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The action of red wine and purple grape juice on vascular reactivity is independent of plasma lipids in hypercholesterolemic patients

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Abstract

Although red wine (RW) reduces cardiovascular risk, the mechanisms underlying the effect have not been identified. Correction of endothelial dysfunction by RW flavonoids could be one mechanism. We measured brachial artery reactivity by high-resolution ultrasonography, plasma lipids, glucose, adhesion molecules (ICAM-1 and VCAM), and platelet function in 16 hypercholesterolemic individuals (8 men and 8 women; mean age 51.6 ± 8.1 years) without other risk factors. Twenty-four normal subjects were used as controls for vascular reactivity. Subjects randomly received RW, 250 ml/day, or purple grape juice (GJ), 500 ml/day, for 14 days with an equal wash-out period. At baseline, all 16 subjects were hypercholesterolemic (mean LDL = 181.0 ± 28.7 mg/dl) but HDL, triglycerides, glucose, adhesion molecules, and platelet function were within normal limits. Brachial artery flow-mediated dilation was significantly decreased compared to controls (9.0 ± 7.1 *vs* 12.1 ± 4.5%; $P < 0.05$) and increased with both GJ (10.1 ± 7.1 before *vs* 16.9 ± 6.7% after: $P < 0.05$) and RW (10.1 ± 6.4 before *vs* 15.6 ± 4.6% after; $P < 0.05$). RW, but not GJ, also significantly increased endothelium-independent vasodilation (17.0 ± 8.6 before *vs* 23.0 ± 12.0% after; $P < 0.01$). GJ reduced ICAM-1 but not VCAM and RW had no effect on either molecule. No significant alterations were observed in plasma lipids, glucose or platelet aggregability with RW or GJ. Both RW and GJ similarly improved flow-mediated dilation, but RW also enhanced endothelium-independent vasodilation in hypercholesterolemic patients despite the increased plasma cholesterol. Thus, we conclude that GJ may protect against coronary artery disease without the additional negative effects of alcohol despite the gender.

Key words
- Atherosclerosis
- Endothelial function
- Flavonoids
- Alcohol
- Red wine and grape juice
- Brachial artery dilation

Introduction

Moderate drinking is associated with a decreased risk of myocardial infarction, stroke, and cardiac death (1,2), as well as macular degeneration, Alzheimer's disease, and cognitive deficits (3-7). Although the benefits of alcohol consumption are partially related to its ability to increase high-density lipoprotein (HDL)-cholesterol and to inhibit

platelets, some studies indicate that the greatest protection is due to red wine (RW), rather than beer or spirits (8-10). This suggests that phenolic acids, polyphenols, and or flavonoids may confer additional benefits.

Flavonoids are polyphenol derivatives of 2-phenyl-1-benzopyran-4-1 that are present in fruits, vegetables, nuts, and seeds (11). In epidemiological studies, flavonoid intake is associated with a reduced risk of coronary events (11,12). In an experimental study (13) we showed that both RW and nonalcoholic wine products can prevent plaque formation in hypercholesterolemic rabbits despite significant increases in low-density lipoprotein (LDL). Apoprotein E (Apo E)-deficient mice also showed reduced atherosclerosis progression when fed RW (14). RW also causes monocyte chemotactic protein-1 expression blockade and reduced neointimal hyperplasia after balloon injury in rabbits (15), inhibition of smooth muscle cell proliferation and of cyclin A expression (16), as well as inhibition of the platelet-derived growth factor receptor by RW flavonoids in cultured smooth muscle cells (17) - all of them factors contributing to atherogenesis.

More recent evidence (18) indicated that RW, but not vodka, inhibited nuclear factor-κB expression in leukocytes of human volunteers fed a high fat diet. Furthermore, RW phenols blocked endothelin-1 production (19) and induced endothelial nitric oxide synthase (eNOS) expression in cultured endothelial cells (20).

