Comparative effects of dietary supplementation with red grape juice and vitamin E on production of superoxide by circulating neutrophil NADPH oxidase in hemodialysis patients

Patricia Castilla, Alberto Dávalos, José Luis Teruel, Francisca Cerrato, Milagros Fernández-Lucas, José Luis Merino, Carolina C. Sánchez-Martín, Joaquín Ortuno, and Miguel A Lasuncio

ABSTRACT

Background: Atherosclerotic cardiovascular disease is the most common cause of death among hemodialysis patients; it has been attributed to increased oxidative stress, dyslipidemia, malnutrition, and chronic inflammation. Activation of neutrophils is a well-recognized feature in dialysis patients, and superoxide-anion production by neutrophil NADPH oxidase may contribute significantly to oxidative stress.

Objective: The aim of the study was to compare the effects of dietary supplementation with concentrated red grape juice (RGJ), a source of polyphenols, and vitamin E on neutrophil NADPH oxidase activity and other cardiovascular risk factors in hemodialysis patients.

Design: Thirty-two patients undergoing hemodialysis were recruited and randomly assigned to groups to receive dietary supplementation with RGJ, vitamin E, or both or a control condition without supplementation or placebo. Blood was obtained at baseline and on days 7 and 14 of treatment.

Results: RGJ consumption but not vitamin E consumption reduced plasma concentrations of total cholesterol and apolipoprotein B and increased those of HDL cholesterol. Both RGJ and vitamin E reduced plasma concentrations of oxidized LDL and ex vivo neutrophil NADPH oxidase activity. These effects were intensified when the supplements were used in combination; in that case, reductions in the inflammatory biomarkers intercellular adhesion molecule 1 and monocyte chemoattractant protein 1 also were observed.

Conclusions: Regular ingestion of concentrated RGJ by hemodialysis patients reduces neutrophil NADPH-oxidase activity and plasma concentrations of oxidized LDL and inflammatory biomarkers to a greater extent than does that of vitamin E. This effect of RGJ consumption may favor a reduction in cardiovascular risk. Am J Clin Nutr 2008;87:1053–61.

INTRODUCTION

Atherosclerosis and cardiovascular complications are the main cause of morbidity in end-stage renal disease (1). Oxidative stress, brought about by an imbalance between the production of oxidants and the availability of antioxidant defense mechanisms, plays a critical role in the pathogenesis of atherosclerosis and vascular disease (2, 3). This may be particularly important in hemodialysis patients, in whom reactive oxygen species (ROS) are overproduced and antioxidant defense mechanisms are impaired (4, 5). Chronic inflammation associated with uremia and the presence of trace amounts of endotoxins in the dialysate may contribute to oxidative stress in hemodialysis patients (6, 7). Furthermore, the hemodialysis procedure itself causes oxidative stress by generating ROS through activation of circulating neutrophils (8, 9). Coating dialysis membranes with vitamin E has been shown to effectively reduce superoxide anion production by neutrophils (9, 10) and to lower the plasma concentration of oxidized LDL (8, 9, 11), an emerging marker for cardiovascular risk in hemodialysis patients (12–14).

NADPH oxidase is a main source of superoxide radicals, which promote LDL oxidation. The enzyme is particularly active in neutrophils and monocytes, where it plays an important role in host defense. Recently, a direct association between increased phagocytic NADPH oxidase activity and elevated circulating oxidized LDL was found in patients with metabolic syndrome (15). Furthermore, NADPH oxidase has been found to be overexpressed in vascular cells from atherosclerotic lesions, which supports a role for this enzyme in the pathogenesis of atherosclerosis (16). In particular, NADPH oxidase–dependent superoxide production appears to be abnormally high in mononuclear cells from patients with early chronic kidney disease (17). Consequently, both modulation of NADPH oxidase to prevent overproduction of ROS (7) and supplementation with antioxidants (18, 19) have been proposed as reasonable strategies to prevent the deleterious effects of oxidative stress in hemodialysis patients.

