DOI:10.1021/jf900576h

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Red Wine Protects against Ethanol-Induced Oxidative Stress in Rat Liver

Marco Assunção,*,† Maria J. Santos-Marques,† Rosário Monteiro,‡,§ Isabel Azevedo,‡ José P. Andrade,† Félix Carvalho,# and Maria J. Martins‡

†Department of Anatomy (U121/94-FCT) and ‡Department of Biochemistry (U38-FCT), Faculty of Medicine, University of Porto, Alameda Professor Hernâni Monteiro, 4200-319 Porto, Portugal, §Faculty of Nutrition and Food Sciences, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal, and #REQUIMTE, Department of Toxicology, Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha 164, 4099-030 Porto, Portugal

Ethanol consumption may be deleterious to the liver. However, alcoholic beverages contain, besides ethanol (EtOH), complex chemical mixtures that can modify EtOH's adverse effects. Red wine (RW) is rich in polyphenolic antioxidants, often reported as hepatoprotective agents. This study aimed to investigate the effects of 6 months of RW ingestion on hepatic oxidative stress and inflammation. Six-month-old Wistar rats were treated with RW or EtOH; controls were pair-fed. EtOH increased 8-hydroxy-2′-deoxyguanosine and decreased reduced and oxidized glutathione. These animals also displayed stimulated superoxide dismutase, catalase, and glutathione reductase activities. RW treatment decreased malondialdehyde and reduced glutathione levels. Glutathione-*S*-transferase and selenium-dependent glutathione peroxidase activities were stimulated and glutathione reductase activity was inhibited by RW intake. No modifications were detected in nuclear factor-κ B or alkaline phosphatase activities. EtOH consumption induced fibrosis in portal spaces and hepatocyte lipid accumulation that were absent with RW treatment. This paper highlights the importance of RW nonalcoholic components and the relevance of biological matrix in the study of EtOH oxidative effects.

KEYWORDS: Red wine; ethanol; oxidative damage; inflammation; liver; rat.

INTRODUCTION

Moderate red wine consumption has been linked to a broad spectrum of health-promoting effects (*1*). Several epidemiological studies have related the moderate ingestion of red wine to the reduction of cardiovascular disease risk (*2*, *3*), decreased incidence of some types of cancer (*4*, *5*), and prevention of dementia (*6*). Most of the protective actions of red wine can be attributed to its high content in polyphenols, which are extracted from grape seeds and skins during winemaking (*7*−*9*). However, apart from these antioxidant molecules, wine also contains a great amount of ethanol (*1*), generating reasonable doubts and concerns as to whether or not it could be considered a suitable polyphenol source.

Ethanol metabolism takes place mostly in the liver, where it is converted to acetaldehyde, a highly toxic metabolite, by three enzymatic pathways: alcohol dehydrogenase, microsomal ethanol oxidizing system, and catalase (CAT) (*10*−*13*). Toxicity resulting from ethanol consumption can be due to the direct action of acetaldehyde or ethanol itself, but can also arise from metabolic alterations with consequent modification of the NAD^+/NADH ratio, production of reactive oxygen and nitrogen species, and occurrence of hypoxia, endotoxemia, and cytokine release (*11*−*15*). Furthermore, ethanol can alter the levels of several metals in the body, thereby facilitating reactive species production thus favoring an oxidative environment (*13*, *15*). Some of these consequences of ethanol intake can translate into changes in gene expression within the liver and contribute to the progression of alcohol-induced hepatic injury (*11*−*13*, *15*). Consequently, reports regarding alterations in lipid accumulation, inflammation, Kupffer and hepatic stellate cell activation, and fibrosis have been related to ethanol ingestion (*11*−*13*, *15*). Moreover, ethanol has been shown to modulate hepatic levels of alkaline phosphatase and antioxidant enzyme activities and to alter pro- and antioxidant molecules in the liver in human or experimental animal models (*11*−*22*).

Apparently controversial data exist regarding hepatic antioxidant and anti-inflammatory activities of red wine intake, due to its relatively high ethanol content (*1*), and this issue requires clarification, particularly concerning long-term consumption (*20*, *22*). Furthermore, most available studies have been performed in young rodents (*20*, *22*), and there is no knowledge if the putative protective effects of red wine are still observed in more advanced ages. As a matter of fact, we began to approach this issue in the central nervous system by treating 6-month-old rats for 6 months with red wine. In those studies, we were able to demonstrate decreased oxidative stress markers in comparison to

*Author to whom correspondence should be addressed (telephone +351-22-5513616; fax +351-22-5513617; e-mail marco_ma@med.up.pt).