In vitro, the flavonoid components of RW and purple grape juice (GJ) induce endothelium-dependent vasodilation of arterial rings (21), a phenomenon mediated by the nitric oxide cyclic guanosine monophosphate (NO-cGMP) pathway (22). Although improved endothelial function and inhibition of platelet aggregation are potential mechanisms by which RW flavonoids may reduce cardiovascular risk in hypercholesterolemic patients, the *in vivo* effects of RW and GJ on these parameters are not well known. Despite favorable clinical and experimental evidence, recommendation for wine or any alcohol drinking is controversial, due to well known untoward side effects of alcohol.

Hence we decided to compare RW and GJ in humans to determine whether the benefits of polyphenols could be achieved without the undesirable side effects of alcohol. The specific purpose of this study was to compare the effects of RW and GJ on arterial reactivity, platelet aggregability, adhesion molecules, glucose, and plasma lipids in hypercholesterolemic subjects.

Patients and Methods

Patients

Sixteen healthy adult subjects with isolated hypercholesterolemia were enrolled. Exclusion criteria included smoking, diabetes mellitus, hypertension, lipid-lowering therapy, and obesity (body mass index >26). In order to establish normal values for vascular reactivity, 24 healthy individuals were also studied. Study subjects were not allowed to consume fruit products, tea, or alcoholic beverages during the study. They were encouraged not to change their diet otherwise and to keep a diary of daily intake of all food and beverages which was reviewed to assure dietary compliance. A complete medical history was obtained and physical examination was carried out at study entry. The study was approved by the Hospital Ethics Committee. Each subject gave written informed consent to participate in the study after a thorough explanation of the study design and protocol. Subjects were provided with commercial purple GJ and instructed to drink 500 ml/day, and with RW Pinot Noir (Aurora Company, Bento Gonçalves, RS, Brazil) and instructed to drink 250 ml/day for 14 days according to the study schedule.

Brachial artery reactivity

Brachial artery (BA) reactivity studies were always performed in the morning after a 10-min rest in a temperature-controlled room (20º to 25ºC). The diameter of the left BA and baseline forearm blood flow velocity were measured with a 7.5-MHz linear array vascular ultrasound transducer part of an APOGEE 800 plus ATL ultrasound system (ATL Ultrasound, Bothell, WA, USA). For the evaluation of endothelial-dependent reactivity (23-25) 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 250 mmHg, followed by deflation after 5 min. The first five blood flow scans were obtained continuously immediately thereafter and blood flow velocities and BA diameters were measured again after 1 min. Twenty minutes were allowed for vessel recovery, and repeat resting BA diameter and blood flow scans were obtained. For endothelial-independent evaluation, sublingual isosorbide dinitrate (5 mg) was administered and final scans were performed after 5 min. A single-lead ECG was monitored throughout the study. Blood pressure was measured in the right upper arm before the first scan, before administration of sublingual nitroglycerin, and every 5 min thereafter until it returned to baseline.

Ultrasound images were recorded in VHS. The BA was imaged 2 to 15 cm above the elbow and scanned in longitudinal section with the focus zone set to the depth of the near wall. During image acquisition, anatomic landmarks such as fascial planes were noted to help maintain the same image of the artery throughout the study. Depth and gain settings were used to optimize the images of the lumen/arterial wall interface. Vessel diameters were measured using a software program, developed at InCor, a semi-automatic approach to measure artery diameter based on active contour technique improved by multiresolution analysis. The operator selected a region of interest (1 cm in longitudinal diameter) in six series of brachial images obtained from b-mode ultrasound. The distance between near wall media-adventitia and far wall media-adventitia was obtained for all images and the mean value was calculated during diastole (26).

Flow-mediated vasodilation (FMD) was calculated as the ratio of the BA diameter after reactive hyperemia to the baseline diameter, expressed as a percent change. Nitroglycerin-mediated vasodilation (NTGD) was calculated in an analogous fashion.

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).

