Red Wine Induced Modulation of Vascular Function: Separating the Role of Polyphenols, Ethanol, and Urates

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Abstract: By using red wine (RW), dealcoholized red wine (DARW), polyphenols-stripped red wine (PSRW), ethanol-water solution (ET), and water (W), the role of wine polyphenols, ethanol, and urate on vascular function was examined in humans (n = 9 per beverage) and on isolated rat aortic rings (n = 9). Healthy males randomly consumed each beverage in a cross-over design. Plasma ethanol, catechin, and urate concentrations were measured before and 30, 60 and 120 minutes after beverage intake. Endothelial function was assessed before and 60 minutes after beverage consumption by normalized flow-mediated dilation (FMD). RW and DARW induced similar vasodilatation in the isolated vessels whereas PSRW, ET, and W did not. All ethanol-containing beverages induced similar basal vasodilatation of brachial artery. Only intake of RW resulted in enhancement of endothelial response, despite similar plasma catechin concentration after DARW. The borderline effect of RW on FMD (P = 0.0531) became significant after FMD normalization (P = 0.0043) that neutralized blunting effect of ethanol-induced basal vasodilatation. Effects of PSRW and ET did not differ although plasma urate increased after PSRW and not after ET, indicating lack of urate influence on endothelial response. Acute vascular effects of RW, mediated by polyphenols, cannot be predicted by plasma catechin concentration only.

Key Words: red wine, endothelium, polyphenols, ethanol, vasodilatation, urates

Wine is an important component in Mediterranean dietary traditions and it is claimed to contribute to the “French paradox.” This beneficial effect has been attributed mainly to the red wine (RW) polyphenols (like catechin and quercetin), as their intake is also inversely associated with the incidence of many diseases, including coronary heart disease. In vitro studies revealed several mechanisms of vascular function that are modulated by RW and its polyphenols: vasodilatation of isolated vessels through increased production of nitric oxide (NO); enhancement of NO bioactivity by antioxidant action, elevation of endothelial NO synthase protein expression, and reduction of endothelin-1 synthesis. As endothelial dysfunction has been proposed to play a significant role in the initiation of vascular diseases, including coronary heart disease, these effects could partially explain the protective mechanisms related to RW consumption. However, the role of wine polyphenols in endothelium modulation has not been confirmed by using polyphenols-stripped RW (PSRW) as a negative control.

Studies on humans showed conflicting results regarding acute effects of RW and its derivatives on endothelial function in healthy subjects. Acute consumption of dealcoholized red wine (DARW) was shown to increase endothelium-derived vasodilatation, assessed by flow-mediated dilation (FMD) method, whereas intake of RW showed no conclusive effects on FMD. It was speculated that the net effect of unchanged RW intake on endothelium function could be blunted owing to ethanol-induced vasodilatation. Ethanol itself, apart from induced vasodilatation, did not influence endothelial function (FMD) in healthy subjects. It was also suggested that concomitant increase in plasma urate levels after RW intake could aggravate the endothelial function, as this urate elevation does not follow consumption of DARW.

To distinguish the role of polyphenols and urate on vascular endothelial functions more clearly, we introduced PSRW group as a negative control for polyphenols-mediated effect which at the same time caused elevation in plasma urate levels. The endothelial function in humans after acute intake of RW and its derivatives was observed by using FMD method. According to recent guidelines for FMD, we normalized it to the shear rate, a particular variable which depends on vessel diameter. In such a way, the endothelium function could be determined independently from any confounding changes in vascular diameter, like the one induced by ethanol. We also measured plasma catechin...
concentrations after beverages intake, and assessed its reliability as an indicator of wine related effects on vascular function.

**SUBJECT AND METHODS**

**Preparation of Beverages**

RW (Dingac 2002, 14% vol/vol ethanol, Croatia) was dealkoholized in a rotary evaporator (Laborota 4000, Heidolph Instruments Inc, Schwabach, Germany) at 30°C for 1 hour. Vacuum was applied progressively and gradually up to a reading of −130 kPa. PSRW was prepared by passage through a column of polyvinylpolypyrrolidone, as previously described by Caccetta et al. Polyvinylpolypyrrolidone (360 g) was used to remove flavonoids from 2.7 L RW. A 14% vol/vol ethanol solution was prepared by diluting 70% vol/vol ethanol solution with tap water.

