r. Linear and step-wise regression analyses of changes in FMD and NTGMD incorporated the following parameters: subject age, BA diameter, total cholesterol, HDL- and LDL-C levels, insulin levels, and subject use of lipid-lowering medications, antioxidant vitamins, and nitrates. The results of these statistical analyses were verified using permutation tests to calculate exact probabilities.33 Intraobserver variability in blood vessel diameter measurements was described as an intraclass correlation coefficient determined from a nested 2-way ANOVA and was determined across all conditions and all readings.

Results

Subject Characteristics
Twelve subjects were male, 3 were female. The average age was 62.5±12.7 years. Ten subjects had a history of hypertension or were receiving antihypertensive medications. Eleven subjects were dyslipidemic and 10 were receiving lipid-lowering therapy (8 with HMG-CoA reductase inhibitors, 4 with nicotinic acid). Twelve subjects were receiving therapy with antioxidant vitamins, 11 with vitamin E (200 to 400 IU daily), and 10 with vitamin C (500 to 1000 mg daily). Subjects ingested 7.7±1.2 mL·kg⁻¹·d⁻¹ of GJ. Forty-four subjects had ultrasound images adequate for interpretation.

Lipid and Insulin Values
At baseline, mean lipid and insulin values were as follows: total cholesterol 4.46±0.76 mmol/L, TG 0.97 mmol/L (median,
Effect of Purple Grape Juice on BA Reactivity

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Grape Juice</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>60.2±10.3</td>
<td>64.3±7.3</td>
<td>0.152</td>
</tr>
<tr>
<td>BA diameter, mm</td>
<td>4.5±0.6</td>
<td>4.4±0.6</td>
<td>0.380</td>
</tr>
<tr>
<td>Hyperemic flow, %</td>
<td>507.4±209.5</td>
<td>672.4±661.5</td>
<td>0.382</td>
</tr>
<tr>
<td>FMD, %</td>
<td>2.2±2.9</td>
<td>6.4±4.7</td>
<td>0.003</td>
</tr>
<tr>
<td>NTG flow, % rest</td>
<td>107.0±141.5</td>
<td>151.1±90.6</td>
<td>0.335</td>
</tr>
<tr>
<td>NTGMD, %</td>
<td>9.4±5.5</td>
<td>12.1±6.6</td>
<td>0.044</td>
</tr>
</tbody>
</table>

BA indicates brachial artery; FMD, flow-mediated vasodilation; NTG, nitroglycerin; and NTGMD, nitroglycerin-mediated vasodilation.

range 0.35 to 2.14 mmol/L, HDL-C 1.11±0.23 mmol/L, LDL-C 2.76±0.75 mmol/L, total cholesterol/HDL-C ratio 4.3±1.4, and insulin 7.52±6.03 μIU/mL. After GJ, mean values were: total cholesterol 5.17±1.42 mmol/L \( (P=0.043) \), TG 1.47 mmol/L (median, range 0.38 to 4.24 mmol/L, \( P<0.001 \)), HDL-C 1.12±0.23 mmol/L \( (P=0.062) \), LDL-C 3.29±1.06 mmol/L \( (P=0.212) \), total cholesterol/HDL-C ratio 4.7±1.3 \( (P=0.103) \), and insulin 20.49±12.55 μIU/mL \( (P=0.004) \).

Brachial Artery Reactivity

The mean baseline BA diameter was 4.5±0.6 mm. After 2 weeks of GJ, the mean baseline diameter was 4.4±0.6 (mean change -0.1±0.2 mm, \( P=0.380 \)). Baseline FMD was impaired (2.2±2.9%) (Table, Figure 1). After GJ, FMD increased to 6.4±4.7% (mean change 4.2%, 95% CI 1.7 to 6.8%, \( P=0.003 \)). At baseline, NTGMD was 9.4±5.5%. After GJ, NTGMD increased to 12.1±6.6% (mean change 2.7%, 95% CI 0.1 to 5.3%, \( P=0.044 \)). Resting BA blood flow (data not shown) and heart rate did not change after GJ.

Step-wise regression analysis identified use of antioxidant vitamins and LDL-C levels as positive contributors to change in FMD. Use of lipid-lowering therapy was a negative contributor. The linear regression model identified significant contributions of all 3 variables (adjusted \( R^2 = 58.2\% \)); however, the change in FMD after ingestion of GJ remained significant after adjusting for these variables (mean change 4.2%, 95% CI 2.1 to 6.3%, \( P<0.001 \)). Changes in insulin levels correlated inversely with changes in FMD \( (r=-0.292) \) and did not affect the regression model. No parameters contributed to the change in NTGMD in either linear or step-wise regression analyses.

