Select Flavonoids and Whole Juice From Purple Grapes Inhibit Platelet Function and Enhance Nitric Oxide Release

Jane E. Freedman, MD; Crawford Parker III, MD; Liqing Li, MS; Jacob A. Perlman; Balz Frei, PhD; Vadim Ivanov, PhD; Leslie R. Deak, BS; Mark D. Iafrati, MD; John D. Folts, PhD

Background—Moderate red wine consumption is inversely associated with coronary ischemia, and both red wine and purple grape juice (PGJ) contain flavonoids with antioxidant and antiplatelet properties believed to be protective against cardiovascular events. Acute cardiac events are also associated with decreased platelet-derived nitric oxide (NO) release. In this study, the effects of PGJ and PGJ-derived flavonoids on platelet function and platelet NO production were determined.

Methods and Results—Incubation of platelets with dilute PGJ led to inhibition of aggregation, enhanced release of platelet-derived NO, and decreased superoxide production. To confirm the in vivo relevance of these findings, 20 healthy subjects consumed 7 mL · kg\(^{-1}\)· d\(^{-1}\) of PGJ for 14 days. Platelet aggregation was inhibited after PGJ supplementation, platelet-derived NO production increased from 3.5 ± 1.2 to 6.0 ± 1.5 pmol/10\(^8\) platelets, and superoxide release decreased from 29.5 ± 5.0 to 19.2 ± 3.1 arbitrary units (\(P<0.007\) and \(P<0.05\), respectively). α-Tocopherol levels increased significantly after PGJ consumption (from 15.6 ± 0.7 to 17.6 ± 0.9 μmol/L; \(P<0.009\)), and the plasma protein–independent antioxidant activity increased by 50.0% (\(P<0.05\)). Last, incubation of platelets with select flavonoid fractions isolated from PGJ consistently attenuated superoxide levels but had variable effects on whole-blood aggregation, platelet aggregation, and NO release.

Conclusions—Both in vitro incubation and oral supplementation with PGJ decrease platelet aggregation, increase platelet-derived NO release, and decrease superoxide production. These findings may be a result of antioxidant-sparing and/or direct effects of select flavonoids found in PGJ. The suppression of platelet-mediated thrombosis represents a potential mechanism for the beneficial effects of purple grape products, independent of alcohol consumption, in cardiovascular disease. (Circulation. 2001;103:2792-2798.)

Key Words: platelets ■ antioxidants ■ nitric oxide ■ thrombosis ■ nutrition

Daily consumption of alcoholic beverages is associated with a reduction in acute cardiovascular events.\(^1\,2\) It has been suggested that red wine intake may reduce the incidence of coronary artery disease\(^3\,4\); however, the source of its cardioprotective effect is unclear. Alcohol in any form appears to raise HDL levels\(^5\), yet survival analysis models show that only half of the observed cardiovascular protection in moderate drinkers is mediated by an increase in HDL levels.\(^6\)

Purple grape products, including red wine and purple grape juice (PGJ), contain flavonoids that are polyphenol derivatives of diphenylpyrans found in plant but not animal food products. Grapes and grape products, especially those made from the skins, seeds, and stems of Concord grapes, are good sources of flavonoids. Compared with white wine, red wine contains an \(\approx\)10-fold increase in flavonoid compounds. Flavonoids may contribute to the cardioprotective effects of grape products, as suggested by several studies associating increased flavonoid intake with reduced risk of coronary events.\(^7\,8\)

Most acute coronary syndromes are caused by platelet adhesion, aggregation, and thrombus formation in areas of ruptured atheromatous plaques.\(^9\,10\) Although ethanol attenuates platelet activity, this inhibition occurs only at high levels not achievable with moderate alcohol consumption.\(^11\) Both wine and grape juice have also been shown in vivo to inhibit platelet activity and thrombosis in stenosed canine coronary arteries.\(^12\) Recently, PGJ was shown to decrease platelet aggregation, although this platelet-inhibitory effect resolved 1 week after completion of consumption of PGJ.\(^13\) Supplementation with red wine or alcohol-free red wine prolongs bleeding times and reduces platelet adhesion, and infusion of
a nitric oxide synthase (NOS) inhibitor prevents these effects.\textsuperscript{14}

Endothelium-dependent vasorelaxing activity in an aortic ring model is increased by incubation with grape products, including PGJ, and these changes appear to be mediated by the NO-cGMP pathway.\textsuperscript{15} Recently, it was also shown that endothelium-dependent vasodilation is enhanced in patients with cardiovascular disease who consumed PGJ for 14 days.\textsuperscript{16} Although these findings suggest that PGJ improves NO-dependent vasodilation, the mechanism by which PGJ alters NO-mediated flow has not been determined. NO is also released directly from stimulated platelets and inhibits platelet recruitment and thrombosis formation.\textsuperscript{17,18} In addition, platelets from patients with unstable coronary syndromes have decreased release of platelet-derived NO compared with patients with stable coronary disease.\textsuperscript{19} The effects of PGJ on platelet-derived NO release, however, are currently unknown. Therefore, the purpose of this study was to characterize the inhibition of platelet function by PGJ and PGJ-derived flavonoids, study the effects on platelet-derived NO release, and explore the mechanism(s) for any observed changes.

