Dealcoholized red and white wines decrease oxidative stress associated with inflammation in rats

D. López1, M. Pavelkova2, L. Gallova2, P. Simonetti3, C. Gardana3, A. Lojek2, R. Loaiza4 and M. T. Mitjavila1,*

1Department of Physiology, Faculty of Biology, University of Barcelona, Av. Diagonal 645, E-08028 Barcelona, Spain
2Institute of Biophysics, Academy of Sciences, Královsqé 135, 612 65 Brno, Czech Republic
3Department of Food Science and Microbiology, University of Milano, Via Celoria 2, 20133 Milan, Italy
4School of Pharmacy, University of Costa Rica, San Pedro de Montes de Oca, San José, Costa Rica

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In vitro experiments have demonstrated that polyphenols exhibit antioxidant and anti-inflammatory activities. The present study was designed to test whether dealcoholized red (DRW) and white (DWW) wines can decrease the oxidative stress associated with inflammation in vivo. Rats were fed for 15 d either a control diet or one supplemented with DRW or DWW. Finally, a granuloma was induced by subcutaneous administration of carrageenan. Although DRW showed higher antioxidant activity in vitro than DWW, both wines decreased the number of cells recruited into the granuloma pouch. Malondialdehyde decreased in plasma and inflammatory exudate from rats fed with DRW- and DWW-rich diets. Moreover, the concentration of NO increased in exudate, which correlates with the increase in the citrulline:arginine ratio. Polymorphonuclear leukocytes from the inflammatory exudate of rats fed dealcoholized wines showed decreased superoxide anion (O2⁻) production and increased NO production ex vivo. This change in NO production resulted from increased expression and activity of inducible NO synthase (EC 1.14.13.39). Moreover, the up regulation of cyclo-oxygenase-2 (EC 1.14.99.1) protein expression observed in rats fed the DRW-rich diet was not related to a direct effect of NO. The present results indicate that the non-alcoholic compounds of wines not only improve antioxidant status in an inflammatory situation, but also limit cell infiltration, possibly through a decrease in O2⁻ and an increase in NO production.

Free radicals: Nitric oxide synthase: Polymorphonuclear leucocytes: Polyphenols

Most of the beneficial effects associated with the moderate consumption of red wine are related to polyphenols, although wines contain a broad range of non-polyphenolic compounds. In vitro studies have shown that polyphenols exhibit antioxidant and anti-inflammatory activities. The anti-inflammatory activities are a consequence of the inhibition of phospholipase A₂ (EC 3.1.1.4), cyclo-oxygenase (COX)-2 and 5-lipoxygenase (EC 1.13.11.34). However, evidence from the in vitro experiments cannot be extrapolated to in vivo settings, since polyphenols are extensively metabolized in vivo, which can affect their activities.

The inflammatory response is characterized by the production of chemical mediators, and the presence of large amounts of exudate, proteins and cells in the inflamed area. Neutrophils are the first blood cells that migrate towards inflammatory lesions and they have the capacity to express NADPH oxidase, inducible NO synthase (iNOS) and COX-2. We have observed, in a carrageenan-induced granuloma model of inflammation, that polymorphonuclear (PMN) leucocytes generate superoxide anion (O2⁻) and NO11 and it has been described that O2⁻, by reacting with NO, generates peroxynitrite12,13. Thus, preventing the generation of O2⁻ by inhibiting the enzymes responsible or scavenging O2⁻ or avoiding peroxynitrite formation may attenuate inflammation.

It is likely that low levels of flavonoids and their metabolites exert biological effects such as altered cell signalling and gene expression that will contribute to the modulation of oxidative stress and inflammation. These activities have been more extensively studied in vitro than in vivo6,14. However, wine also contains alcohol that has been reported to enhance lipid peroxidation15 and can thus mask the effects of the non-alcoholic compounds. Our purpose was to explore the effects of dealcoholized red (DRW) and white (DWW) wines on rats with carrageenan-induced granulomas, because of the wines’ absence of alcohol and also their differing polyphenol content. This model allowed us to examine the effects of both dealcoholized wines on oxidative stress and whether such effects would be beneficial in situations of inflammation.