 Published on Web 06/23/2009 © 2009 American Chemical Society

ethanol-drinking animals, which were, in turn, accompanied by functional improvements (*7*, *23*). To further pursue the question of whether or not red wine intake contributes to beneficial effects on oxidative stress and overall health, we aimed to evaluate the effects of prolonged red wine ingestion on hepatic oxidative stress markers and inflammation and compare them to those observed in control animals ingesting the same amount of ethanol and in pair-fed controls.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemical substances used in the experiments were of analytical grade. Reduced (GSH) and oxidized glutathione (GSSG), glutathione reductase (GR; EC 1.6.4.2), NADPH, 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB), 2-vinylpyridine, bovine serum albumin (BSA), xanthine, xanthine oxidase (EC 1.17.3.2), 1-chloro-2,4-dinitrobenzene, and nitroblue tetrazolium (NBT) for oxidative stress determinations were purchased from Sigma-Aldrich (St. Louis, MO). Pentobarbital, 8-hydroxy-2′-deoxyguanosine (8-OHdG), nuclease P1, *p*-nitrophenol (*p*-NP), *p*-nitrophenylphosphate (*p*-NPP), levamisole, and L-phenylalanine (L-Phe) were also obtained from Sigma. Ethanol for drinking solutions and morphological methods, borax, propylene oxide, and sucrose were obtained from Merck (Darmstadt, Germany). Alkaline phosphatase (ALP; EC 3.1.3.1) for determination of 8-OHdG was purchased from AppliChem (Darmstadt, Germany). Glutaraldehyde, osmium tetroxide, and Epon 812 were purchased from TAAB Laboratories (Berkshire, U.K.). Toluidine blue was purchased from Difco (Voigt Global Distribution, Lawrence, KS) and cacodylate buffer from BDH Chemicals (Poole, U.K.).

Animals and Treatments. Studies were carried out in adult male Wistar rats obtained from Charles River Laboratories (Barcelona, Spain). All animals were maintained on a daily photoperiod of 12 h lighting schedule at constant room temperature (20−22 °C) with free access to food and tap water. At 6 months of age (average body weight = 624 ± 24 g), rats were individually housed and randomly assigned to one of the following groups of six animals each: (1) ethanol (EtOH), (2) red wine [RW; red table wine produced in 2004 containing 15% (v/v) ethanol], or (3) pair-fed control (PFC). The first group of rats had unrestricted access to a 20% (v/v) aqueous ethanol solution as the only available liquid source, beginning with a 5% (v/v) ethanol solution and progressively increasing 1% per day until the final concentration of 20% (v/v) that was achieved 15 days later. The second group of animals had free access to RW in which the EtOH concentration was increased from the original 15 to 20% (v/v) to ensure that the liquid diets used in RW and EtOH groups had similar content of EtOH. RW was also gradually introduced, starting with an amount of RW that could supply 5% (v/v) EtOH at the beginning of treatment and increasing progressively 1% per day until a final concentration of 20% (v/v). RW was supplied to the animals as the sole drinking fluid in dark bottles to avoid degradation of polyphenolic compounds. The third group of rats received a drinking solution in which sucrose was added to replace the isocaloric value of the ethanol and were staggered one day behind and fed an amount equal to the average consumed in the previous day by the EtOH and RW groups, taking into account that 1 g of sucrose corresponds to 16.7 kJ and 1 g of ethanol to 29.3 kJ and that the density of ethanol is 0.79 g/mL. EtOH and RW rats had unrestricted access to standard laboratory pellet food (Letica, Barcelona, Spain), whereas PFC animals, as described above, were staggered one day behind and received the average amount of rat chow consumed by the former groups to obtain similar caloric intakes among groups throughout the experiment. Animal food supplied 15397 kJ/kg and was composed of 154 g of protein/kg (21% of energy supplied), 29 g of fat/kg (7% of energy supplied), 41 g of cellulose/kg, and 59 g of mineral salts/kg. Detailed RW polyphenol composition was determined by HPLC after a prepurification of the wine by chromatography using a Toyopearl gel, as previously described (*7*) and is displayed in **Table 1**. Consumption of food and fluid was monitored every other day, and rats were weighed weekly. The handling and care of the animals were conducted in conformity with the European Community Council guidelines for the use of experimental animals (86/609/EEC) and Portuguese Act 129/92. Serum ethanol concentrations were measured in all ethanol-ingesting animals, using blood samples collected from the dorsal tail vein 2−4 h after the beginning of the dark period. Serum ethanol levels were determined weekly in the first month of treatment and monthly thereafter using a commercial enzymatic assay kit (Sigma-Aldrich, St. Louis, MO) and are reported as grams per liter.