Polyphenols comprise a wide variety of compounds that occur naturally in foods, which are absorbed to a significant extent (20). They are thought to contribute to the putative beneficial effects

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on cardiovascular disease attributed to consumption of grape derivatives (21–23). In studies conducted in vitro, polyphenols have been shown to down-regulate the expression of NADPH oxidase subunits p22^phox and p47^phox in endothelial cells (24). In animal models, quercetin reduces p47^phox expression in spontaneously hypertensive rats (25), and red wine polyphenols prevent angiotensin II–induced hypertension and endothelial dysfunction in rats by reducing the expression of p22^phox and Nox-1 (26). In humans, grape juice ingestion has been shown to increase serum antioxidant capacity, reduce peroxide formation, decrease platelet aggregation, and improve flow-mediated vasodilation (27–30).

Malnutrition and dietary restrictions in patients undergoing hemodialysis may limit the intake of certain micronutrients, such as polyphenols. We recently reported that ingestion of concentrated red grape juice (RGJ) as a polyphenol-rich dietary supplement exerts hypolipidemic, antioxidant, and antiinflammatory effects in those patients (30). In the present study, we investigated whether RGJ ingestion, either alone or in combination with vitamin E, affects neutrophil NADPH-oxidase activity and other plasma biochemical variables in hemodialysis patients.

**SUBJECTS AND METHODS**

**Subjects and study design**

Thirty-two clinically stable patients (16 M, 16 F; age range, 33–79 y) were recruited from those attending the morning shift in our dialysis unit at Hospital Ramón y Cajal (Madrid, Spain). They were undergoing maintenance hemodialysis initiated ≥3 mo previously at a rate of 3 times/wk for 3.5–4.5 h per session. Exclusion criteria were diabetes mellitus, a cardiovascular event within the previous 12 mo, and the use of cholesterol-lowering medication. All patients were receiving erythropoietin and a multivitamin supplement (Becozyme C Forte; Roche Diagnostics), containing vitamin C (200 mg/d) as the only antioxidant, to compensate losses of low-molecular-weight molecules as a result of the dialysis procedure. A diet consisting of 15% protein, 50% carbohydrates, 35% lipids (35% saturated fatty acids, 52% monounsaturated fatty acids, and 13% polyunsaturated fatty acids), 320 mg cholesterol, 25 g fiber, and 1.2 L of water was recommended in all subjects. To limit the intake of potassium, patients were instructed to avoid foods with high potassium content, such as green leaf vegetables and dried fruit, and to restrict the consumption of fruit and vegetables in general. During the course of the study, patients were also recommended not to change their diet or level of physical activity.

Patients were randomly distributed in 4 groups (n = 8/group): RGJ, vitamin E, RGJ + vitamin E, and control. Participants in the RGJ group agreed to consume an oral supplement of 50 mL concentrated RGJ twice a day, at lunch and at dinner, for 2 wk. Patients in the vitamin E group were instructed to take 800 IU vitamin E during each hemodialysis session for 2 wk. The third group of patients (RGJ + vitamin E) received both 50 mL RGJ twice daily and 800 IU vitamin E as before, and the control group received neither supplement nor placebo during the study period. Blood samples were collected immediately before supplementation was begun (baseline) and on days 7 and 14 during the intervention period. Blood samples were taken at the beginning of the hemodialysis session, after an overnight fast.

Written informed consent was obtained from all patients. All procedures were approved by the Ethics Committee on Clinical Research of Hospital Ramón y Cajal. The study was undertaken in accordance with the principles outlined in the Declaration of Helsinki.

Concentrated RGJ, prepared from the bobal grape variety, was purchased from Dream Fruits (Quero, Toledo, Spain); detailed specifications are given elsewhere (30). The daily ingested dose of RGJ (100 mL) contained 84 g sugar, approximately equal amounts of glucose and fructose, 6.8 mEq potassium, and 0.64 g total polyphenols (gallic acid equivalents), with particularly high concentrations of quercetin, myricetin, and anthocyanidins (31).