Abbreviations: COX, cyclo-oxygenase; DRW, dealcoholized red wine; DWW, dealcoholized white wine; iNOS, inducible NO synthase; O2⁻, superoxide anion; ORAC, oxygen radical absorbance capacity; PMN, polymorphonuclear.

* Corresponding author: Dr M. T. Mitjavila, fax +34 93 4110358, email mmitjavila@ub.edu
Materials and methods

Animals and diets

Male Sprague–Dawley rats weighing approximately 125 g were used (Harlan Interfauna Ibérica, Barcelona, Spain). They were housed in temperature-controlled rooms (21–23°C), with 40–60 % humidity, and exposed to a 12 h light–dark cycle.

Rats were randomly divided into three groups of seven rats and fed one of the following semi-purified diets for 15 d: (1) a control diet; (2) a 35 % (v/w) DRW diet; or (3) a 35 % (v/w) DWW diet (Table 1). Chow was prepared weekly and stored at −20°C under vacuum to prevent oxidation and loss of antioxidants. Fresh food and water were provided in excess every day, and food consumption was recorded for every cage. All experimental protocols were reviewed and approved by the Ethical Committee of the University of Barcelona, in accordance with European Community guidelines.

Dealcoholized wines were prepared from commercial wines available in Spain. Alcohol was removed using a rotary evaporator at a maximum temperature of 30°C. Vacuum was applied progressively to avoid mechanical stress. Evaporated ethanol was replaced by acidulated distilled water and the pH was adjusted to that of the original wine. GC was used to identify any traces of ethanol in dealcoholized wines. We have also evaluated the ascorbic acid and fructose content in wines by fluorimetric and colorimetric techniques (Boehringer Mannheim, Darmstadt, Germany), respectively.

Liquid chromatography–mass spectrometry analysis, total phenol content and antioxidant activity of wines

Wines were diluted in methanol–formic acid 1 % (90:10, v/v) and the resulting solutions were sonicated for 10 min and centrifuged at 1000 g for 10 min. One sample of the supernatant fraction was filtered through a Millipore 0·2 μm disk and 20 μl was used for analysis.

The chromatographic system for liquid chromatography–MS analysis of wines included an Alliance model 2695 (Waters, Milford, MA, USA) coupled with a diode array detector (model 2996; Waters) and a triple-quadrupole mass spectrometer (model Quattro micro; Micromass, Beverly, MA, USA). The analytical column was a 3.5 μm C18 Symmetry 150 × 2.1 mm (Waters). The column was maintained at 30°C. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both with 0.1 % formic acid. The flow rate was set at 200 μl/min. The separation was carried out with a linear solvent gradient program starting at 0 % B and ramping to 20 % B in 30 min and to 60 % in 10 min. The mass spectrometer was equipped with an electrospray source operating both in the positive (anthocyanin analysis) and negative (polyphenols) ion mode. The capillary voltage was set to 3.2 kV, the sampling cone voltage to 40 V, and the source temperature to 130°C. The analysis was carried out in full scan mode in the range 100–1000 atomic mass units, while UV-Vis chromatograms were acquired in the range 200–600 nm. All data were acquired by Masslink 4.0 software (Micromass).

Anthocyanin calibration curves were obtained from stock solutions prepared by dissolving 5 mg standard powder (Extra-synthese, Genay, France) in 5 ml methanol–formic acid 10 % (90:10, v/v). Gallic, p-coumaric, ferulic and caffeic acid, catechin, epicatechin, trans-resveratrol (Sigma, St Louis, MO, USA), myricetin, quercetin (Extra-synthese) and stock solutions were prepared by dissolving 20 mg standard powder in 20 ml methanol. The working solutions were prepared in the range 1–50 μg/ml.

Total phenols in dealcoholized wines were measured following the Folin–Ciocalteau colorimetric method, and utilizing gallic acid as a standard.