Table 1. Catechins, Procyanidins, and Anthocyanins Present in Red Wine[a]

polyphenol	red wine (g/L)
monomeric catechins	
(+)-catechin	0.026
(−)-epicatechin	0.009
(−)-epicatechin gallate	nd
oligomeric procyanidins (OPC)	
B1	0.104
B2	0.027
B3	nd
B4	0.012
B5	nd
B6	nd
B7	nd
B8	nd
C1	0.003
B2-gallate	0.012
total catechins and OPC	0.193
polymeric procyanidins	1.530
anthocyanins	
delphinidin-3-glucoside	0.009
petunidin-3-glucoside	0.024
peonidin-3-glucoside	0.005
malvidin-3-glucoside	0.361
petunidin-3-acetylglucoside	nd
delphinidin-3-coumaroylglucoside	nd
malvidin-3-acetylglucoside	0.159
peonidin-3-coumaroylglucoside	nd
malvidin-3-coumaroylglucoside	0.090
total anthocyanins	0.648

[a] These results correspond to the mean of two replicate analyses. nd, not detected.

Collection of Samples. At the end of the experimental period, at 12 months of age, all rats were deeply anesthetized with sodium pentobarbital [80 mg/kg of body weight (bw)]. Blood was collected from the left ventricle to measure blood lipid profile [triglycerides, total cholesterol, and high-density lipoprotein (HDL) cholesterol] through enzymatic colorimetric tests, using commercially available kits (Cholesterol, Triglycerides, HDL Cholesterol Direct, ABX Diagnostics, Northampton, U.K.), in an auto-analyzer (Cobas Mira Plus, ABX Diagnostics). Animals were transcardiacally perfused with an ice-cold isotonic sodium chloride solution. After perfusion, the liver was rapidly removed from the abdominal cavity, washed in the same solution, and cut in several fragments. For biochemical analyses, liver fragments were immersed in liquid nitrogen and stored at −80 °C until biochemical processing. For assessment of oxidative stress, hepatic tissue was sliced and homogenized in cold phosphate buffer (pH 7.4) containing 0.1% Triton X-100 in a glass−Teflon homogenizer. Homogenates were centrifuged (16000*g*, 10 min, 4 °C) and the supernatants separated for the different biochemical assays of oxidative stress (see details below). Liver fragments were stored at −80 °C for determination of DNA oxidative damage and ALP and nuclear factor-kappa B (NF-κB) activities. For morphological evaluation, liver fragments were immediately immersed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) without prior immersion in liquid nitrogen and then processed for morphological evaluation.

Assessment of Oxidative Stress Markers. *Malondialdehyde Levels.* The extent of hepatic lipid peroxidation was estimated by measuring the concentration of thiobarbituric acid-reactive substances (TBARS) at 535 nm, as described elsewhere (*24*, *25*). The results were expressed as malondialdehyde (MDA) equivalents using a molar extinction coefficient (ε) of 1.56×10^5 mM^{-1} cm^{-1}.

Oxidative Damage to DNA. Oxidative damage to cellular DNA was determined through measuring 8-OHdG as a cellular marker. Liver DNA was extracted, from liver fragments kept at −80 °C, using a commercial extraction kit (V-gene, Bioron, Ludwigshafen, Germany) according to the producer's instructions, dissolved in water, and

spectrophotometrically quantified at 260 nm. DNA samples were digested with 8 U of nuclease P1 (prepared in 20 mM sodium acetate buffer, pH 5.0) at 37 °C for 30 min. The solution was then incubated with 1.3 U of ALP at 37 °C for 1 h in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 7.5) (*26*). For enzyme extraction, 50 μL of chloroform was added, and samples were vortexed for 10 s and centrifuged at 5000*g* for 5 min (*27*). Supernatants were collected and analyzed by HPLC-EC (Gilson 302, Gilson Medical Electronics, Villiers le Bel, France) on a 25 × 3.2 mm i. d., Superspher RP18-4 End-cap column (HiCHROM, Merck, Darmstadt, Germany), and electrochemical detection (Gilson 141) was carried out at 10 V. Solvent was 50 mM potassium phosphate buffer, 10% (v/v) methanol (pH 5.5), at a flow rate of 0.5 mL/min. 8-OHdG concentration was calculated from a calibration curve, using commercial 8-OHdG as standard and normalized for the DNA concentration of each sample.