**Analytic procedures**

Blood samples were collected in tubes containing EDTA, maintained on ice, and rapidly centrifuged to obtain plasma. Plasma was immediately distributed in aliquots for the different determinations and stored at −70 °C until analysis. Routine biochemical analysis was performed with a RA-1000 Autoanalyzer (Technicon Ltd, Dublin, Ireland). Cholesterol and triacylglycerols were assayed enzymatically (Menarini Diagnostic, Florence, Italy), HDL-cholesterol concentrations were measured after precipitation of apolipoprotein (apo) B–containing lipoproteins (Roche Diagnostics, Madrid, Spain). LDL-cholesterol concentrations were calculated by using the Friedewald formula. ApoA-I and apoB concentrations were measured by immunonephelometry (Dade Behring, Frankfurt, Germany). Plasma concentrations of α-tocopherol were measured by reversed-phase HPLC, as described previously (32).

Oxidized LDL was measured with a commercial sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Merckodia AB, Uppsala, Sweden). The capture antibody used in the assay is the murine monoclonal antibody 4E6, which is directed against a conformational epitope in the apoB moiety of LDL that is generated by the replacement of lysine residues by aldehydes (13). A peroxidase-conjugated anti-apoB antibody is used for detection. Serum concentrations of soluble vascular cell adhesion molecule 1 (VCAM-1) and soluble intercellular adhesion molecule 1 (ICAM-1) were measured by using ELISA kits (Diaclone SAS, Besançon, France). Monocyte chemoattractant protein 1 (MCP-1) concentration was also measured with an ELISA kit (PetroTech EC Ltd, London, UK). Concentrations of high-sensitivity C-reactive protein (hs-CRP) and complement C3 protein were measured by immunonephelometry (Dade Behring).

**Neutrophil NADPH oxidase activity**

Blood samples for analysis of NADPH oxidase activity in isolated neutrophils were collected in sodium heparin tubes, and neutrophils were isolated with Histopaque (Sigma, St Louis, MO). Briefly, 3 mL Histopaque-1119 was placed in the bottom of a 15-mL conical sterile tube, and 3 mL Histopaque-1077 was carefully layered on top. Then, 6 mL blood diluted 1:1 in Ca^2+-, and Mg^2+-free phosphate-buffered saline (PBS) was carefully layered over the Histopaque-1077. Centrifugation was performed at 700 × g for exactly 30 min at room temperature. The polymorphonuclear leukocyte layer was carefully aspirated with a sterile Pasteur pipette and transferred to another sterile tube. The cell solution was diluted with isotonic PBS supplemented with 5 mmol glucose/L and centrifuged at 250 × g for 10 min at
FIGURE 1. Mean (±SD) in vitro effects of different NADPH oxidase inhibitors (A), concentrated red grape juice (RGJ) (B), and α-tocopherol (C) on NADPH oxidase activity in human neutrophils. n = ≥4. A: Cells were incubated with 5 μmol diphenylene iodonium/L (DPI), 250 μmol apocynin/L (Apo), or vehicle (control). 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was added; after 15 min, the cells were treated with (activated) or without (resting) 50 ng phorbol myristate acetate/L (PMA) and incubated for an additional 45 min. After incubation, flow cytometry was performed, and the median intensity of fluorescence (MIF) was calculated; only data corresponding to PMA-activated cells are shown. B and C: Cells were incubated with increasing concentrations of RGJ or α-tocopherol for 30 min at 37 °C and then extensively washed. DCFH-DA was added, and the cells were treated and processed as before. *, **, ***Significant differences between treatments (one-factor RM-ANOVA; the Tukey test was used for post hoc statistical comparisons with controls): *P < 0.05, **P < 0.01, ***P < 0.001.