Lipids and platelet aggregation

Plasma samples were collected in EDTA after a 12-h fast by venipuncture performed on the same day as the BA studies.

Plasma total cholesterol and triglycerides were determined using enzymatic methods (Roche Laboratories, Basel, Switzerland) (27). HDL-cholesterol was determined by the same method as for total cholesterol, and after chemical precipitation of apolipoprotein B100-containing lipoproteins with MgCl and phosphotungstic acid. VLDL-cholesterol and LDL-cholesterol were estimated by the Friedewald formula. Apo B and Apo AI were also determined by immunoturbidimetry (Roche). All lipid and apolipoprotein determinations were performed automatically using a COBAS-MIRA analyzer (Roche). Lipoprotein (a) (Lp(a)) was measured by immunoprecipitation analyses and immunoturbidimetric methods. Platelet aggregation was estimated by the Born and Cross turbidimetric methods. The results are reported as normal, increased or decreased platelet aggregation.

Adhesion molecules

Serum was separated from whole blood following centrifugation in a benchtop centrifuge for 15 min at 750 *g* and was stored at -80ºC until assayed. Serum levels of intercellular adhesion molecule 1 (h-sICAM-1 ELISA, anti-sICAM-1: monoclonal antibody from murine, parameter R&D Systems, Minneapolis, MN, USA) and vascular cell adhesion molecule (h-sVCAM-1 ELISA, anti-sVCAM-1 biotin, monoclonal antibody from mouse, clone M-1.G11-B1, and anti-sVCAM-1 peroxidase, monoclonal antibody from mouse, clone M-DDV-1 fab fragments) were determined by ELISA.

Protocol

Sixteen subjects were randomized to receive either 500 ml/day GJ or 250 ml/day RW for 14 days as first treatment; a wash-out period of equal duration was then observed, followed by the second treatment modality.

Patients were examined every 2 weeks after an overnight 12-h fast. Endothelial function was assessed four times: 1) at baseline, 2) at the end of the first treatment period, 3) after a 14-day washout, and 4) after the second treatment period. At all 4 points, blood was collected and vital signs were measured with automated monitor after 20 min of rest.

Statistical analysis

Continuous variables are reported as means ± SD. Changes in FMD and NTGD are described as means with 95% confidence. FMD and NTGB were compared by ANOVA with repeated measures and categorical variables were compared by the Fisher test. The level of significance was set at $P \leq 0.05$ for all analyses.

Table 1. Characteristics of hypercholesterolemic patients at baseline and after the ingestion of red wine or grape juice.

	Red wine		Grape juice	
	Before	After	Before	After
Body mass index (kg/m^2)	24.8 ± 1.5	24.8 ± 1.4	24.8 ± 1.5	24.8 ± 1.2
Heart rate (bpm)	62.1 ± 8.0	62.9 ± 9.0	60.6 ± 6.3	61.4 ± 8.5
Systolic blood pressure (mmHg)	123.0 ± 9.9	128.2 ± 10.5	122.0 ± 10.3	124.7 ± 12.0
Diastolic blood pressure (mmHg)	76.6 ± 5.3	77.8 ± 6.2	74.5 ± 5.6	78.2 ± 7.0
Mean blood pressure (mmHg)	92.1 ± 6.1	94.6 ± 4.7	90.3 ± 6.6	93.7 ± 8.4
Total cholesterol (mg/dl)	252.7 ± 35.1	258.0 ± 42.2	259.0 ± 35.1	253.0 ± 40.4
LDL cholesterol (mg/dl)	175.1 ± 28.7	178.2 ± 32.7	179.0 ± 27.7	176.0 ± 31.4
HDL cholesterol (mg/dl)	55.6 ± 17.7	55.9 ± 17.0	56.0 ± 15.9	53.0 ± 12.5
Triglycerides (mg/dl)	110.1 ± 40.6	118.2 ± 57.9	124.0 ± 46.8	117.0 ± 50.9
Lp(a) (mg/dl)	35.5 ± 34.5	26.2 ± 18.8	35.5 ± 34.5	30.5 ± 24.5
Apo A (mg/dl)	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.3
Apo B (mg/dl)	1.3 ± 0.3	1.4 ± 0.2	1.3 ± 0.3	1.5 ± 0.2
Glucose (mg/dl)	88.6 ± 6.5	93.0 ± 8.6	90.0 ± 7.3	90.0 ± 6.9
VCAM-1 (ng/ml)	363.3 ± 127.3	380.7 ± 81.8	384.7 ± 105.4	363.8 ± 82.5
ICAM-1 (ng/ml)	140.6 ± 46.6	130.6 ± 55.5	146.8 ± 44.9	115.9 ± 21.8*