Glutathione Content. Glutathione was assessed in liver supernatants as reported before (*24*) by measuring total glutathione (GSX) and GSSG through the DTNB−GR recycling assay. All samples, formerly precipitated with perchloric acid, were neutralized with potassium bicarbonate, centrifuged, and kept on ice until transfer into a 96-well microplate. Appropriate standards and blanks were accomplished for each experiment receiving the same treatment as samples. For GSSG estimation, samples were previously treated with 2-vinylpyridine to achieve GSH derivatization. Both GSSG and GSX levels were measured by using the same procedure from appropriate standard curves, and GSH content was calculated by subtracting the double of GSSG from GSX content.

Antioxidant Enzyme Activities. Glutathione-*S*-transferase (GST; EC 2.5.1.18) activity in the supernatant was determined according to the method of Warholm et al. (*28*) using 1-chloro-2,4-dinitrobenzene as substrate. GST activity was monitored at 340 nm, calculated with an ε of 9.6 mM^{-1} cm^{-1} and expressed as nanomoles of *S*-2,4-dinitrophenylglutathione per minute per milligram of protein. Selenium-dependent glutathione peroxidase (Se-GPX; EC 1.11.1.9) activity was determined in the supernatant by using the Flohé and Günzler (*29*) method. The Se-GPX assay is based on the oxidation of NADPH to $NADP^+$, catalyzed by a limiting concentration of GR, with maximum absorbance at 340 nm. The results were expressed as nanomoles of NADPH oxidized per minute per milligram of protein (ε = 6.2 mM^{-1} cm^{-1}). GR activity was measured according to the method of Carlberg and Mannervik (*30*) by monitoring the oxidation of NADPH at 340 nm. GR activity was expressed as nanomoles of NADPH oxidized per minute per milligram of protein using an ε of 6.2 mM^{-1} cm^{-1}. CAT (EC 1.11.1.6) activity was determined according to the method described by Aebi (*31*). CAT activity was measured at 240 nm using an ε of 3.94 μM^{-1} cm^{-1} and expressed as units per milligram of protein. One unit of CAT was defined as the amount of enzyme that catalyzes the decomposition of 1 μmol of H_2O_2/min. Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined in liver homogenates as previously described (*32*). SOD assay is based on the ability of SOD to inhibit the reduction of NBT by the xanthine−xanthine oxidase system as a superoxide generator, with maximum absorbance at 560 nm. SOD activity was expressed as units per milligram of protein (one unit is the amount of SOD inhibiting the reaction rate by 50% in the given assay conditions).

Assessment of Inflammation Markers. *ALP Activity*. The homogenization solution, composed of 4 volumes of phosphate-buffered saline (PBS, pH 7.4) and 1 volume of 0.5% Triton X-100 solution, was added to liver fragments (2 mL/g tissue), just before storage at −80 °C. On the day of ALP quantification, tissue samples were homogenized (final dilution 1:5) in homogenization solution and kept on ice. The reaction mixture contained, in a final volume of 500 μL, 80 mM tris(hydroxymethyl)aminomethane free base (pH 10.4), 0.4 mM $MgCl_2$, 0.376 mg *p*-NPP, 60 μL of liver homogenate, and one of the two ALP classic inhibitors to be tested: levamisole (0.1 or 0.5 mM, inhibitor of tissue nonspecific ALP) or L-Phe (2, 5, or 10 mM, inhibitor of intestinal ALP). The reaction was started by the addition of the ALP substrate (*p*-NPP). Incubation took place at 37 °C for 12 min, and the reaction was stopped by adding 2 mL of ice-cold 20 mM NaOH. ALP activity was determined by reading, at 405 nm, the absorbance of the *p*-NP produced. The effect of each ALP inhibitor tested was determined as a percentage of the correspondent ALP control activity. ALP activity was expressed in nanomoles of *p*-NP produced per minute per milligram of protein. ALP activity assays were carried out in triplicate, as previously described (*33*−*35*).

NF-κB Activity. Protein extraction from liver fragments was performed using the Tripure method (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. NF-κB activity evaluation was performed using an ELISA kit from Active Motif (TransAM Flexi, NF-κB Family Kit, Carlsbad, CA), according to the manufacturer's instructions. The NF-κB p50 antibody was used in a 1:1000 dilution, and the colorimetric reaction took place for 3.5 min.