**Methods**

**Preparation of Samples**
Venous blood was obtained from healthy subjects who were not receiving vitamin supplements and had not used any platelet inhibitor for \textsuperscript{27} days. In some experiments, whole blood was incubated with PGJ for 30 minutes at room temperature, and platelets were isolated and aggregations were performed as previously described.\textsuperscript{20} Whole-blood aggregation studies were performed with an impedance aggregometer (Chronolog).

To study the effect of PGJ supplementation, 20 healthy, normal subjects (mean age 30.6±1.8 years, range 20 to 45 years, 12 male, 8 female) consumed PGJ after having given informed consent according to the policies of the Institutional Review Board at Georgetown University Medical Center. Each donor consumed 7 mL·kg\textsuperscript{-1·d\textsuperscript{-1}} of PGJ (Welch’s) for 14 days. The subjects kept a daily log to confirm the amount of PGJ consumed and to record any side effects. For the duration of the study, subjects took no drugs and refrained from consuming other grape products, caffeine, alcohol, supplemental vitamins, garlic, and herbs. Vegetarians and persons on macrobiotic diets were excluded from the study.

**Measurement of Platelet NO and Superoxide Release**
NO was measured with an NO-selective microelectrode, and platelet NO production was quantified as the integrated signal detected by the microelectrode after platelet activation.\textsuperscript{21} Aggregation-dependent platelet superoxide production was measured in a lumiaaggregometer in the presence of lucigenin as previously described.\textsuperscript{21}

**Protein Determination in Megakaryocytic Cells**
Human erythroleukemic (HEL) cells are a permanent cell line exhibiting features of megakaryocyte differentiation. Protein samples were separated by gel electrophoresis, and proteins were electrochemically transferred onto nitrocellulose and incubated with antibody to human endothelial NOS (eNOS) (1:2500, Transduction Laboratories). The nitrocellulose membrane was exposed to goat anti-mouse antibody linked to horseradish peroxidase (1:2000). Antibody binding was detected by use of the ECL system from Amersham.

**Measurement of Plasma Antioxidant Levels**
Plasma levels of α-tocopherol and γ-tocopherol were determined by use of high-performance liquid chromatography (HPLC) with electrochemical detection as previously described.\textsuperscript{22} Ascorbate and urate levels were measured by paired-ion reverse-phase HPLC with electrochemical detection.\textsuperscript{23}

**Total Antioxidant Capacity**
The whole-plasma oxygen radical absorbance capacity (ORAC) assay and perchloric acid (PCA)–plasma ORAC assay (protein-independent antioxidant activity) were performed by the method of Cao et al\textsuperscript{24} with minor modifications. Two hundred microliters of β-phycoerythrin in PBS (final concentration 16.7 pmol/L) was added to 30 μL of sample or standard, followed by addition of 70 μL 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) in PBS (4 mmol/L, final concentration). Trolox (in PBS or in PBS containing corresponding amounts of PCA) was used as a reference antioxidant for calibration. Calculations of area under the curve parameters were made as described.\textsuperscript{24}

Sigma cholesterol (Procedure 352) and HDL cholesterol (Procedure 352-3) kits were used for total plasma, HDL plasma, and LDL plasma cholesterol measurements.

**Phosphorylation of Platelet Proteins**
Platelet-rich plasma from 5 subjects was incubated with a 1:1000 dilution of PGJ, 500 μmol/L quercetin (a flavonoid found in PGJ), or vehicle control. The platelets were centrifuged and resuspended in phosphate-free HBSS and incubated with [\textsuperscript{32}P]orthophosphate as previously described.\textsuperscript{20} After removal of free [\textsuperscript{32}P] by gel filtration, the samples with incubated with PMA (27 nmol/L) for 1 minute. Samples were analyzed by electrophoresis on a 10% polyacrylamide gel and analyzed by autoradiography.