**Total Phenolic Compound Concentration**

The total phenolic compound concentration in tested beverages was measured by the Folin-Ciocalteau method, as previously described. All measurements were carried out in triplicate. Results are expressed as milligram gallic acid equivalents per liter.

**Ethanol Content**

Ethanol content in tested beverages was determined in a routine analysis performed in the enological-analytical laboratory of local wine-producer “Dalmacija-vino,” Split, Croatia. All measurements were carried out in triplicate. Results are expressed as percentages (vol/vol).

**Animal Study**

The study was approved by the Ethical Committee of University of Split School of Medicine. Twenty male Wistar rats, 3-month old and 330 ± 20 g of body weight, were used for this study. Animals received intraperitoneal injection of urethane (1.2 g/kg). After becoming unresponsive to noxious stimulation, they were decapitated. The descending thoracic aorta was dissected free from connective tissue and placed in modified Krebs-Henseleit solution. The aorta was carefully cleaned of adhering fat and cut into rings (3 to 4 mm in length). Four rings from each animal were prepared as described previously. After wash-out and stabilization in modified Krebs-Henseleit solution, each ring was precontracted with test dose of norepinephrine (NE 10\(^{-7}\) M). When the contraction reached the plateau phase, endothelium-dependent relaxation was induced by acetylcholine (10\(^{-6}\) M). The functionality of endothelium was confirmed if 10\(^{-6}\) M acetylcholine induced more than 70% relaxation of precontracted rings. The rings that relaxed less than 70% were excluded from the study. After triple wash-out and tension stabilization, the aortic rings were again precontracted with NE (10\(^{-7}\) M). After the stable plateau was reached, the rings were randomly exposed to cumulative concentrations (1:10 000 to 1:100 final dilution in organ baths) of one of the tested beverages [(1) RW, (2) the same red wine but dealcoholized, DARW, (3) the same red wine but with the polyphenols removed, PSRW, (4) 14% vol/vol ethanol-water solution (ET), and (5) water (W)]. The vasodialatory effect was expressed as the percentage decrease in NE induced vasconstriction. Because the tested beverages differed significantly in total phenolic concentration and ethanol content, we used logarithm of dilution, instead of concentration, to calculate pD\(_2\) (−log EC\(_{50}\)), which is in accordance with previous report by Fitzpatrick et al (eg, 1:1 000 dilution = 0.001 = log of −3). pD\(_2\) was calculated using nonlinear regression analysis (GraphPad Prism, version 4.03 for Windows, GraphPad Software, San Diego, CA).

**Human Study Design**

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Committee of University of Split School of Medicine, and all subjects gave a written consent before their participation in the study. Nine healthy male volunteers aged 25 to 40 years (mean BMI 25 ± 4 kg/m\(^2\)) who were normolipidemic, nonsmokers, and taking no medications were recruited. Subjects were asked to abstain from exercise, fruits, vegetables, dietary supplements, tea, alcoholic beverages, and caffeine-containing or theobromine-containing foods for 24 hours before each visit.

After an overnight fast, the subjects randomly consumed 1 of 5 tested beverages (3 mL/kg body wt) in a cross-over design. Each beverage was consumed only once by each subject and the period before drinking next beverage was 1 week. The following beverages were consumed: (1) RW, (2) DARW, (3) PSRW, (4) 14% vol/vol ET, and (5) W. Endothelial function was assessed immediately before and 60 minutes after beverage consumption. Heart rate (HR) and mean arterial blood pressure were measured before, 30 and 60 minutes after beverage consumption by Polar HR monitor (Polar Vantage, Finland) and Tango BP monitor (Sun-Tech Medical Instruments, Raleigh, NC). Cubital vein blood samples were collected into heparin vacutainers immediately before (control) and 30, 60, and 120 minutes after beverage consumption. After the centrifugation, plasma was immediately analyzed or stored at −80°C. The subjects remained in the supine position and abstained from any food or beverages during the 2-hour study.