Data are reported as means ± SD (N = 16). Lp(a) = lipoprotein a; Apo = apolipoprotein; VCAM-1 = vascular cell adhesion molecule 1; ICAM-1 = intercellular adhesion molecule 1.
*P < 0.05 compared to baseline grape juice (Fisher test).

Results

Baseline characteristics

For the specific purpose of establishing normal values for vascular reactivity for our laboratory, 24 healthy subjects were studied. Their mean age was 42.6 ± 7.1 years, and blood pressure was 124/80 ± 12/5 mmHg, total cholesterol was 180 ± 9.1 mg/dl, LDL 111 ± 7.9 mg/dl, HDL 53 ± 2.2 mg/dl, triglycerides were 155 ± 10.1 mg/dl, and fasting glucose was 89 ± 12 mg/dl. The brachial artery diameter averaged 3.55 ± 0.6 mm, FMD was 12.1 ± 4.5% and NTGD was 16.6 ± 9.5%. These subjects were not included in the RW/GJ study.

Of the 16 randomized subjects 8 were males and 8 were females. Their average age was 51.6 ± 8.1 (SD) years. All subjects were hypercholesterolemic and none was receiving lipid-lowering therapy. Table 1 describes the demographic data of the subjects.

Comparisons were made at different times in the same patients and between treatment groups. There were no statistically significant differences between baseline, pretreatment and washout values for any parameter. There was no interaction between beverage order and treatment effects (P = 0.93).

Brachial artery reactivity

Red wine effects. In the 16 hypercholesterolemic subjects the mean baseline BA diameter was significantly increased compared with control (3.8 ± 0.7 *vs* 3.55 ± 0.6 mm, P < 0.05). After 2 weeks of RW, the mean baseline diameter remained unchanged (3.9 ± 0.8 *vs* 3.8 ± 0.7 mm, P = ns).

Baseline FMD was significantly impaired in hypercholesterolemic subjects compared with controls (9.0 ± 7.1 *vs* 12.1 ± 4.5%, P < 0.05). After RW, FMD increased significantly (10.1 ± 6.4 *vs* 15.6 ± 4.6%, P < 0.05) in hypercholesterolemic subjects (Figure 1A). Red wine had no effect on the intensity of reactive hyperemic blood velocity (data not shown).

At baseline, NTGD did not differ between hypercholesterolemic and control subjects (18.9 ± 7.6 *vs* 16.6 ± 9.5%, P = ns). However, after RW, NTGD increased from 16.6 ± 9.5 to 23.0 ± 12.0%, P < 0.05 (Figure 1A).

These effects on vascular reactivity occurred in the absence of any significant changes in heart rate and blood pressure, as shown in Table 1.

Grape juice. Similar to RW, the mean baseline BA diameter of hypercholesterolemic subjects remained unchanged after 2 weeks of GJ consumption (3.9 ± 0.8 *vs* 3.8 ± 0.7 mm; P = ns). On the other hand, in hypercholesterolemic subjects FMD increased significantly (10.9 ± 7.4 *vs* 16.9 ± 6.7%, P < 0.05) with GJ (Figure 1B). GJ had no effect on the intensity of reactive hyperemic blood velocity (data not shown).