The polymorphonuclear leukocyte pellet was washed gently with 10 mL PBS glucose and centrifuged again. Neutrophils were resuspended in Krebs-Ringer solution phosphate buffer (Sigma) containing 10 mmol glucose/l and supplemented with Ca²⁺ and Mg²⁺. NADPH oxidase activity was assayed by using the method described by Bass et al (33), with minor modifications. For this, cells were incubated (1 × 10⁶ cells/mL) with 5 μmol 2′7′-dichlorodihydrofluorescein diacetate/L (DCFH-DA) Molecular Probes, Leiden, Netherlands) for 15 min at 37 °C. Then, cells were incubated in the absence (resting) or the presence (activated) of 50 ng/mL phorbol myristate acetate/L (PMA; Sigma) and incubated for 45 min at 37 °C in an atmosphere of 5% CO₂. Immediately after incubation, flow cytometry was performed (FACScalibur; Becton-Dickinson, San Jose, CA). Data were analyzed by using CELL QUEST software (version 3.2.1; Becton-Dickinson), and the median intensity of fluorescence (MIF) was used to evaluate the fluorescence of each tube.

To assay neutrophil NADPH oxidase activity in whole blood samples, a flow cytometry assay was used according to the method described by Richardson et al (34). Whole blood aliquots (100 μL) were incubated in the absence or presence of PMA (50 ng/mL) for 45 min at 37 °C in a 5% CO₂ atmosphere. A solution of dihydrorhodamine 123 (DHR) (Sigma, St. Louis, MO) was added at a final concentration of 30 μg/mL and incubated for 5 min at 37 °C. Red blood cells were lysed with 2 mL FACS lysis solution (Becton-Dickinson) for 15 min at room temperature. Subsequently, cells were centrifuged at 400 × g for 5 min at 4 °C, the pellet was washed twice with 3 mL isotonic saline solution, and white blood cells were resuspended in 0.5 mL PBS and analyzed by flow cytometry. In both cases, data were analyzed by using CELL QUEST software, and the MIF was used to evaluate the fluorescence of each tube. NADPH oxidase activity was calculated as the difference between values obtained in PMA-activated and resting cells.

With each set of samples, 2 types of polystyrene-bead standards were run to standardize FACS fluorescence response measurements over the intervention study period. Fluorescence was standardized with either the LinearFlow Green Flow Cytometry Intensity Calibration Kit (Molecular Probes) for the DCFH-DA method or the LinearFlow Orange Flow Cytometry Intensity Calibration Kit (Molecular Probes) for the DHR method.

Statistical analysis
The results are presented as means ± SEMs. Data from in vitro studies were analyzed by one-factor repeated-measures analysis of variance (RM-ANOVA); when a significant difference was found, a Tukey post hoc test was performed for comparisons between subgroups and controls. In human studies, data were analyzed by a multifactor RM-ANOVA; the factors were RGJ treatment, vitamin E treatment, and time (study periods). When the second-order interaction (RGJ × vitamin E × time) was significant, the effect of each treatment over time was assessed by one-factor RM-ANOVA. When the second-order interaction was not significant, first-order interactions (RGJ × time and vitamin E × time) were then analyzed; in the case that one of them was significant and the other was not, the main effect of time was assessed by using one-factor RM-ANOVA, in which all of the patients with the corresponding supplement were considered as a whole group. When no interaction was significant, all the patients were considered as a whole group. In every case, post hoc multiple comparisons were performed by using the Tukey test. Differences between groups at baseline were analyzed by one-factor ANOVA and post hoc Tukey test for multiple comparisons. When baseline differences were noted for a specific variable, baseline values were included as a covariate in the factor analyses. Statistical significance was set at P < 0.05. Calculations were performed by using SIGMASTAT statistical software (version 1.00; Jandel Corporation, San Rafael, CA), STATGRAPHICS PLUS software (version 5.0; Statistical Graphics, Rockville, MD), and SPSS software (version 12; SPSS Software, Chicago, IL) programs.