**Isolation of Flavonoids From PGJ**
There are many subclasses of flavonoids in PGJ.\textsuperscript{25–28} The major groups of polyphenolic compounds were separated by HPLC analysis done on a reverse-phase column (C18, 10 μmol/L particle size, 4.5 mm×25 cm inner diameter by length) with a linear gradient from water/trifluoroacetic acid to 100% methanol/trifluoroacetic acid. The first fraction of polyphenolic compounds was eluted with 100 mL water followed by 50 mL each of 3% acetonitrile in water (second fraction), 16% acetonitrile in water (third fraction), methanol (fourth fraction), and 1% HCl in methanol (fifth fraction). The pH of the first fraction (water) was adjusted to 3.5 with 0.5 mol/L HCl, and the acidified material was run through the C18 column. The methanol fractions collected from the first fraction were combined and evaporated. Fractions 2 to 5 collected in the initial fractionation were evaporated directly without additional processing. A second C18 solid-phase extraction column was equilibrated with methanol and water for a second pass of the fractions.

Qualitative identification of phenolic compounds was carried out by analysis of the UV/visible spectrum of major peaks in each fraction with a Waters diode array detector and Millennium chromatography software.

The phenolic compounds were characterized as belonging to 4 major classes: cinnamic acids (ie, esters of ferulic and coumaric acid) and 3 types of flavonoids: anthocyanins (ie, cyanidin and delphinidin glycosides), flavonols (ie, quercetin glycosides), and polyflavan-3-ols (also known as proanthocyanidins or condensed tannins). In addition, some of the poorly eluting polyflavan-3-ol fractions had an

**TABLE 1. Platelet NO, Superoxide, and Aggregation Before and After Supplementation With PGJ**

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<th>Before Supplementation</th>
<th>After Supplementation</th>
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<tr>
<td>Platelet aggregation, %</td>
<td>57.6±3.3</td>
<td>38.5±3.7*</td>
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<tr>
<td>Platelet NO, pmol/10\textsuperscript{8} platelets</td>
<td>3.5±1.2</td>
<td>6.0±1.5†</td>
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<tr>
<td>Platelet superoxide, arbitrary units</td>
<td>29.5±5.0</td>
<td>19.2±3.1†</td>
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\textsuperscript{n=20. *P<0.002 vs before supplementation, †P<0.05 vs before supplementation.}
absorbance of \(\sim 520\) nm, which indicates that these may also be pigmented (dark red or burgundy).

Statistics

All data are presented as mean ± SEM. Paired samples were compared by Student’s \(t\) test. Dose-response curves were evaluated with a post hoc Newman-Keuls or Dunnett’s comparison where appropriate. Statistical significance was accepted if the null hypothesis was rejected, with \(P < 0.05\).

Results

Effect of PGJ on Platelet Aggregation

As shown in Figure 1, incubation of platelets (in whole blood followed by separation) with dilutions of PGJ (1:1000, 1:500, 1:250) led to a dose-dependent inhibition of extent of aggregation. Overall, the maximal extent of aggregation in ADP-stimulated platelets decreased from 89.3 ± 3.3\% to 50.5 ± 3.7\% (\(P < 0.01\)).

Effect of PGJ on Platelet-Derived NO Production

To determine whether PGJ alters platelet NO release, platelets were incubated with dilutions of PGJ (1:1000, 1:500, 1:250), and stimulation-dependent platelet NO production was determined. As shown in Figure 2, platelet-NO release increased with increasing concentration of PGJ. Maximal platelet NO release increased from 0.53 ± 0.11 pmol/10\(^8\) platelets at baseline to 1.78 ± 0.15 pmol/10\(^8\) platelets after incubation with a 1:250 dilution (\(P < 0.001\)).

Effect of PGJ on Platelet Superoxide Release

Aggregating platelets produce superoxide,\(^{27}\) which is known to react readily with NO and reduce its bioactivity. With increasing concentrations of PGJ, there was a dose-dependent decrease in superoxide release (Figure 3). Platelet superoxide release decreased from 268.5 ± 13.7 arbitrary units at baseline to 127.8 ± 16.1 arbitrary units after incubation with a 1:250 dilution of PGJ (\(P < 0.001\)).