In contrast, NTGD remained unchanged (19.8 ± 8.8 *vs* 18.0 ± 9.4%, P = ns) after GJ (Figure 1B). These effects were not accompanied by any significant changes in heart rate and systolic, diastolic or mean blood pressure (Table 1).

A comparison of the final FMD values in both RW and GJ treatments showed no sig-

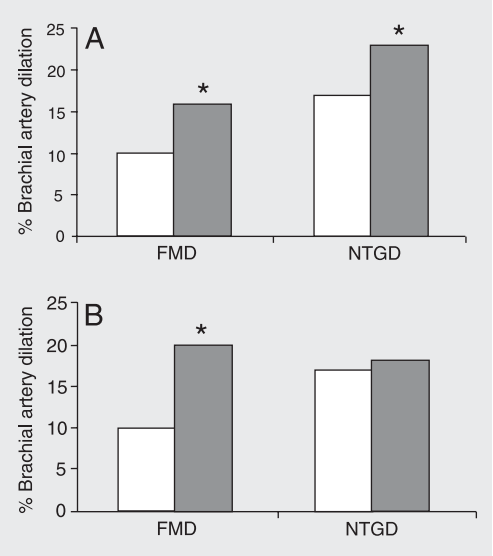

Figure 1. Effect of ingestion of red wine (RW) and grape juice (GJ) on flow-mediated dilation and endothelium-dependent dilation. Twelve hypercholesterolemic patients received 250 ml RW/day (A) or 500 ml GJ/day (B) for 14 days. FMD = flow-mediated dilation; NTGD = nitroglycerin-mediated dilation. Open columns = before treatment; filled columns = after treatment. *P ≤ 0.05 compared to before treatment (ANOVA).

nificant difference (unpaired *t*-test), indicating that the magnitude of response to both treatments was similar.

Lipid and glucose values

As shown in Table 1, subjects were clearly hypercholesterolemic, but triglycerides, HDL-cholesterol and glucose levels were normal. Also, Apo A, Apo B, and Lp(a) were normal. After RW or GJ no effect on fasting plasma lipids, Lp(a), Apo A, Apo B, or glucose was observed (Table 1).

Adhesion molecules

At baseline, mean adhesion molecule values were within normal ranges and RW had no effect on VCAM-1 or ICAM-1 (Table 1). In contrast to RW, GJ ingestion significantly decreased ICAM-1, but had no effect on VCAM-1.

Platelet aggregation

At baseline, platelet aggregation was normal in 12 subjects, decreased in 3 and increased in 1. Neither RW nor GJ produced any significant change in platelet aggregation.

Discussion

This study demonstrates comparable improvement in arterial endothelial dilation after short-term ingestion of both GJ and RW in hypercholesterolemic individuals, without affecting plasma lipids, glucose levels or platelet aggregability. RW, but not GJ, also increased endothelium-independent vasodilation. In addition, GJ reduced ICAM-1 but did not affect VCAM. Since endothelial dysfunction is an early event in atherosclerosis (28) and may be of considerable prognostic value (29,30), these findings may be relevant for vascular protection in patients at risk.

Since FMD increments were similar in RW and GJ, such improvement cannot be attributed to alcohol, nor can it be attributed to lipid, glucose or platelet effects, because none changed significantly. Therefore, the improvement in endothelial function is most probably due to other substances present in both RW and GJ. Although various chemicals are present in RW and GJ, several lines of evidence point to flavonoids as the most likely to play a role in vascular function.

In vitro the NO-cGMP pathway mediates the endothelium-dependent vasodilating effects of RW and GJ. Ethanol, at the same concentration as contained in red and white wines, does not cause vasorelaxation (31). RW or extracts obtained from it caused endothelium-dependent, NO-mediated vasorelaxation of rat (32) or rabbit (33) aorta preconstricted with norepinephrine. Also, in human coronary arteries and rat aortic rings *in vitro*, short-term incubation with RW induced FMD and increased vascular cGMP content (31). These effects were abolished after endothelial denudation and reversed by NOS inhibition. Ethanol did not affect vascular tension or cGMP content.