RESULTS
We performed in vitro experiments to compare the effects of RGJ and α-tocopherol on NADPH oxidase activity in neutrophils isolated from healthy volunteers. Preliminary experiments
TABLE 1
Plasma lipids and apolipoproteins in hemodialysis patients receiving a concentrated red grape juice (RGJ) supplement, vitamin E, or both, as compared with control treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Study period</th>
<th>P for interaction&lt;sup&gt;2&lt;/sup&gt;</th>
<th>P for time&lt;sup&gt;5&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>7 d</td>
<td>14 d</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGJ</td>
<td>2.07 ± 0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.50 ± 0.37</td>
<td>1.72 ± 0.18</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.40 ± 0.22</td>
<td>1.40 ± 0.18</td>
<td>1.45 ± 0.32</td>
</tr>
<tr>
<td>RGJ + vitamin E</td>
<td>1.55 ± 0.37</td>
<td>1.51 ± 0.27</td>
<td>1.34 ± 0.17</td>
</tr>
<tr>
<td>Control</td>
<td>1.60 ± 0.24</td>
<td>1.54 ± 0.24</td>
<td>1.72 ± 0.34</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGJ</td>
<td>4.34 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.36 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.01 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>4.74 ± 0.34</td>
<td>4.62 ± 0.33</td>
<td>4.64 ± 0.36</td>
</tr>
<tr>
<td>RGJ + vitamin E</td>
<td>4.69 ± 0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.29 ± 0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.07 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>3.96 ± 0.34</td>
<td>3.82 ± 0.39</td>
<td>4.10 ± 0.35</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RGJ</td>
<td>0.95 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.73 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.70 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.84 ± 0.04</td>
<td>0.79 ± 0.04</td>
<td>0.81 ± 0.05</td>
</tr>
<tr>
<td>RGJ + vitamin E</td>
<td>0.84 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.74 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.70 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>0.70 ± 0.08</td>
<td>0.71 ± 0.07</td>
<td>0.73 ± 0.07</td>
</tr>
<tr>
<td>HDL phospholipids (mmol/L)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RGJ</td>
<td>0.70 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.83 ± 0.10</td>
<td>0.86 ± 0.08</td>
<td>0.80 ± 0.12</td>
</tr>
<tr>
<td>RGJ + vitamin E</td>
<td>0.78 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.98 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.99 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>0.74 ± 0.04</td>
<td>0.72 ± 0.05</td>
<td>0.71 ± 0.05</td>
</tr>
<tr>
<td>ApoA-I (g/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGJ</td>
<td>1.11 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.18 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.14 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.10 ± 0.07</td>
<td>1.09 ± 0.06</td>
<td>1.11 ± 0.05</td>
</tr>
<tr>
<td>RGJ + vitamin E</td>
<td>1.09 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.20 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.25 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>1.01 ± 0.05</td>
<td>0.96 ± 0.06</td>
<td>1.02 ± 0.07</td>
</tr>
<tr>
<td>Oxidized LDL (U/L)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>RGJ</td>
<td>126 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>149 ± 16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100 ± 9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>RGJ + vitamin E</td>
<td>147 ± 17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>84 ± 14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>121 ± 19</td>
<td>125 ± 16</td>
<td>126 ± 19</td>
</tr>
</tbody>
</table>

<sup>1</sup> n = 8/group. Apo, apolipoprotein. Means within a row that do not share a common superscript letter are significantly different, P < 0.05.
<sup>2</sup> Multifactor repeated-measures ANOVA was used to evaluate interactions. E, vitamin E; t, time. NS = P > 0.05.
<sup>3</sup> The effect of time was analyzed by using one-factor repeated-measures ANOVA. When a significant result was obtained, comparisons between the study periods were performed by using the Tukey test.
<sup>4</sup> ± SEM (all such values).
<sup>5</sup> Statistical comparisons among groups at baseline were performed by using one-factor ANOVA. NS = P > 0.05.
confirmed that PMA-stimulated superoxide production in neutrophils was inhibited by diphenylene iodonium (5 μmol/L), a flavoprotein inhibitor, and by apocynin (250 μmol/L), an intracellular inhibitor of NADPH oxidase assembly (Figure 1A). Thus, the activity evaluated in the present study was specific for the neutrophil NADPH oxidase system. Using this assay system, we found that both RGJ and α-tocopherol inhibited PMA-stimulated NADPH oxidase activity in a dose-dependent manner (Figure 1B, C).