**Endothelial Function**

Endothelial function was measured according to the method of Raitakari and Celermajer. This method determines the arterial response to reactive hyperemia, FMD. The subjects were placed in a quiet room with temperature about 20°C and were resting for 15 minutes on the bench in a supine position before measurement. Participants were tested at the same time of day (10 AM) and were always at least 12-hour postprandial. Measurements were performed with 5.7 to 13.3 MHz linear transducer using a Vivid 3 Expert ultrasonic scanner (GE, Milwaukee). Brachial artery diameter was measured.
from longitudinal images with lumen-intima interface visualized on both (anterior and posterior) walls. Images were acquired using electrocardiogram gating during acquisition, using the onset of R wave to identify end diastole. When the images were chosen for analysis, the boundaries for diameter measurement were identified manually with an electronic caliper. Together with imaging the brachial artery, mean blood velocity (MBV) was obtained using the duplex function of the linear array vascular probe. Pulsed Doppler measurements for measuring blood flow velocity were performed with the sample volume placed in mid-artery. The position of the transducer was marked at 3 to 5 cm proximal to the antecubital fossa to ensure the same position of the transducer for all measurements. Once the control basal measurements were obtained, arterial occlusion was created by inflating a cuff placed on the forearm to 240 mm Hg for 5 minutes. After 5-minute inflation, the cuff was deflated producing a brief high-flow state resulting in artery dilatation (reactive hyperemia). Flow and diameter of brachial artery were measured at time of cuff deflation, and at points of every 30 seconds for first 3 minutes, and at fourth and fifth minute. FMD was calculated as the percent of increase in the brachial artery diameter from the resting state to maximal dilatation, FMD = (diameter during reactive hyperemia – diameter during basal measurements)/diameter during basal measurements. Blood flow was calculated from the mean velocity measurements and the vessel diameter, assuming that the vessel was circular. All raw data were saved on ultrasound hard disk as still and cine-loop images for later reviewing lasting between 5 to 7 seconds. The accuracy of the method was assessed in our previous study.

Blood velocity measurements were acquired 5 to 7 seconds after cuff release. Measurements of peak beat and 5-second average flow were calculated (vessel cross-sectional area \times MBV) and used to quantify the hyperemic response. Also, the peak beat and 5-second average shear rate were determined as (4 \times MBV)/mean diameter.

**Normalized FMD**

Measurements of FMD were normalized to average shear rate, because the extent of dilation has been shown to depend on the hyperemic flow stimulus as represented by shear rate (normalized FMD = FMD/average shear rate). Values are expressed as %/s\(^{-1}\).

**Plasma Catechin Concentration**

Plasma catechin concentration was measured by HPLC (Series 200, PerkinElmer Inc, Shelton) coupled with fluorescence detector, as described by Carando et al. A LiChrosphere C\(18\), 4 \times 250 mm, 5\(\mu\)m particle size analytical column was used as the stationary phase. The mobile phase was heated to 40°C, delivered at 0.5 mL/min and consisted of 2 solvents: solvent A = 50 mM ammonium dihydrogen phosphate (NH\(_4\)H\(_2\)PO\(_4\)) at pH 2.60, solvent B = 20% solvent A in acetonitrile. Briefly, 500\(\mu\)L of plasma was mixed with 25\(\mu\)L ascorbic acid-ethylenediaminetetraacetic acid solution (20% ascorbic acid, 0.1% ethylenediaminetetraacetic acid), 25\(\mu\)L of \(\beta\)-glucoronidase and sulphatase (Sigma G 7017), and 250\(\mu\)L of 0.6 M CaCl\(_2\), incubated at 37°C for 45 minutes, to release conjugated forms of catechin. After incubation, the sample was treated with 700\(\mu\)L of acetonitrile, and centrifuged at 5000g for 5 minutes, to precipitate proteins. The supernatant was filtered through 0.45\(\mu\)m pore size membrane and injected into high performance liquid chromatography system. Catechin was determined by fluorescence detection at an excitation wavelength of 280 nm and an emission wavelength of 310 nm. All measurements were carried out in triplicate. Results are expressed as milligrams of catechin per liter.