Because of the relatively small sample size, permutation tests were performed to verify the above analyses and to determine the exact probabilities that the observed changes in FMD and NTGMD were related to ingestion of GJ. The exact probability that the observed change in FMD was related to the intervention was 0.009. The weak effect of GJ ingestion on NTGMD was not verified \( (P=0.288) \).

The intraobserver reliability for measurement of BA diameters was 0.987. This value reflects the interclass correlation coefficient across all readings and all conditions (baseline, reactive hyperemia, prenitroglycerin, and postnitroglycerin).

LDL Susceptibility to Oxidation

At baseline, the lag time to conjugated diene formation was 87±29 minutes (Figure 2). After 2 weeks of ingesting GJ, the lag time increased to 117±23 minutes (34.5%, \( P=0.015 \)). Patients taking vitamin E had similar responses (baseline 86±33 minutes, change +40.7%, \( P=0.020 \)). Baseline, on GJ, and changes in lag times correlated weakly with lipid values and FMD and were not statistically related. Change in lag time correlated weakly with change in FMD \( (r=0.155) \), but addition of this parameter to the regression model had no impact on the adjusted \( R^2 (58.8\%, P=0.309) \).

Discussion

In this study, short-term ingestion of purple GJ improved FMD and decreased LDL susceptibility to oxidation in CAD patients. These benefits were observed despite (1) use of lipid-lowering and antioxidant therapies that have been shown to improve endothelium-dependent vasodilation in patients with dyslipidemia and CAD\textsuperscript{30–39} and (2) small increases in total cholesterol and TG levels, changes that adversely affect endothelial function and LDL susceptibility to oxidation.\textsuperscript{40–43}

Alcohol Consumption, Coronary Heart Disease, and the French Paradox

The French Paradox—that the rate of coronary heart disease mortality in France has been lower than observed in other industrialized countries with a similar coronary risk factor profile—has been attributed to frequent consumption of red wine.\textsuperscript{5,11–12} Mechanisms by which red wine consumption may reduce coronary risk include alcohol-related increases in HDL-C levels and platelet inhibition.\textsuperscript{7–14,44–46} It is not known,
however, if the effects of alcohol, per se, mediate the reduction in CHD events, or if red wine and the flavonoids contained within it offer a particular advantage. Purple GJ also inhibits platelet aggregation and in vitro LDL oxidation. Because purple GJ does not contain ethanol, it has been postulated that its antiplatelet and antioxidant effects are due to flavonoids. Indeed, increased intake of dietary flavonoids has been associated with a reduced risk of CHD events.

Purple Grape Products, Flavonoids, and Endothelial Function

In in vitro studies, the NO-cGMP pathway mediates the endothelium-dependent vasodilating effects of red wine and purple GJ. These effects are more dramatic than observed with white wine but also depend on the fermentation process. Ethanol, at the same concentration as contained in the red and white wine (0.12%), does not cause vasorelaxation. This observation suggests that the nonalcoholic components of red wine, most likely flavonoids, are responsible for the changes in vessel tension observed in vitro. This study is the first in vivo demonstration of improved endothelial function after administration of a grape product, namely, purple GJ. The small increase in NTGMD probably was spurious. Relative to baseline NTGMD, the magnitude of the change was small and of marginal statistical significance. Permutation tests, which calculate the exact probability that the observed changes were a result of the intervention rather than chance, did not support the test finding of a significant improvement in NTGMD. The superiority of permutation tests, compared with t- and F tests, has been reviewed.

Flavonoids that are abundant in grape products such as red wine and purple GJ include quercetin, catechin, myricetin, kaempferol, and tannic acid. Quercetin is a powerful antioxidant that relaxes aortic rings in vitro; however, it is not clear whether this is an endothelium-dependent process or if significant amounts of quercetin are absorbed after drinking grape products. Catechins are well-absorbed from the gastrointestinal tract and are powerful inhibitors of LDL oxidation. Their effects on endothelial function are not known. Intakes of myricetin, kaempferol, and quercetin have been associated with reduced rates of coronary heart disease in epidemiological studies. Tannins exhibit powerful endothelium-dependent vasodilating activity in experimental arterial preparations. Resveratrol, a hydroxystilbene compound more abundant in red than white wines may inhibit platelet function, but it does not affect endothelial function, nor does malvidin, an anthocyanin component of purple grape skin that provides some of the dark color of grape products.

