Grape Juice, But Not Orange Juice or Grapefruit Juice, Inhibits Human Platelet Aggregation[1]

(Manuscript received 7 April 1999. Initial review completed 28 May 1999. Revision accepted 8 September 1999.)

Jon G. Keevil,* Hashim E. Osman,* Jess D. Reed and John D. Folts*[2]**

**Cardiology Section of Department of Medicine and **Department of Animal Sciences, University of Wisconsin, Madison, WI 53792*

ABSTRACT Coronary artery disease is responsible for much mortality and morbidity around the world. Platelets are involved in atherosclerotic disease development and the reduction of platelet activity by medications reduces the incidence and severity of disease. Red wine and grapes contain polyphenolic compounds, including flavonoids, which can reduce platelet aggregation and have been associated with lower rates of cardiovascular disease. Citrus fruits contain different classes of polyphenolics that may not share the same properties. This study evaluated whether commercial grape, orange and grapefruit juices, taken daily, reduce ex vivo platelet activity. In a randomized cross-over design, ten healthy human subjects (ages 26–58 y, five of each gender) drank 5–7.5 mL/(kg · d) of purple grape juice, orange juice or grapefruit juice for 7–10 d each. Platelet aggregation (whole blood impedance aggregometry, Chronolog Model #590) at baseline was compared to results after consumption of each juice. Drinking purple grape juice for one week reduced the whole blood platelet aggregation response to 1 mg/L of collagen by 77% (from 17.9 ± 2.3 to 4.0 ± 6.8 ohms, *P* = 0.0002). Orange juice and grapefruit juice had no effect on platelet aggregation. The purple grape juice had approximately three times the total polyphenolic concentration of the citrus juices and was a potent platelet inhibitor in healthy subjects while the citrus juices showed no effect. The platelet inhibitory effect of the flavonoids in grape juice may decrease the risk of coronary thrombosis and myocardial infarction. J. Nutr. 130: 53–56, 2000.

KEY WORDS: • platelet aggregation • fruit bioflavonoids • humans

Platelets participate in fatal and nonfatal myocardial infarction due to coronary thrombosis. They also contribute to the development and progression of coronary artery atherosclerosis (Fuster 1994). Inhibition of platelet activity by aspirin significantly reduces the incidence of first myocardial infarction, recurrent infarction and vascular death among patients with cardiovascular disease (Antiplatelet Trialists' Collaboration 1994, Hennekens et al. 1997).

Flavonoids are polyphenolic compounds, widely distributed in the plant kingdom, many of which have antioxidant and antithrombotic properties (Beretz et al. 1982, deWhalley et al. 1990, Demrow et al. 1995, Folts et al. 1994, Osman et al. 1998, Wang et al. 1996). Epidemiological studies showed an inverse correlation between the level of intake of dietary flavonoids and death from coronary artery disease (CAD)[5] (Hertog et al. 1993 and 1995, Knekt et al. 1996, Rimm et al. 1996). Hertog et al. (1993) found lower rates of myocardial infarction and cardiac death in the upper tertials of flavonoid intake among 805 elderly men in Zutphen, The Netherlands. In a larger group of 16 cohorts in seven countries, flavonoid intake was inversely related to CAD mortality (Hertog et al. 1995). In Finland, a study of over 5,000 men and women suggested increased incidence of CAD in the population with the lowest flavonoid intake (Knekt et al. 1996).

In the early 1990s, television reports on the "French Paradox" noted that while smoking and fat intake in France are higher than in the United States, the incidence of myocardial infarction is one-third that of the United States (Renaud and de Lorgeril 1992). This suggested that the higher intake of flavonoids in red wine and fresh fruit in France may provide protection from the development of CAD (Renaud and de Lorgeril 1992). Red wine and purple grape juice contain many of the same biologically active flavonoids, primarily of the flavonol type. In animals, red wine and purple grape juice significantly inhibited ex vivo platelet activity and experimental coronary thrombosis in the Folts cyclic flow model (Demrow et al. 1995). Neither orange nor grapefruit juice had any platelet inhibitory effect (Osman et al. 1998). In human subjects, 350 mL of red wine or 700 mL of purple grape juice given once inhibited ex vivo platelet aggregation (Folts et al. 1994, Folts 1998) while 350 mL of white wine had no significant effect on ex vivo platelet aggregation (Folts et al. 1994, Folts 1998).