**Other Plasma Measurements**

Plasma urate concentration was determined by enzymatic laboratory kit (Olympus, OSR6236) with uricase as the enzyme. Plasma ethanol concentration was measured by enzymatic laboratory kit (Behring Diagnostics, DF18) with alcohol dehydrogenase as the enzyme.

**Statistics**

Data are expressed as mean ± SEM. Student paired \(t\) test, 1-way, and 2-way analysis of variance were used for statistical analysis. Tukey test was applied for post hoc analysis when statistical significance (\(P < 0.05\)) was reached by analysis of variance.

**RESULTS**

**Chemical Analysis of Tested Beverages**

Values of total phenolic compound concentration and ethanol content in tested beverages are shown in Table 1. Procedure of RW dealcoholization was efficient in elimination of ethanol without changing concentration.

<table>
<thead>
<tr>
<th>TABLE 1. Phenolic and Ethanol Content of Tested Beverages</th>
</tr>
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<tbody>
<tr>
<td><strong>RW</strong></td>
</tr>
<tr>
<td>Total phenolic concentration (mg GAE/L)</td>
</tr>
<tr>
<td>Ethanol content (% vol/vol)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. \(* P < 0.001\) versus RW group. \(† P < 0.05\) versus RW group.

GAE indicates gallic acid equivalent; ND, could not be determined.

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of polyphenols. However, procedure of polyphenols-stripping substantially reduced concentration of phenolic compounds in RW with only slightly reducing ethanol content.

### Vascular Effects of Beverages on Rat Aortic Rings

Dose response curves of beverages-induced relaxation (n = 9 per beverage) are shown in Figure 1. Only polyphenols-containing beverages, that is RW and DARW, induced considerable vasodilatation, without significant difference between them. Maximum relaxation values were 82.54 ± 3.87%, 88.90 ± 2.83%, 10.33 ± 4.78%, and 10.73 ± 5.50% (P < 0.0001), for RW, DARW, PSRW, and ET, respectively. pD2 values for RW, DARW, PSRW, and ethanol were 3.22 ± 0.06, 3.25 ± 0.04, 3.04 ± 0.33, and 3.29 ± 0.44, respectively (P > 0.05).

### Cardiovascular Effects of Beverages Consumption in Human Subjects

Before intake there was no significant difference between groups for HR, mean arterial pressure, brachial artery diameter, and FMD. Table 2 shows initial control values and related changes in brachial artery diameter and FMD values after beverages consumption. All beverages induced no change in HR and arterial pressure after consumption (data not shown). However, all ethanol-containing beverages induced an increase in vessel diameter after consumption in contrast to DARW and W, which caused no change. Although wine showed the most pronounced tendency in increasing FMD response (P = 0.0531), no beverages caused a significant change in FMD value. However, after normalization of FMD to mean shear rate, it was revealed that RW induced a significant increase in vasodilatory response (P = 0.0043). Other beverages had no significant effect.

### Biochemical Parameters in Plasma After Beverages Consumption

Initial control values of plasma catechin, ethanol, and urate, and their relative change after beverages consumption, are shown in Table 3. Before intake there was no significant difference between groups for any parameter. After ingestion there was a substantial and beverage-dependent change in plasma values for all 3 parameters. RW and DARW consumption resulted in a similar increase in catechin plasma concentration, whereas PSRW, AL, and W intake expectedly had no such effect. DARW and W consumption did not induce increase in ethanol values, whereas RW, PSRW, and ET intake resulted in nearly comparable increase in ethanol plasma concentration. ET and W consumption did not induce increase in plasma urate values, DARW intake slightly reduced it, and RW and PSRW consumption resulted in nearly equal increase in plasma urate concentration.