Effect of PGJ Supplementation on Platelet Aggregation, NO Production, and Superoxide Release

Consumption of PGJ led to a reduction in phorbol 12-myristate 13-acetate (PMA)–dependent platelet aggregation (maximal extent) from 57.6 ± 3.3\% to 38.5 ± 3.7\% (Table 1; \(P = 0.002\)). With ADP and collagen used for agonists, mean platelet aggregation decreased by 18.2 ± 7.4\% and 12.8 ± 5.3\% (\(P = 0.09\) and \(P = 0.08\), respectively, data not shown). Similar to the previous in vitro findings, supplementation with PGJ led to an increase in platelet-derived NO release (Table 1). The mean increase in platelet NO levels was 2.5 pmol/10\(^8\) platelets (\(P = 0.012\)). Supplementation with PGJ also significantly decreases platelet superoxide production (Table 1).

Effect of PGJ Supplementation on Antioxidant Levels

To determine whether supplementation with PGJ in healthy volunteers alters antioxidant content, plasma levels of \(\alpha\)-tocopherol, \(\gamma\)-tocopherol, ascorbate, and urate were determined.
The effect of PGJ on total antioxidant activity was determined by quantification of the oxygen-radical–absorbing capacity of antioxidant in plasma with the ORAC assay. As shown in Figure 4, the antioxidant activity of plasma increased from 0.6±0.1 to 0.9±0.1 ORAC units×1000, as measured by the protein-free ORAC level (ORAC PCA-plasma). Total plasma ORAC values did not change significantly (from 5.3±0.5 to 5.7±0.6, P=NS; Figure 4), suggesting that ingestion of PGJ increases low-molecular-weight antioxidants in plasma. There was no significant change in plasma cholesterol levels after supplementation with PGJ (data not shown).

Effect of PGJ on eNOS Content in Megakaryocytic Cells
A cultured megakaryocytic cell line was incubated for 4 to 24 hours with increasing dilutions of PGJ (1:1000, 1:500, 1:250), after which the cells were lysed and protein samples separated by gel electrophoresis and transferred onto nitrocellulose. After exposure to the antibody, expression of eNOS was determined. Incubation of megakaryocytes with PGJ did not result in any change in eNOS protein content (P=NS, n=3, data not shown).

Effect of PGJ on Platelet Protein Kinase C Activity
Protein kinase C (PKC) activity is an important determinant of NO production. Therefore, we studied the effect of PGJ and the flavonoid quercetin on platelet PKC activity. Stimulation of platelets by the agonist PMA induces the phosphorylation of a 47-kDa platelet protein, which is a well-known substrate of PKC. As shown in Figure 5, vehicle-treated platelets show the expected increase in phosphorylation of the 47-kDa protein, but incubation with PGJ or quercetin causes a partial decrease in phosphorylation. Thus, incubation with PGJ is associated with partial inhibition of PKC stimulation.

Effect of Isolated Flavonoids on Platelet Aggregation, NO Production, and Superoxide Release
PGJ contains many subclasses, and the bioactivity of most of these compounds has not been studied previously. The specific characterizations of the 5 flavonoid fractions are shown in Table 3. As seen in Table 4, there are marked differences in the platelet-dependent effects between the 5 fractions studied. These responses were seen in washed, resuspended platelets. Whole-blood impedance aggregometry showed that fractions 3, 4, and 5 were significant inhibitors of platelet activity. These fractions contain primarily proanthocyanins.

Discussion
In this study, the in vitro and ex vivo effects of PGJ and fractions of flavonoids obtained from PGJ on platelet function and platelet NO production were determined. Incubation of platelets with dilute PGJ or consumption of PGJ by healthy volunteer subjects led to a dose-dependent inhibition of aggregation and enhanced release of platelet NO. Platelets and megakaryocytes are known to contain constitutive NOS (eNOS), and stimulated platelets release NO. Platelet release of NO has been found to have modest effects on aggregation as well as more profound effects on platelet recruitment to a growing thrombus. Thus, enhanced release of platelet-derived NO may have contributed to inhibition of aggregation detected after both in vitro incubation with PGJ and oral supplementation. How PGJ enhances platelet release of NO is unknown; however, NO interacts with superoxide anion, which may alter the antithrombotic properties of NO. By reacting with superoxide or lipid peroxyl radicals (LOO·), NO can form peroxynitrite (OONO·) and lipid peroxynitrites (LOONO), respectively, with a resultant loss in NO bioactivity. Superoxide is produced by platelets and in this study, superoxide release was significantly decreased by incubation or supplementation with PGJ (Figure 3
In addition, superoxide itself has also been shown to augment platelet aggregation responses.\textsuperscript{30}

In addition to possible enhancement of NO bioactivity via suppression of superoxide production, detectable NO levels could be enhanced by increased levels of cNOS. No detectable change was noted in protein content after incubation with PGJ. Because platelets contain no DNA and minimal RNA, it is unlikely that PGJ is altering cNOS levels directly in the platelet. In addition, brief in vitro incubations with PGJ led to significant changes in platelet NO release, and these changes were similar to those observed after oral supplementation. Isolated platelets were used to measure NO release, suggesting that increases in NO are most likely due to direct PGJ-dependent platelet effects and not alterations of plasma components.