This study compared the ex vivo platelet activity in human volunteers before and after drinking purple grape juice, orange juice or grapefruit juice for 7–10 d each.

MATERIALS AND METHODS

Ten healthy subjects (five males and five females) from the University of Wisconsin community volunteered for the study. They were on no scheduled medications except hormonal replacement therapy (n = 1). The average age was 42 y (range 26–58 y) and average weight was 76 kg (range 52–91 kg). Eight subjects were predominantly Caucasian and two were of North African origin. They agreed

[1]Data from this study were presented in poster form at the American College of Cardiology Annual Meeting, Atlanta, GA, April 1998. Keevil, J., Osman, H., Maalej, N. and Folts, J. (1998) Grape juice inhibits ex vivo platelet aggregation while orange juice and grapefruit juice do not. 31:2(suppl A) Feb. P172A.

[2]To whom correspondence should be addressed.

[3]Abbreviation used: CAD, coronary artery disease.

to abstain from aspirin, nonsteroidal antiinflammatory drugs, tea, wine, beer, citrus fruits, fruit juices, grape products and alcohol for the 6-wk period of enrollment. The Institutional Review Board at the University of Wisconsin approved the protocol and subjects signed informed consent prior to participation.

The subjects drank each of the three juices, one at a time, for a week. Each juice was separated by a 1-wk washout period with no juice consumption. The juice order varied and the daily dose was selected based on body weight (5–7.5 mL/kg). The average intake was 450 ± 120 mL/d (about two cups). The three juices consumed were: Welch's® (Concord, MA) 100% Purple Grape Juice (250 mL = 711 kJ, 76 mg vitamin C), America's Choice® (Montvale, NJ) 100% Orange Juice (250 mL = 531 kJ, 63 mg vitamin C) and Ocean Spray® (Lakesville-Middleboro, MA) 100% Grapefruit Juice (250 mL = 444 kJ, 63 mg vitamin C). The same lot of each of the three brands of juices was used throughout the study. The grape juice was supplied by one of the study's sponsors, and the orange and grapefruit juices were selected from stock at a local grocery store.

At study entry and after each week of juice consumption or washout, blood was drawn between 0800 and 1000 h and platelet aggregation studied using whole blood aggregometry. Subjects fasted for at least 14 h before the blood draw, except for the current study juice, which they drank ~2 h before the blood draw. Using minimal tourniquet time, blood was gently withdrawn through 19 or 21G needles into a 20 mL syringe containing tri-sodium citrate (anticoagulant final concentration 8.4 mmol/L). A separate 5-mL tube was drawn for measurement of hematocrit and platelet counts. The blood was then diluted 50% with preservative-free saline and held at room temperature. Platelet aggregation was studied in a four-channel whole-blood aggregometer (Chronolog® Model #590; Havertown, PA), using previously published methods (Cardinal and Flower 1980,Demrow et al. 1995). Chart recorders graphed impedance (ohms) as a function of time (min). One-mL blood aliquots were placed in cuvettes with a stir bar and warmed to 37°C. The aggregometer's impedance probe was placed into the warmed blood, and the baseline impedance was set to zero on the chart recorders. Collagen (1.0, 2.0 and 12.5 mg/L), ADP (5 and 20 μmol/L) or thrombin (150 U/L) was added to the cuvette at time zero and the impedance increased proportional to the amount of platelet aggregation on the impedance probe. Measurements from chart recordings were taken as ohms at 6 min using a transparent template to minimize observer bias. For each different agonist concentration, the aggregation response was analyzed in duplicate and averaged. All measurements were completed within 2 h from the blood draw.

The total polyphenolic content of each of the three juices was assayed by the Folin-Ciocalteu assay (Folin and Ciocalteu 1927, Singleton and Rossi 1965). The reagent was prepared by diluting a stock solution (Fisher Scientific, Pittsburgh, PA) with distilled water (1:10, v/v). A sample of juice or gallic acid standards (50 μL) were added to 5 mL of reagent in a test tube followed by 4 mL of Na_2CO_3 (75 g/L). The tubes were stirred and kept at ambient temperature for 2 h. Absorbance at 675 nm was recorded for the juices and gallic acid standards.

Qualitative analysis of the major types of flavonoids in the juices was performed by HPLC using a diode array detector. Reversed-phase HPLC (C-18 column, 5 mm particle size, 4.6 × 250 mm) was performed using a linear gradient from water/acetic acid (975:25, v/v; solvent A) to 100% methanol (solvent B) over 40 min at a flow rate of 1 mL/min. The UV spectra of major peaks were used to classify the flavonoids as flavonols, anthocyanidins, flavanones, flavones and proanthocyanidins.