The antioxidant potential of dealcoholized wines was determined by using four methods. The reducing power was determined in 150 μl according to the method described by Oyaizu, using quercetin as a standard. The oxygen radical absorbance capacity (ORAC) assay was used to elucidate the antioxidant behaviour of dealcoholized wines (dilutions 1:25, v/v) against the peroxyl radicals by using R-phycoerythrin as a redox-sensitive fluorescent indicator and 2,2′-azobis-(2-amidinopropane) dihydrochloride as a chemical peroxyl radical generator. R-phycoerythrin fluorescence decline was measured at 37°C as the difference between 5 and 30 min (excitation wavelength 530 nm, emission wavelength 585 nm). The O2−-scavenging activity was quantified in serial dilution of wines using the hypoxanthine–xanthine oxidase (EC 1.17.3.2) system, whereby the reduction rate of nitroblue tetrazolium to a dark-blue formazan is measured at 540 nm every 1 min for a 20 min period. Maximal velocity was computed to determine the necessary concentration for inducing a 50 % scavenging of the O2− produced. NO scavenging was determined according to the method described by Vriesman et al. with some modifications. Briefly, NO was chemically generated by dilution of 50 μl 50 mm-NaNO2 (giving 250 nmol NO/l) in 10 ml 0.1 m-H2SO4 and 0·1 m-KI (pH 1·0). After 2 min, DRW and DWW were added to a final concentration of 1:100 to 1:1000, with Hb used as the standard. During measurement, the test vial was kept under a stream of N2. NO concentration was monitored using an Iso-NOP NO sensor (World Precision Instruments, Sarasota, FL, USA). The natural logarithm of the electrode values was plotted vs. time and the (pseudo) first-order reaction constant for scavenging was calculated.

### Table 1. Composition of semi-purified diets (g/kg)*

<table>
<thead>
<tr>
<th>Component</th>
<th>Control</th>
<th>DRW</th>
<th>DWW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>225</td>
<td>225</td>
<td>225</td>
</tr>
<tr>
<td>Potato starch</td>
<td>446</td>
<td>446</td>
<td>446</td>
</tr>
<tr>
<td>Sucrose</td>
<td>223</td>
<td>223</td>
<td>223</td>
</tr>
<tr>
<td>Cellulose</td>
<td>31</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mineral mix†</td>
<td>14</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Vitamin mix‡</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Maize oil</td>
<td>50</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>Water</td>
<td>350</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wine</td>
<td>0</td>
<td>350</td>
<td>350</td>
</tr>
</tbody>
</table>

DRW, dealcoholized red wine; DWW, dealcoholized white wine.

* Pellets were dried at room temperature for 12 h.
† AIN-93MX ICN Pharmaceuticals, Costa Mesa, CA, USA.
‡ AIN-93VX ICN Pharmaceuticals.
Induction of inflammation and evaluation of inflammatory parameters

After day 15 of the experimental feeding period, a granuloma was induced by subcutaneous administration of 6 ml air, followed 24 h later by 4 ml carrageenan (Hispanoland, Barcelona, Spain) 2 % (w/v) in sterile saline, into the dorsum of rats as previously described24. Rats were anaesthetized with isoflurane (Abbott Laboratories, Madrid, Spain) 24 h after carrageenan injection. Blood, extracted by heart puncture, and the inflammatory ptery chyons were harvested following overnight fast using heparin-treated syringes. The pouch cavity was then rinsed with 3 ml saline to recover all cells and the granulomatous tissue extracted and weighed. Blood and exudates were centrifuged at 800 g for 15 min. Supernatant fractions (50 ml) by 20 % TCA (1:1) at 4 °C and centrifuged at 800 g for 15 min. Supernatant fractions (50 ml) were incubated in 5 ml of a solution (10 mM-KI, 10 mM-H2SO4) for 10 min. The absorbance of the upper phase was measured at 540 nm. The resulting dried residues were re-suspended for PGE2 immunoassay analysis (Cayman Chemicals, Ann Arbor, MI, USA).

Parameters related to oxidative stress in plasma and supernatant fraction of the exudates

We measured the total phenol and the antioxidant potential, both in plasma and exudate supernatant fractions. For antioxidant potential, we used three methods described elsewhere.