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**TABLE 2. Vascular Effects of Beverages Consumption on Human Subjects**

<table>
<thead>
<tr>
<th></th>
<th>RW</th>
<th>DARW</th>
<th>PSRW</th>
<th>Ethanol</th>
<th>W</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>3.956 ± 0.143</td>
<td>3.933 ± 0.100</td>
<td>4.033 ± 0.165</td>
<td>3.989 ± 0.184</td>
</tr>
<tr>
<td>60 min</td>
<td>4.333 ± 0.167</td>
<td>3.956 ± 0.109</td>
<td>4.389 ± 0.187</td>
<td>4.233 ± 0.162</td>
<td>4.111 ± 0.181</td>
</tr>
<tr>
<td></td>
<td>0.0080</td>
<td>0.3466</td>
<td>0.0012</td>
<td>0.0009</td>
<td>0.7287</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>7.110 ± 0.607</td>
<td>8.802 ± 0.895</td>
<td>7.332 ± 1.393</td>
<td>6.458 ± 0.620</td>
</tr>
<tr>
<td>60 min</td>
<td>8.832 ± 0.635</td>
<td>7.466 ± 0.688</td>
<td>5.573 ± 1.055</td>
<td>4.913 ± 0.935</td>
<td>7.477 ± 0.548</td>
</tr>
<tr>
<td></td>
<td>0.0531</td>
<td>0.1460</td>
<td>0.1102</td>
<td>0.1975</td>
<td>0.2146</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.06256 ± 0.00593</td>
<td>0.08878 ± 0.01148</td>
<td>0.0643 ± 0.01117</td>
<td>0.06289 ± 0.00650</td>
</tr>
<tr>
<td>60 min</td>
<td>0.09889 ± 0.00755</td>
<td>0.07533 ± 0.01015</td>
<td>0.0643 ± 0.01117</td>
<td>0.05833 ± 0.01218</td>
<td>0.06800 ± 0.00348</td>
</tr>
<tr>
<td></td>
<td>0.0043</td>
<td>0.2013</td>
<td>0.3664</td>
<td>0.7542</td>
<td>0.3463</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. Initial control values before beverages consumption and changes in brachial artery diameter, FMD, and “normalized” FMD, 60 min after beverage consumption, n = 9 subjects per group.
DISCUSSION

We investigated the effects of RW and its derivatives on endothelial function, both in human subjects and isolated rat vessels, to distinguish the role of wine polyphenols and ethanol. We also evaluated the modulating potential of urates on human vascular response, as an acute elevation of plasma urate is a consequence of RW intake.30

In the study on isolated vessels, we did not find significant difference in vasodilatation induced by unchanged and DARW. Ethanol itself did not induce significant vascular effect when applied in vitro. However, the elimination of polyphenolic compounds from RW resulted in lack of the observed vasodilatation, confirming the essential role of polyphenols as mediators for this vascular effect. This finding supports results from previous studies on isolated vessels where endothelium-dependent vasodilatation induced by RW or polyphenols from RW was reported and well characterized5–8 and contrasts the results of the study where RW showed no effects on the isolated rabbit coronary vessels.30 Use of KCl in the study on isolated rabbit coronary vessels, instead of NE or phenylephrine, could be a possible explanation for such difference. It was recently shown by de Moura et al8 that RW-induced vasodilatation was significantly reduced after addition of KCl and potassium-induced depolarization, in comparison to receptor-mediated vasoconstriction, as induced by NE.

In the human subjects, all ethanol-containing beverages induced similar vasodilatation of brachial artery. This in vivo effect of ethanol differs from its in vitro ineffectiveness on isolated vessels. We assume that this vasodilatation is not mediated by direct effect of ethanol on endothelium and vascular wall and is rather a consequence of modulation of central vasomotor control mechanism, as suggested previously by Malpas et al.31 However, few studies reported ethanol induced relaxation of pulmonary artery by release of NO and prostaglandin,32 and increase of NO production in endothelial cells.33 Therefore, possibility that ethanol-induced vasodilatation observed in vivo is mediated by increased endothelial production of NO can not be eliminated and should be further investigated.