Each subject served as his or her own control in a three-arm crossover design. Platelet aggregation after each juice was compared to baseline with a paired Student's *t* test using the spreadsheet program Excel® (Microsoft Corp., Redmond, WA). A *P*-value of <0.05 was considered significant. Values are reported as means ± SD

RESULTS

All 10 subjects completed three cycles of juice consumption. No one had complications or was intolerant to juice.

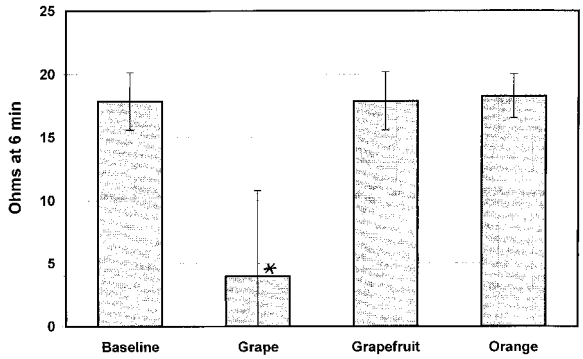

FIGURE 1. Platelet aggregation response in humans after drinking grape, orange or grapefruit juices for 1 wk. The *y*-axis shows the platelet aggregation in ohms, 6 min after exposure to 1 mg/L of collagen. Data after consumption of each juice are compared to baseline using a paired two-tailed *t* test. *Significantly different from baseline, $P = 0.0002$. Values are means ± SD, $n = 10$.

Average hematocrit was 0.41 ± 0.04, and platelet counts were within normal limits.

The baseline platelet aggregation response to 1.0 mg/L of collagen was 17.9 ± 2.3 ohms. After drinking purple grape juice, the platelet aggregation was reduced to 4.0 ± 6.8 ohms, 77% less than baseline ($P = 0.0002$). The platelet aggregation response to a higher collagen concentration (12.5 mg/L) was also significantly inhibited by 21% after grape juice consumption (from 24.5 ± 3.7 to 19.3 ± 4.6 ohms, $P = 0.003$). No significant change from baseline was found after drinking orange or grapefruit juice (**Fig. 1**) at either collagen concentration. Drinking any of the three juices did not affect the aggregation response when ADP or thrombin was used as an agonist. After a 1-wk washout period after consumption of each juice, platelet aggregation of all the subjects returned to their baseline level.

Purple grape juice had a total polyphenolic concentration of almost three times that of orange or grapefruit juice. The gallic acid equivalents were 2.26, 0.75 and 0.86 g/L, respectively. There were large differences in the classes of flavonoids present in the juices. Grape juice contained flavonols, anthocyanidins and proanthocyanidins, and there was no evidence that these polyphenolic compounds were present in the orange or grapefruit juice. The major peaks in orange and grapefruit juices were flavanones and flavones.

DISCUSSION

In a small group of healthy subjects, this study showed substantial inhibition of platelet activity after drinking about two cups of purple grape juice daily for 1 wk. Drinking the same amount of orange or grapefruit juice resulted in no platelet inhibition. The difference in the platelet inhibitory effect between purple grape juice and orange or grapefruit juice may well be due to the different classes of flavonoid compounds they contain. The flavonoids in grapes are primarily flavonols, anthocyanidins and proanthocyanidins (RibÄreau-Gayon 1982, Silva et al. 1991, Singleton and Esau 1969), while the flavonoids in orange and grapefruit juices are primarily flavanones and flavones (Kefford and Chandler 1970). In vitro studies showed that the flavonols are more potent antioxidants and antiplatelet agents than the flavanones (Landolfi et al. 1984).

Some epidemiological studies (Hertog et al. 1993 and 1995, Knekt et al. 1996), but not all (Rimm et al. 1996), demon-

strated that diets high in five specific flavonoids (quercetin, kaempferol, myricetin, luteolin and apigenin) are inversely related to the incidence of myocardial infarction. Quercetin and kaempferol, flavonols found in grapes, made up ~95% of the flavonoids studied (Hertog et al. 1993 and 1995, Knekt et al. 1996, Rimm et al. 1996). The present study suggests that the flavonols in grape juice may be strong platelet inhibitors, while the flavones in citrus fruits may have little or no effect on platelet aggregation. In addition, polyphenolics are in higher total concentrations in purple grape juice so the same citrus juice doses may have too low a total polyphenolic content to detect an effect. A recent study of the antioxidant capacity of five commercial juices (Concord grape, grapefruit, orange, tomato and apple) showed that grape juice had more than three times the antioxidant capacity of the other four juices (Wang et al. 1996). This is similar to purple grape juice having three times the total polyphenolic concentration of orange or grapefruit juice, as seen in the present study.