Malondialdehyde was assayed as an endproduct of lipid peroxidation. Malondialdehyde equivalents were measured in 20 ml samples using the thiobarbituric acid-reactive substances method, with tetraethoxypropane as a standard25. Thiobarbituric acid-reactive substances were extracted with butanol. The tubes were centrifuged at 450 g for 10 min and the absorbance of the upper phase was measured at 540 nm.

NO released in plasma or in exudate supernatant fractions is oxidized to nitrates. NO was thus measured in deproteinized samples (100 ml) by 20 % TCA (1:1) at 4 °C and centrifuged at 18 600 g for 15 min. Supernatant fractions (50 ml) were incubated in 5 ml of a solution (10 mM-KI, 10 mM-H2SO4) converting nitrates to NO, which was evaluated by the 2-0 mm NO sensor (ISO-NOP). The maximal signal was recorded and compared with a NaNO2 standard.

The relationship between free L-citrulline v. free L-arginine was also measured in deproteinized and ultramicrofiltrated exudate supernatant fractions. Amino acid analysis was conducted by cation-exchange chromatography coupled to an auto-analysers (Alpha Plus; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) according to the method of Moore et al.26 with post-column derivatisation with ninhydrin.

Lithium citrate buffers were used as eluents. Identification and quantification of amino acids was performed according to the retention time and peak areas, respectively, of standards eluted in the same conditions. Norleucine was used as an internal standard. The detection limit was 1 nmol.

Free radical production by polymorphonuclear leucocytes and indolubile nitric oxide synthase and cyclo-oxygenase-2 assays

Cells isolated from inflammatory exudates were used to measure O2·- and NO production as previously described11. The viability of the cells was above 90 %, as assessed by the Trypan blue exclusion test, and 85–90 % of the cells were PMN cells. Less than 1 h elapsed between harvesting the exudates and the start of incubation. PMN cells (1 × 10⁶ cells per tube) were incubated for 1 h in PBS, at a pH of 7.4 with 2 mM-CaCl2, 0.5 mM-MgCl2, and phorbol 12-myristate 13-acetate (100 μg/l). O2·- generation was assayed by measuring superoxide dismutase (EC 1.15.1.1)-inhibitable reduction of cytochrome c (0.15 mm; horse heart type VI) in the presence of N-iminoethyl-L-ornithine (0.6 mM; Sigma), an NOS inhibitor.

Nitrites plus nitrates were measured as indicators of NO generation by PMN cells, and assays were performed in the presence of L-arginine (0.6 mM) and 150 international units superoxide dismutase. In addition, N-iminoethyl-L-ornithine (0.6 mM) was used as a negative control for each experiment. Incubation medium (60 μl) was treated with 10 μl nitrate reductase (EC 1.7.1.3) (Cayman Chemicals) followed by Griess reagent.

To evaluate iNOS protein expression (Immun-Star Anti-Rabbit Detection kit; Cayman Chemicals), 10⁷ PMN cells were incubated in 500 μl lysis buffer containing 50 mM-tri (hydroxymethyl)-aminomethane-HCl (pH 7.4), 500 μM-Na2EDTA, 500 mM-ethylene-glycol-bis(2-aminoethyl ether)-N,N,N’,N’-tetra-acetic acid, 7 mM-glutathione, 10 % glycerol (v/v), 20 mM-3-[3-cholamidopropyl]dimethylammonio]-2-hydroxy-1-propanesulfonate, 100 mM-dithiothreitol, pepstatin (1 μg/ml), aprotinin (1 μg/ml), leupeptin (1 μg/ml) and 2 mM-phenylmethylsulfonyl fluoride for 30 min at 4 °C. Samples were sonicated and boiled at 95 °C for 3 min. Protein concentration was determined using the Bradford technique. Homogenate samples (20 μg protein per lane) were submitted to gel electrophoresis and blotted onto a nitrocellulose membrane. Purified murine iNOS (Cayman Chemicals) was also loaded (1 μg) as a positive control, and a pre-stained protein standard was used to monitor transfer efficiency. Membranes were then exposed to a 1:1000 ratio of rabbit anti-human iNOS polyclonal antibody (Cayman Chemicals) and a 1:1000 ratio of rabbit anti-chicken polyclonal γ-tubulin (Sigma) as housekeeping for 1 h. The relative densitometric units with regard to murine iNOS were evaluated.