Ingestion of RW resulted in enhancement of endothelial response and it was clearly visible only if normalized FMD was analyzed. Normalization of FMD to mean share rate neutralizes changes in basal vessel diameter, induced by ethanol, which apparently interfere with measurement of net endothelial response, estimated by FMD. In the present study, the borderline effect of RW on FMD (P = 0.0531) became clearly significant after FMD normalization (P = 0.0043).

Effect of RW on human endothelial response was abolished after polyphenols removal, which confirms their essential role in endothelial modulation, and this is in agreement to the findings on the isolated vessels. Interestingly, DARW in our study did not induce increase in FMD values, which is in contrast with previous studies of Agewall et al14 and Hashimoto et al,15 who reported that DARW intake resulted in the increase of FMD values.

Presently, we are unable to give an exact explanation for this discrepancy but we can postulate that there is a possibility that a process of wine dealcoholization affects differently the physical and chemical properties of polyphenolic compounds in different wines and wines of different ages (Cabernet Sauvignon14 and 7-year-old Chateau Beychevelle15 vs. 3-year-old Dingac that we
have previously analyzed\textsuperscript{21} and used in this study). Namely, the size and the shape of wine high molecular weight polyphenols (tannins) depend on their chemical composition and it changes as the wine ages. These characteristics of wine polyphenols and their solubility are highly sensitive to the changes of the medium in which they are contained.\textsuperscript{34} Furthermore, Serafini et al\textsuperscript{35} showed that ethanol reduced interaction between protein and tannins, which may interfere with their absorption and biologic effectiveness.

In our pilot study, we found that relatively small remains of ethanol in DARW may significantly interfere with its effects on FMD. Intake of DARW into which 3\% (vol/vol) ethanol was added resulted in increase of FMD, detectable even without FMD normalization (n = 2). This would implicate that some presence of ethanol in the beverage is essential for the absorption of the vasoactive polyphenols, at least for the wine that we used in the present study. This notion is in agreement with the report by Matsuo et al,\textsuperscript{36} who showed an increase of plasma NO production in human subjects after acute intake of RW, but not after consumption of polyphenolic beverage without ethanol.

Removal of ethanol, however, did not influence catechin plasma concentration after DARW intake in comparison to unchanged RW, which is in agreement with previous studies by Donovan et al\textsuperscript{37} and by Bell et al.\textsuperscript{38} This implies that catechin is not a reliable marker for the absorption of polyphenols responsible for endothelial modulation. Even more, in vitro application of catechin failed to induce vasodilatation in rat aorta.\textsuperscript{39} Maximal induction of endothelial NO synthase in human endothelial cells was achieved by synergistic effect of blend of different wine polyphenols.\textsuperscript{39} It is plausible that similar synergism also occurs on endothelium after wine consumption in vivo. Therefore, measuring levels of only one polyphenolic compound in plasma could be misleading.

Interestingly, vascular effect of consumption of PSRW did not differ from the one after ethanol intake, although plasma urate increased significantly after PSRW and not after ethanol. This would suggest that urates do not affect vascular and endothelium function significantly, as it was previously hypothesized.\textsuperscript{17} Although an acute increase in plasma urate after wine intake could be a potential risk for gout, recent epidemiologic studies have shown that consumption of RW is not associated with higher incidence of gout, in contrast to consumption of red meat and beer.\textsuperscript{46}

In conclusion, the present study confirmed the pivotal role of polyphenols in RW-mediated acute vascular effects in both human subjects and isolated vessels. However, some presence of ethanol compound seems to be required for the full extent of polyphenols-induced effects in vivo. Isolated measurements of plasma catechin concentration are not reliable predictor of biologic effects of polyphenolic beverages on vasculature. It seems that an acute elevation of plasma urate does not significantly interfere with vascular endothelial function in vivo. Studies focusing on the assessment of endothelial modulation in human subjects should use FMD normalized to mean shear rate to neutralize factors affecting basal vessels diameter.

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