The difference in platelet inhibitory effects among the three juices could have other explanations. Variations in digestion, absorption and hepatic processing may limit the bioavailability of the flavonoids in the citrus juices. It is possible that compounds in grape juice other than the polyphenolic flavonoids could be responsible for the platelet inhibitory and antioxidant effects seen with purple grape juice. The small variation in vitamin C concentrations among the three juices is unlikely to have a significant effect (Wang et al. 1996).

There has been debate as to the reasons why red wine might reduce the risk of CAD. The alcohol may reduce risk by increasing HDL concentration (Gaziano et al. 1993), while the nonalcoholic constituents in red wine and grapes have protective antioxidant and antiplatelet properties (Demrow et al. 1995, Folts 1998, Folts et al. 1994, Havsteen 1983, Maxwell et al. 1994, Renaud and de Lorgeril 1992). This study strongly supports the existence of a platelet inhibitory effect by some constituent in grape juice. A platelet inhibitory effect is likely to reduce the risk of CAD (Hennekens et al. 1997).

The mechanism by which purple grape juice inhibits platelet activity is not known, but numerous studies suggest possible mechanisms. The flavonoids in grapes were shown to inhibit cyclooxygenase and phosphodiesterase enzymes (Havsteen 1983 Laughton et al. 1991). Sauter et al. (1998) incubated platelets in a 1:1000 grape juice dilution and showed three times the nitric oxide release (a platelet inhibitor) and a 55% decrease in platelet release of the free radical, super oxide, a platelet stimulator, ($P < 0.01$) compared to vehicle controls. In addition, incubating arterial rings in a tissue bath with grape juice increased endothelial-dependent vasorelaxation by a nitric oxide-dependent mechanism (Fitzpatrick et al. 1993).

The present study contrasts with a recent study (Pace-Asciak et al. 1996), showing no significant change in platelet aggregation after 24 healthy males drank red grape juice for 2 wk. Several important methodological differences could explain the different results. First, the previous study used only ADP and thrombin as agonists (to which platelet aggregation was unchanged in both studies) while we also used collagen. Second, there are likely to be differences in polyphenolic concentration of red and purple grape juices. Third, the previous study used platelet-rich plasma aggregation, which may be less sensitive than the whole blood methods used in the present study (Riess et al. 1986). Fourth, the subjects' diets were not as well controlled for other flavonoid sources such as tea or apples. Finally, the juice dose was not titrated to body weight (Pace-Asciak et al. 1996).

Aspirin has become a standard therapy for prevention of recurrent ischemia in patients with known CAD (Antiplatelet Trialists' Collaboration 1994, Hennekens et al. 1997). The platelet inhibitory effect of aspirin is believed to be the mechanism by which it offers protection. While not compared directly here, the degree of purple grape juice's platelet inhibitory effect is similar to that reported for aspirin (Ingerman-Wojenski and Silver 1984). In animal studies, the in vivo platelet inhibitory effect of aspirin can be reversed with an IV infusion of epinephrine 0.2 μg/(kg · min) (Folts 1995). However, the platelet inhibitory effect of grape juice is not reversed by an epinephrine infusion (Demrow et al. 1995, Folts 1995, Osman et al. 1998).

Our results suggest that purple grape juice or its constituents may have a role in preventing the development and progression of CAD. Limitations of this study include its small size and inclusion of only healthy subjects. Patients with multivessel CAD have increased platelet activity (Gorog et al. 1995) and may present a greater challenge to grape juice's inhibitory effect. In addition, because grape juice was not compared to aspirin in the same subjects, the comparative conclusions are speculative. Before grape juice can be recommended to patients with cardiovascular disease, studies comparing the platelet inhibition of grape juice and aspirin, as well as the combined effect of the two interventions, should be performed in this population.

ACKNOWLEDGMENTS

Grant support came from Welch's Foods, Concord, the Rennebohm Foundation and the Nutricia Research Foundation.

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