The effects of RGJ ingestion on neutrophil NADPH oxidase activity in vivo were studied in patients affected by end-stage renal disease treated with hemodialysis. All 32 participants successfully completed the study. Both RGJ and vitamin E provided as oral supplements were well tolerated, and no side effects were reported. Patients declared that no changes were made to their diet or level of physical activity during the study. No significant changes in the plasma concentrations of glucose, uric acid, bilirubin, albumin, or total proteins were observed as a result of dietary supplementation with RGJ, vitamin E, or both; nor were any such changes observed in the control group (data not shown).

In terms of the lipid profile (Table 1), the plasma concentration of cholesterol but not triacylglycerols decreased significantly during the study period in response to RGJ supplementation, either alone or in combination with vitamin E. This change was paralleled by decreases in the concentrations of both LDL cholesterol and apoB. Furthermore, significant increases in plasma concentrations of HDL cholesterol, HDL phospholipids, and apoA-I were observed in patients receiving RGJ but not vitamin E. Oxidized LDL concentration was significantly decreased during the study period in all groups receiving RGJ or vitamin E (or both) as compared with control treatment (data not shown).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Study period</th>
<th>P for interaction</th>
<th>P for time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>7 d</td>
<td>14 d</td>
</tr>
<tr>
<td>α-Tocopherol (μmol/mmol cholesterol)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGJ</td>
<td>5.88 ± 0.33</td>
<td>6.04 ± 0.39</td>
<td>6.08 ± 0.57</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>5.01 ± 0.50</td>
<td>5.77 ± 0.79</td>
<td>5.95 ± 0.73</td>
</tr>
<tr>
<td>RGJ + vitamin E</td>
<td>5.14 ± 0.65</td>
<td>5.93 ± 0.69</td>
<td>6.93 ± 0.71</td>
</tr>
<tr>
<td>Control</td>
<td>6.69 ± 0.93</td>
<td>6.54 ± 0.82</td>
<td>6.51 ± 1.15</td>
</tr>
</tbody>
</table>

1 n = 8/group. Means within a row that do not share a common superscript letter are significantly different, P < 0.05.
2 Multifactor repeated-measures ANOVA was used to evaluate interactions. E, vitamin E; t, time. NS = P > 0.05.
3 The effect of time was analyzed by using one-factor repeated-measures ANOVA. When a significant result was obtained, comparisons between the study periods were performed by using the Tukey test.
4 ± SEM (all such values).
5 Statistical comparisons among groups at baseline were performed by using one-factor ANOVA. NS = P > 0.05.

DISCUSSION

In the present study, we showed that ingestion of a polyphenol-rich RGJ supplement with a high antioxidant capacity reduces the NADPH oxidase-dependent production of superoxide in circulating neutrophils of hemodialysis patients. These findings, together with the observation of reduced plasma concentrations of oxidized LDL and inflammatory biomarkers, indicate that dietary supplementation with RGJ may be beneficial for the prevention of cardiovascular disease in hemodialysis patients.

Previous in vitro studies showed that polyphenols decrease both NADPH oxidase activity (35, 36) and the expression of p22phox and p67phox, 2 components of the NADPH oxidase system (24). Administration of polyphenols reduced the expression...
of NADPH oxidase subunits in vascular cells from hypertensive rats (25, 26) and prevented increased cardiac expression of gp91phox in a rat model of metabolic syndrome (37). The present work shows that RGJ, as a source of polyphenols, affects NADPH oxidase activity in human subjects. Exposure of human neutrophils to RGJ in vitro resulted in a decrease in superoxide production by NADPH oxidase. Given that RGJ polyphenols are absorbed by the intestine and circulate in plasma (30, 38, 39), we propose that the reduction of NADPH oxidase activity observed in circulating neutrophils as a result of RGJ ingestion is due to absorbed polyphenols.