PMN iNOS activity in rats fed the different diets was measured by quantifying the amount of [3H]-citrulline formed from [3H]-L-arginine in 10⁷ cells. [3H]-citrulline content (Cayman Chemicals) was measured by liquid scintillation counting using a Packard Top-Count instrument (Packard Instrument Company, Meriden, CT, USA). To selectively measure iNOS, a Ca2+-free buffer was used with ethylene-glycol-bis(2-aminoethyl ether)-N,N,N’,N’-tetra-acetic acid present in a final concentration of 1 mM. Non-enzymic conversion
was determined by using a heat-inactivated (2 min at 100°C) cell suspension containing $10^7$ cells. As a negative control for each experiment, 1 mM $N^\xi$-nitro-l-arginine methyl ester-HCl (Sigma), an inhibitor of NOS, was added to the incubation medium for 30 min.

To evaluate COX-2 protein expression, $10^7$ PMN cells were incubated in 500 μl of the lysis buffer used for iNOS protein expression for 30 min at 4°C. Samples were treated as for iNOS expression. In addition, purified murine COX-2 was loaded (2 μg) as a positive control and a pre-stained protein standard was used to monitor electrophoresis and transfer efficiency. Membranes were then exposed to a 1:2000 ratio of rabbit anti-murine COX-2 polyclonal antibody (Sigma) and a 1:1000 ratio of rabbit anti-chicken γ-tubulin polyclonal antibody for 1 h and then processed as for iNOS expression.

COX-2 activity in PMN cells was measured by quantifying the amount of PGE$_2$ that can be formed from arachidonic acid (Cayman Chemicals). Cells ($10^5$) were incubated in 500 μl buffer (pH 7.4) containing 0.14 M NaCl, 1.59 mM Na$_2$HPO$_4$, 8.8 mM Na$_2$PO$_4$, 2 mM EDTA, 100 mM di-thiothreitol, leupeptin (1 μg/ml), aprotonin (1 μg/ml) and 2 mM-phenylmethylsulfonyl fluoride for 30 min at 4°C. After centrifugation at 14,000 g for 10 min, protein concentration was evaluated in homogenates. Homogenate samples (10 μl) were incubated in 1 ml 0.1 M tri(hydroxymethyl)amino-methane-HCl (pH 8.0), 5 mM-EDTA and 2 mM-phenol, in the presence of haeme group and arachidonic acid. A COX-inhibitor, indomethacin (10 mM), was added for sample control. After 2 min of incubation at 37°C, the reaction was stopped with 50 μl HCl.

### Statistical methods

Statistical analysis of the data was performed using SPSS software, version 11.5 (SPSS Inc., Chicago, IL, USA). The results are expressed as mean values with their standard errors. Data were evaluated using either the unpaired Student’s t test or one-way ANOVA. Homogeneity of variances was tested by the Bartlett’s test. The Student–Newman–Keuls multiple comparison test was used to detect differences among groups ($P<0.05$). Correlations between variables were studied by linear regression.

### Results

#### Dealcoholized wines and diets

According to GC, the ethanol content of DRW and DWW was less than 3 g/l. Red wine had a higher concentration of polyphenolic compounds than white (Table 2). The dealcoholization process did not alter the polyphenolic composition of wines. The total polyphenol content was also higher in DRW than in DWW (8.64 and 1.82 mM gallic acid equivalents/m, respectively; $P<0.001$) (Table 3).

The antioxidant activities *in vitro* were lower in DWW than in DRW (Table 3). Hypoxanthine–xanthine oxidase and ORAC were better correlated with total phenol ($r = 0.9378$ and $r = 0.9577$, respectively; $P<0.001$) than with the reducing power ($r = 0.9015$ and $r = 0.9011$, respectively; $P<0.005$). The largest difference observed between the two wines was the 12-fold higher NO scavenging activity of DRW (2.72 mmol Hb equivalents/M) v. DWW (0.23 mmol Hb equivalents/M) ($P<0.001$) (Table 3).