The bioactivity of NO is also dependent on antioxidant status. Flavonoids have antioxidant capacity and could be affecting thrombosis by influencing levels of other antioxidants. Although PGJ did not alter levels of the watersoluble antioxidants ascorbate and urate, consumption of PGJ significantly increased $\alpha$-tocopherol and nonsignificantly increased $\gamma$-tocopherol levels. Procyanidins isolated from grapes decrease membrane lipid peroxidation and prevent loss of vitamin E.\textsuperscript{31} The increase in $\alpha$-tocopherol is especially interesting, because $\alpha$-tocopherol is an antioxidant with well-characterized direct antiplatelet and anti thrombotic effects.\textsuperscript{20} The absolute change in $\alpha$-tocopherol levels after PGJ consumption, however, was relatively modest and unlikely to be the sole explanation for the platelet-inhibitory effects. The total antioxidant capacity in plasma was significantly increased after PGJ consumption, as determined by the ORAC assay. Total and protein-free assays showed that PGJ consumption increased the aqueous but not lipid-soluble antioxidant pool (Figure 4). This assay has high specificity; however, it reflects numerous antioxidants and does not determine the precise antioxidant responsible for the changes.

We have previously shown that $\alpha$-tocopherol inhibits platelet function by a PKC-dependent mechanism.\textsuperscript{20} After supplementation with PGJ, the greatest changes in platelet function were detected by use of the PKC-dependent agonist PMA. In addition, as seen in Figure 4, incubation of platelets

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<th>TABLE 3. Characterization of Flavonoid Fractions Derived From PGJ*</th>
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*Although there is considerable overlap in the first 2 fractions, they are low in the oligomeric and polymeric proanthocyanidins. The main phenolic components in fractions 4 and 5 are the oligomeric and polymeric proanthocyanidins.

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<th>TABLE 4. Effect of PGJ-Derived Flavonoid Fractions on Platelet Aggregation, NO Production, and Superoxide Release</th>
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<td>PGJ Fraction</td>
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$P<0.05$ vs vehicle control.
with PGJ also inhibits PKC activity. Reactive oxygen species may also cause activation through regulation of PKC\(^{32}\); therefore, antioxidant metabolism of both exogenous and platelet-derived reactive oxygen species by PGJ may influence platelet PKC activity and, potentially, platelet-derived NO release. Finally, plant flavonoids, including quercetin, have previously been shown to directly inhibit PKC.\(^{33}\)

To characterize the other potential compounds in PGJ that may be responsible for our observed changes in platelet function and NO release, groups of flavonoids were isolated from PGJ. As seen in Table 4, there are marked differences in the platelet-dependent effects between the 5 fractions studied. Although superoxide release was attenuated by incubation with all of the flavonoid fractions, NO production was not uniformly increased. Only fraction 3, containing primarily polymeric anthocyanins, significantly enhanced platelet NO release, and this was associated with a marked decrease in platelet superoxide production and inhibition of platelet aggregation. Fractions 3, 4, and 5 led to inhibition of whole-blood aggregation, suggesting enhanced bioactivity in whole blood. Conversely, flavonoid fraction 2 (containing mainly catechins and other monomers) increased platelet aggregation in washed, resuspended platelets, significantly decreased NO release, and compared with the other fractions, caused less inhibition of superoxide production. This fraction, however, caused only minimal changes to whole-blood aggregation (Table 5). Thus, these PGJ-derived flavonoid fractions do not have uniform bioactivity.

In summary, both in vitro incubation and oral supplementation with PGJ decrease platelet aggregation, enhance platelet-derived NO release, and decrease superoxide production. Oral supplementation with PGJ had a modest effect on antioxidant levels that cannot entirely account for the alterations in platelet function and NO release. Because PGJ did not enhance cNOS protein levels, the changes appear to be direct platelet effects and may be partially regulated via the PKC pathway. Although flavonoids are presumed to be the active component of PGJ, select flavonoids isolated from PGJ had varied effects on platelets. Therefore, the inhibition of platelet function and superoxide release, as well as the increase in NO, is probably a result of both antioxidant-sparing and/or direct effects of select flavonoids found in PGJ. The suppression of platelet-mediated thrombosis represents a potential mechanism for the beneficial effects of purple grape products, independent of alcohol consumption, in cardiovascular disease.

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### References


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