Endothelial Function, Grape Juice, and Antioxidants

Endothelial dysfunction is a critical event in the pathogenesis of atherosclerosis and its clinical manifestations, including myocardial ischemia. Endothelial function in the human BA is closely related to endothelial function in the human coronary artery. Although the causes of endothelial dysfunction are numerous, oxidized LDL is toxic to endothelial cells and plays a significant role in the initiation and perpetuation of atherogenesis.

In this study, significant endothelial dysfunction was observed at baseline, despite use of statins and antioxidant vitamins, therapies previously shown to improve endothelial dysfunction in CAD patients. Ingestion of purple GJ also decreased the susceptibility of LDL to copper-catalyzed oxidation, despite the fact that 12 subjects in this study were taking antioxidant vitamins. The improvement in endothelial function associated with GJ did not correlate with the decrease in LDL susceptibility to oxidation; however, LDL resistance to oxidation is neither necessary nor sufficient to preserve or normalize endothelial function. The effects of antioxidants on vascular function appear to be independent of their ability to reduce LDL oxidative susceptibility. The severe baseline endothelial dysfunction, despite antioxidant and lipid-lowering therapies, is also not surprising, because the improvements in endothelial function attributed to these therapies only have been observed in the short-term (<12 months), and no studies have evaluated whether this benefit persists.

Further evidence that the effects of purple GJ on endothelial function may be mediated through the NO-cGMP pathway comes from studies of purple GJ and platelet function. Purple GJ enhances endothelial vasodilation by increasing NO production. Recently, incubation of human blood with a 1/1000 dilution of purple GJ increased NO release from aggregating platelets by 3-fold (P = 0.01). Because NO interacts with superoxide, release of superoxide by aggregating platelets also was measured before and after treatment with purple GJ; a 55% decrease in superoxide release was observed (P < 0.01). In human subjects who ingested 7 mL kg·d of purple GJ for 14 days, significant increases in NO release and decreases in superoxide release also were observed. If similar mechanisms were occurring in endothelial cells, as suggested in laboratory studies, increased NO release might explain part of the improved FMD observed in this study.

Limitations

Although the number of subjects in this study was relatively small, the BA technique for evaluating endothelial function is very sensitive, reproducible, and each subject is used as his or her own control. The sample size for this study was based on published nomograms for interventions assessed by this technique. The relatively small sample size, however, limited the applicability of multivariate statistical techniques to the data in this study, so the effects of purple GJ on FMD and NTGMD were verified by permutation tests. Although the small effect of purple GJ on NTGMD was not verified, a small, nonspecific vasodilating effect of purple GJ cannot be excluded. Because of the short duration of this study, it is also not known whether the observed vasorelaxing and antioxidant properties of purple GJ would persist with chronic ingestion.

In this study, each subject’s baseline BA reactivity (ie, FMD) served as a control value for FMD after GJ ingestion. Significant regression to the mean is unlikely given the magnitude of the treatment effect and the results of the
regression analysis, which controlled for baseline FMD. Furthermore, only a very small part of the variance in FMD observed with this technique can be attributed to time.58 The vast majority of variance in FMD measurements is between patients (72% to 99%), rather than between time points or observers.58

The small but statistically significant increases in total cholesterol and TG levels are not surprising given the carbohydrate content of the GI ingested by the subjects; however, hypercholesterolemia and hypertriglyceridemia tend to increase the susceptibility of LDL to oxidation and worsen endothelial function.30,31,40–43 That we observed improvements in these parameters, despite small but adverse effects on the lipid profile, is further evidence of the potential usefulness of purple GI as antiatherosclerotic agent. Although hyperinsulinemia has been associated with increased blood flow and vasodilation mediated by the sympathetic nervous system, these observations were made in subjects with much higher insulin levels than measured in this study.59,60 Furthermore, increased insulin levels after GI ingestion were inversely associated with changes in FMD. Finally, the resting heart rates, baseline BA diameters, and forearm blood flow rates did not change after ingestion of GI, suggesting that insulin-mediated sympathetic activation did not affect the conclusions of this study.

Conclusion

Short-term ingestion of purple GI improves endothelial function and reduces the susceptibility of LDL to oxidation in CAD patients. These benefits were observed despite use of antioxidant vitamins, lipid-lowering medications, and small increases in total cholesterol and TG levels. Improved endothelium-dependent vasodilation and prevention of LDL oxidation are potential mechanisms by which flavonoids in purple grape products may prevent cardiovascular events, independent of alcohol content.

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