Ascorbic acid and fructose content in both dealcoholized wines was of the order of 6 μM and 0.25 g/l. According to the wine maker the concentration of sulfites was 110 and 120 mg/l for the red and white wine, respectively. Feeding rats a wine-rich diet for 15 d had no effect on food or water ingestion, and the body-weight gain in the treated groups was similar to the control group.

### Inflammatory parameters in exudates

There were no significant differences in the weight of granuloma between dietary groups. Similar volumes of exudate, osmolality and protein concentration in supernatant fractions of the exudates were observed in the three groups of rats. However, the number of cells in inflammatory exudates decreased by about 47% in both groups of treated rats. PGE$_2$ concentration increased in exudates from DRW-fed rats (Table 4).

### Biomarkers of oxidative stress in plasma and supernatant fraction of exudates

Reducing power, ORAC activities and O$_2^-$ were higher in plasma than in exudate supernatant fractions (Table 5). However, the DRW diet significantly increased ORAC (23%) and the O$_2^-$ scavenging activity (50% scavenging...
concentration decreased by 42% in plasma, and the reducing power (41%), ORAC (93%) and O$_2^-$ scavenging activity (50% scavenging concentration decreased by 27%) in exudate supernatant fractions. Feeding rats a DWW-rich diet increased the reducing power and O$_2^-$-scavenging activities in plasma (32 and 13%, respectively) and in supernatant fractions of exudates (41 and 24%, respectively) and the ORAC (23%) in plasma. Dietary administration of dealcoholized wines

<table>
<thead>
<tr>
<th>Table 3. Total phenols and antioxidant activities of dealcoholized wines†</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Mean values with their standard errors for two replicates from three bottles of dealcoholized wine)</td>
</tr>
<tr>
<td>DRW</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Total phenols (mM gallic acid equivalents/l)</td>
</tr>
<tr>
<td>Reducing power (mM quercetin equivalents/l)</td>
</tr>
<tr>
<td>Hypoxanthine–xanthine oxidase SC50 (µM/l)</td>
</tr>
<tr>
<td>ORAC (mM trolox equivalents/l)</td>
</tr>
<tr>
<td>NO scavenging (mM Hb equivalents/l)</td>
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</table>

DRW, dealcoholized red wine; DWW, dealcoholized white wine; SC50, 50 % scavenging concentration; ORAC, oxygen radical absorbance capacity.

† Mean value was significantly different from that of DRW (P<0.001; Student’s unpaired t test).

<table>
<thead>
<tr>
<th>Table 4. Body weight, exudate parameters and granuloma weight after 24 h of carrageenan granuloma induction*</th>
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</thead>
<tbody>
<tr>
<td>(Mean values with their standard errors for two replicates from seven rats)</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Body weight (g)</td>
</tr>
<tr>
<td>Weight of granuloma (g)</td>
</tr>
<tr>
<td>Volume of exudates (ml)</td>
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<td>Osmolarity of exudate (mOsmol/l)</td>
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<tr>
<td>Protein concentration in exudate supernatant fractions (g/l)</td>
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<tr>
<td>PGE2 in exudate supernatant fractions (pg/ml)†</td>
</tr>
</tbody>
</table>

DRW, dealcoholized red wine; DWW, dealcoholized white wine.

* Mean values within a row with unlike superscript letters were significantly different (P<0.05).

† For details of diets and procedures, see Table 1 and Materials and methods.

<table>
<thead>
<tr>
<th>Table 5. Oxidative stress in plasma and supernatant fraction of exudates after 24 h of carrageenan granuloma induction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Mean values with their standard errors for two replicates from seven rats)</td>
</tr>
<tr>
<td>Plasma</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Total phenols (µM gallic acid equivalents/l)</td>
</tr>
<tr>
<td>Reducing power (µM quercetin equivalents/l)</td>
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<tr>
<td>O$_2^-$ scavenging (hypoxanthine–xanthine oxidase SC50) (µM/l)</td>
</tr>
<tr>
<td>ORAC (µM)</td>
</tr>
<tr>
<td>TBARS (µM)</td>
</tr>
</tbody>
</table>

DRW, dealcoholized red wine; DWW, dealcoholized white wine; ORAC, oxygen radical absorbance capacity; TBARS, thiobarbituric acid-reactive substances.