α-Tocopherol has been shown to inhibit NADPH oxidase activity in different cell systems (40, 41), and the administration of vitamin E in mice lowers p47phox and p67phox protein expression in tissues (42). In hemodialysis patients, coating of the dialyzer membranes with vitamin E has been used to prevent the neutrophil activation otherwise induced by the hemodialysis procedure (9, 10). The results of this study confirm and extend these observations to show that administration of vitamin E at a dose of 800 IU every other day causes a significant reduction in NADPH oxidase–mediated superoxide production in neutrophils, as measured by the DHR method. This effect was further increased by dietary supplementation with RGJ, which suggests that increasing the polyphenol content of the diet may counteract the increase in oxidative stress that is due to neutrophil activation in these patients.

The superoxide anion has been shown to be involved in LDL oxidation, and an increasing amount of data indicate that a higher plasma concentration of oxidized LDL is a risk factor for atherosclerosis (43). Several studies have shown an association between increased concentrations of circulating oxidized LDL, as measured by using different antibodies, and coronary artery disease (44–46), and there is a significant positive correlation between circulating oxidized LDL concentration and the severity of stenosis and clinical manifestations (45, 47–49). Prospective studies in patients with coronary artery disease have yielded conflicting results: some studies show that elevated concentrations of oxidized LDL have predictive value for future cardiac
Table 4

NADPH oxidase activity in neutrophils from hemodialysis patients receiving a concentrated red grape juice (RGJ) supplement, vitamin E, or both, as compared with control treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Study period</th>
<th>P for interaction&lt;sup&gt;2&lt;/sup&gt;</th>
<th>P for time&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>7 d</td>
<td>14 d</td>
</tr>
<tr>
<td>ΔDHR (MIF)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGJ</td>
<td>61.6 ± 8.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.7 ± 9.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.8 ± 6.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>60.0 ± 12.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.4 ± 9.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>39.4 ± 7.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RGJ + vitamin E</td>
<td>57.0 ± 11.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.5 ± 6.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.0 ± 5.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>60.4 ± 9.7</td>
<td>60.5 ± 10.1</td>
<td>58.4 ± 9.2</td>
</tr>
<tr>
<td>P&lt;sup&gt;5&lt;/sup&gt;</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔDCFH-DA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGJ</td>
<td>68.0 ± 5.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.9 ± 3.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.4 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>70.1 ± 6.1</td>
<td>57.5 ± 3.9</td>
<td>58.5 ± 5.8</td>
</tr>
<tr>
<td>RGJ + vitamin E</td>
<td>70.5 ± 6.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.8 ± 4.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.6 ± 3.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>66.7 ± 10.9</td>
<td>64.2 ± 10.9</td>
<td>62.3 ± 10.0</td>
</tr>
</tbody>
</table>

<sup>1</sup> n = 8/group. DHR, dihydrorhodamine 123; MIF, median intensity of fluorescence; DCFH-DA, 2′,7′-dichlorofluorescein diacetate. Means within a row that do not share a common superscript letter are significantly different, P < 0.05.

<sup>2</sup> Multifactor repeated-measures ANOVA was used to evaluate interactions. NS = P > 0.05.

<sup>3</sup> The effect of time was analyzed by using one-factor repeated-measures ANOVA. When a significant result was obtained, comparisons between the study periods were performed by using the Tukey test.

<sup>4</sup> ± SEM (all such values).

<sup>5</sup> Statistical comparisons among groups at baseline were performed by using one-factor ANOVA. NS = P > 0.05.

Events (50–52), whereas others found no such association (53, 54). However, differences in methods and study populations may underlie these seemingly contradictory findings. The strong positive correlation between apoB and plasma concentrations of oxidized LDL found in the general population (43, 50) may reduce the predictive value of oxidized LDL, as measured with monoclonal antibody E46, when lipid-normalized values are introduced in the multivariate model (54). It is noteworthy that the elevated plasma concentrations of oxidized LDL that hemodialysis patients usually have (10, 12–14, 30) cannot be attributed to apoB, because the regression line between apoB and oxidized LDL observed in these patients is well above that in controls (30). Thus, factors other than apoB must explain these differences.