These rapid effects of RW are likely to involve an acute activation of eNOS. Wallerath et al. (20) provided evidence that RW can stimulate the expression of the eNOS gene leading to enhanced production of NO. This upregulation was seen in both EA.hy 926 cells and primary human umbilical vein endothelial cells; such phenomenon is mediated in part by increases in Ca^{2+} in endothelial cells (34).

Flavonoids may also affect endothelin-1 production. Corder et al. (19) found that RW polyphenols decreased ET-1 synthesis in cultured bovine aortic endothelial cells by suppressing transcription of the ET-1 gene.

In human studies, Stein et al. (35) showed that short-term ingestion of purple GJ improves FMD and reduces LDL susceptibility to oxidation in coronary patients. On the other hand, Hashimoto et al. (36) demonstrated that endothelium-dependent vasodi-

lation improves after acute intake (120 min) of RW or RW without alcohol in men. But endothelium-independent vasodilation remained unchanged. In contrast, the present study showed that endothelium-independent vasodilation also improves after RW. Therefore, not only endothelial but also smooth muscle cell reactivity function is improved by RW. The mechanisms underlying the latter effect are not clear. One possible explanation is the activation of the parasympathetic nervous system and relative inactivation of the sympathetic nervous system which are known to occur after alcohol consumption (37). Regarding the overall effects, the actions of flavonoids and ethanol on NO production can be invoked. Thus, Matsuo et al. (38) showed an increased acute production of NO with RW and ethanol in the plasma of healthy subjects using diaminofluoresceins as new selective fluorescent indicators.

Hence, the most plausible mechanism responsible for the observed improvement in endothelium-dependent vasodilation is the action of flavonoids. On the one hand, they can decrease endothelin production which may prevent vasoconstriction and on the other, they increase NO production by endothelial cells, thus facilitating vasodilation. Regarding endothelium-independent vasodilation, the mechanisms remain speculative.

It is noteworthy that these vasodilatory effects occurred independently of changes in lipids, glucose or platelet aggregation. This supports the hypothesis of flavonoid mechanisms. Our findings regarding lipids are consistent with those of Hayek et al. (14), who also did not find changes in HDL or LDL after giving RW or the polyphenols quercetin or catechin to mice. However, prolonged alcohol ingestion is known to increase HDL in humans (39); thus, the absence of an RW effect on HDL in the present investigation might have been due to the short period of administration.

The present study also provides evidence that GJ, but not RW, can diminish circulating ICAM-1, perhaps because phenolic compounds can suppress cytokine-induced activation of nuclear factor κB and subsequent expression of adhesion molecules. However, VCAM-1 was not significantly affected by GJ. The reasons for this selective effect of GJ on ICAM-1 remain unknown.

Limitations

Although the number of subjects in this study was relatively small, the BA ultrasonic technique for evaluating endothelial function is very sensitive and reproducible, and each subject is used as his own control. The sample size was based on published nomograms for interventions assessed by this technique. Because of the short duration of this study, it is also not known whether the observed vasorelaxing properties of GJ and RW would persist with chronic ingestion. No direct measurement of flavonoids was made; therefore, their influence on reactive vascular is only deductive.

We have shown that short-term ingestion of GJ and RW improves endothelial function and only GJ reduces ICAM-1 in hypercholesterolemic patients. Also, endothelium-independent vasodilation was increased by RW. Improved endothelium-dependent vasodilation is a potential mechanism by which flavonoids in purple grape products may prevent cardiovascular events, as suggested by several epidemiological studies (40). Of particular interest is the demonstration that the improvement in vascular function can be obtained without alcohol. This is of potential clinical relevance for certain sub-groups of patients at risk of cardiovascular events, for whom even moderate alcohol intake is not advisable.

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