* Mean values for plasma or supernatant fraction of exudates with unlike superscript letters were significantly different (P<0.05).

† For details of diets and procedures, see Table 1 and Materials and methods.
significantly decreased thiobarbituric acid-reactive substances in plasma from both groups of dealcoholized wine-fed rats and in the supernatant fraction of exudates from DRW-fed rats (Table 5). Values in exudate supernatant fractions were about 4.5-fold higher than in plasma (60 μM in plasma of control rats).

NO concentration was about 5-fold higher in the supernatant fraction of exudates than in plasma, and significantly greater in dealcoholized wine-fed rats (Table 5). The NO levels in exudate supernatant fractions, which are indicative of NO production by inflammatory cells in situ, are consistent with the ratios of free citrulline:free arginine found in this compartment (0.76 (SE 0.08), 1.08 (SE 0.12) and 1.34 (SE 0.12) for control, DRW and DWW, respectively), which is equivalent to increases of 43 and 77% in DRW- and DWW-treated rats, respectively, as compared with rats fed the control diet. No correlation between NO and number of cells in exudates was observed. However, a positive correlation between NO and citrulline:arginine in the supernatant fraction of exudates was detected (r 0.5304, P=0.0235).

Free radical production by polymorphonuclear leucocytes and inducible nitric oxide synthase and cyclo-oxygenase-2 assays

The ex vivo O$_2^·$ production by PMN leucocytes isolated from the inflammatory exudates decreased by 56% in DRW- and by 44% in DWW-treated rats (Table 6), while NO generation increased by 220 and 200% in DRW- and DWW-fed rats, respectively (Table 6).

iNOS protein expression increased by 47 and 28% in DRW- and DWW-fed rats, respectively (Fig. 1 (a) and 1 (b)). However, iNOS activity was more enhanced by the administration of dietary wines (67 and 75% for DRW and DWW, respectively) than was its expression (Fig. 1 (b)).

COX-2 expression (Fig. 2 (a) and 2 (b)) and activity (Fig. 2 (b)) significantly increased only in rats fed the DRW-rich diet (36 and 30%, respectively).

Discussion

Polyphenols are important non-alcoholic antioxidant compounds in wine. Most studies on their biological activities have focused on the prevention of atherosclerosis or on the effect on macrophages and platelets, and few studies have been performed using PMN leucocytes as cells present in an acute inflammatory stage. The present paper demonstrates that the administration of DRW- and DWW-rich diets to rats modulates the oxidative stress and the inflammatory response in the carrageenan-induced granuloma pouch, used as a model of acute inflammation.

It has been assumed that red wine shows more protective effects than white wine in vitro because of its high content of polyphenolic antioxidants. Moreover, wine also contains non-polyphenolic compounds with antioxidant activity, such as ascorbic acid and sulfites. These two compounds were present at similar concentrations in DRW and DWW and, thus, the differences observed in the antioxidant activities of these wines should be attributed to their qualitative and quantitative differences in polyphenols. However, the similarities in plasma and supernatant fraction of inflammatory exudates from the two treated groups support the view that the bioavailable polyphenols and some non-polyphenolic compounds such as ascorbic acid and sulfites can be responsible for the low oxidative stress observed in vivo. We can rule out the contribution of uric acid generated from fructose in vivo as its concentration in white wine was lower than in red wine, and also because rats have uricase. Small differences

Table 6. Superoxide anion (O$_2^·$) and nitric oxide production by polymorphonuclear cells* (Mean values with their standard errors for two replicates from seven rats)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DRW</th>
<th>DWW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>O$_2^·$ production (nmol/10$^6$ cells per h)</td>
<td>4.45$^a$</td>
<td>0.49</td>
<td>1.95$^a$</td>
</tr>
<tr>
<td>NO production (nmol/10$^6$ cells per h)</td>
<td>0.91$^a$</td>
<td>0.29$^a$</td>
<td>3.17$^a$</td>
</tr>
</tbody>
</table>

DRW, dealcoholized red wine; DW, dealcoholized white wine.