In the present study, treatment with 800 IU vitamin E every other day led to a significant reduction in the plasma concentration of oxidized LDL. This finding is consistent with the results of previous studies in dialysis patients showing a reduction in plasma concentration of oxidized LDL after oral vitamin E supplementation (18) or coating of dialyzer membranes with vitamin E (8, 9, 11). Furthermore, grape polyphenols have been shown to reduce LDL peroxidation in various systems, both in vitro (55) and in vivo (21, 29, 30, 36). In the present study, intake of RGJ was accompanied by a reduction in oxidized LDL that was even greater than that achieved with vitamin E. Because circulating oxidized LDL concentration correlates with atherosclerotic disease in hemodialysis patients (13), dietary supplementation with RGJ may benefit these patients.

The finding that RGJ supplementation has no effect on α-tocopherol plasma concentration suggests that the reduction in oxidized LDL was not mediated by this antioxidant. Other investigators have shown that both catechin and quercetin are incorporated into circulating LDL after consumption of red wine (56) and that the LDL polyphenol content is directly correlated with the resistance of LDL to ex vivo oxidation (57). We previously showed that quercetin from RGJ is absorbed and circulates in plasma (30, 39). Therefore, it may be possible that grape polyphenols directly increase the resistance of LDL particles to oxidation in plasma. However, other mechanisms may also participate in this effect, such as the reduction in NADPH oxidase activity discussed above, given its direct correlation with the decrease in plasma concentrations of oxidized LDL shown here and the importance of this enzyme activity to the oxidative stress affecting hemodialysis patients.

Other findings deserve some special comment. RGJ consumption, either alone or in combination with the vitamin E supplement, decreased circulating LDL particles, a finding that is in agreement with previous results from our laboratory (30) and other laboratories (29). This is likely to be due to stimulation of LDL receptor activity, as observed in cultured cells in response to red grape polyphenols (31, 58); however, other causes, such as an imbalance in nutrient intake produced by the carbohydrate content in RGJ, cannot be ruled out.

MCP-1 has been recognized as an important factor in the progression of atherosclerosis (59). We found that the concentration of MCP-1 was significantly decreased after 2 wk of intervention with a combination of RGJ and vitamin E, whereas treatment with either supplement alone was ineffective. In a previous study, a significant reduction in MCP-1 concentration was observed after RGJ consumption for 3 consecutive weeks (30). Therefore, an intense antioxidant intervention appears to be required for an effective reduction of MCP-1 concentration. Other authors reported an inhibition of the expression of MCP-1 after the administration of red wine in rabbits for several weeks (60) or continued consumption in humans (61). This finding is consistent with the involvement of a redox-sensitive mechanism in the expression of vascular inflammatory gene products, such as MCP-1, in response to diverse proinflammatory stimuli, as proposed previously (62). Given that the serum concentration of MCP-1 is usually elevated in hemodialysis patients (63), the observed effect of combined administration of RGJ and vitamin...
E must be considered to be beneficial in the reduction of cardiovascular risk.

The results of the present study show for the first time that oral supplementation with concentrated RGI decreases NADPH oxidase–dependent superoxide production in patients with end-stage renal disease. By reducing overproduction of ROS, this nutritional intervention may ameliorate the devastating consequences of vascular disease in hemodialysis patients. However, long-term studies to assess clinical events will be required to confirm this hypothesis and to evaluate the effect of RGI supplementation on overall nutrient intake due to the significant proportion of sugars, in addition to polyphenols, that RGI contains.

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The authors’ responsibilities were as follows—MAL and JLT: study design; PC: the measurements of oxidized LDL, vitamins, and antioxidant markers; AD and CSM: participation in the measurements of NADPH oxidase activity; FC: the lipid and apolipoprotein measurements; JLT, MFL, JLM, and JO: patient care; and MAL: study supervision and writing of the manuscript, which was reviewed by all authors. None of the authors had a personal or financial conflict of interest.

REFERENCES