*For details of diets and procedures, see Table 1 and Materials and methods.

![Fig. 1. Inducible NO synthase (iNOS) protein. (a) Representative iNOS expression by Western blot. (b) iNOS expression (exp) and activity (act) in polymorphonuclear cells from the inflammatory exudate of rats fed a control diet, or diets supplemented with dealcoholized red wine (DRW) or dealcoholized white wine (DWW) for 15 d. Values are means with their standard errors represented by vertical bars (two replicates from four rats). $^a$ and $^b$ Mean values for exp or act with unlike letters were significantly different (P<0.05).](https://www.cambridge.org/core/terms). Subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. IP address: 54.70.40.11, on 26 Jun 2019 at 23:41:07.
in plasma concentration of polyphenols in subjects given red
and white wine for 15 d were also observed by Pignatelli
et al.\textsuperscript{29} while the total polyphenolic content of red wine
was approximately 7-fold higher.

The effect of DRW and DWW dietary administration on
oxidative stress associated with an inflammatory process has
also been tested in the present study in PMN cells isolated
from inflammatory exudates. When activated, PMN cells
release $O_2^-$ (generated by NADPH oxidase and xanthine
oxidase) and NO (generated by iNOS). $O_2^-$ has been associated
with tissue damage\textsuperscript{30} and NO is recognized to be either a
protective or a harmful agent\textsuperscript{11} depending on the amount pro-
duced, the site of production and the presence of other reactive
species\textsuperscript{2,2}. In this sense, NO is a good target for $O_2^-$-yielding
peroxynitrite\textsuperscript{12,13}. Its generation should be deleterious unless
an excess of NO remains acting as a protective agent. This
latter possibility is confirmed by the high levels of NO in the
supernatant fraction of exudates observed in DRW-treated rats, but not in
DWW-treated rats, indicate that under our conditions NO is
not critical for COX-2 activation. The paper by Fujimoto
et al.\textsuperscript{39} suggests that PGE\textsubscript{2} production is complex and that
H\textsubscript{2}O\textsubscript{2}, NO and peroxynitrite can have different modulatory
effects on COX activities depending on their concentration in
cells. The inhibitory effect of polyphenols on COX observed
\textit{in vitro}\textsuperscript{1} is not observed \textit{in vivo} in the present
study and this can be attributed to their metabolism or
bioavailability. In this model of acute inflammation, the up
regulation of COX-2 could indicate that the DRW was pro-
inflammatory. However, the decreased PMN cell number
and oxidative stress, the increased NO concentration in super-
natant fraction of exudates and the decreased capability of
PMN cells to generate $O_2^-$ and the increased NO production
can all be considered as anti-inflammatory effects of both
DRW- and DWW-rich diets.

During the past decade, we have come to appreciate the prominent role played by inflammation and oxidative stress
in several diseases. Our observations indicate that the non-alcoholic compounds of red and white wines are similarly
effective \textit{in vivo}. By decreasing $O_2^-$ production and increasing
NO production they control cell infiltration and prevent
physiopathological processes associated with inflammation.
Moreover, NO, by its antioxidant activity, will terminate
the lipid radical chain reaction\textsuperscript{40,41}.

\textbf{Fig. 2.} Cyclo-oxygenase (COX)-2 protein. (a) Representative COX-2
expression by Western blot. (b) COX-2 expression (exp) and activity (act) in
polymorphonuclear cells from the inflammatory exudate of rats fed a control
diet, or diets supplemented with dealcoholized red wine (DRW) or dealcohol-
ized white wine (DWW). Values are means with their standard errors
represented by vertical bars (two replicates from four rats). *\textsuperscript{a},*\textsuperscript{b} Mean values for
exp or act with unlike letters were significantly different ($P$<0.05).
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References