Protein Determination. Protein concentration for ALP activity quantification was determined as described by Bradford (*36*). For assessment of oxidative stress markers in liver homogenates and supernatants, protein concentration was determined as described by Lowry et al. (*37*). In both methods, BSA was used as standard. For NF-κB activity evaluation protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Morphological Evaluation. After perfusion, the liver fragments were cut with a razor blade and kept in the described fixative for 90 min. The tissues were then washed overnight in cacodylate buffer with 0.6 M sucrose at 4 °C, postfixed in a 2% solution of osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3) at 4 °C for 1 h, dehydrated with ethanol and propylene oxide and embedded in Epon 812. Semithin sections were obtained from three blocks of each animal, taken at random, with an LKB Ultratome (LKB, Bromma, Sweden), stained with 1% toluidine blue in 1% borax, and examined using a light microscope (Laborlux K, Leitz, Wetzlar, Germany) (*38*). A qualitative evaluation was blindly performed by two independent observers. The existence of fibrosis in portal spaces and lipid deposits was registered.

Statistical Analysis. The significance of differences among the groups was evaluated using ANOVA followed by Newman−Keuls post hoc test for multiple comparisons. Student's *t* test was used to assess statistical significance between two groups when appropriate. Statistical analysis was performed using GraphPad Prism software (GraphPad version 3.0, La Jolla, CA). Values are presented as mean ± standard deviation (SD). Differences were considered to be significant when $P < 0.05$.

RESULTS

Animals and Treatments. Data concerning the amounts of food and fluid ingestion by all groups of rats throughout the entire experimental feeding period can be found in **Table 2**. There were no significant differences in daily food and energy intakes among PFC, EtOH, and RW groups. However, although similar between EtOH and RW animals, fluid intake was significantly higher in PFC rats ($P < 0.001$). The mean daily ethanol intake by EtOH and RW animals was constant during the treatment and did not differ significantly between the two groups. Likewise, mean ethanol levels measured in blood samples collected from both groups were similar during the experiment (0.45 ± 0.35 and 0.42 ± 0.28 g/L for EtOH- and RW-treated rats, respectively). Furthermore, no differences were noted in the normal behavior of the animals from the treatment groups in comparison to PFC animals. The mean daily sucrose intake in the PFC animals was 8.55 ± 1.03 g/kg of bw. Body weight did not differ among groups at the beginning of treatments and increased gradually throughout the 6 months of the experiment (**Figure 1**). After completion of the protocol, no significant differences were observed when the mean body weights of PFC (696 ± 33 g), EtOH (686 ± 30 g), and RW (684 ± 28 g) rats were compared. Results from circulating

Table 2. Food and Fluid Intakes[a]

group	food (g/day/kg of bw)	fluid (mL/day/kg of bw)	ethanol (g/day/kg of bw)	energy (kJ/day/kg of bw)
PFC	32.60 ± 2.59	69.17 ± 6.52		644.43 ± 34.81
EtOH	31.93 ± 2.85	30.05 ± 3.42***	4.75 ± 0.54	623.46 ± 38.82
RW	32.51 ± 1.57	31.44 ± 2.20***	4.97 ± 0.35	645.50 ± 25.08

[a] Values represent mean ± SD. bw, body weight; PFC, pair-fed control; EtOH, ethanol; RW, red wine. ***, $P < 0.001$ vs PFC; $N = 6$ in each group; Newman−Keuls post hoc test.

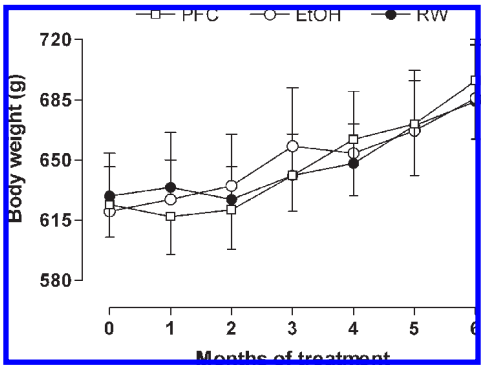

Figure 1. Time course of body weight (g) data of pair-fed control (PFC), ethanol (EtOH), and red wine (RW) rats throughout the 6 months of treatment. Results represent mean ± SD; $N = 6$ in each group.

Table 3. Circulating Lipid Profiles[a]

group	triglycerides (mg/dL)	total cholesterol (mg/dL)	HDL-cholesterol (mg/dL)
PFC	160.4 ± 32.0	75.8 ± 9.7	14.0 ± 2.6
EtOH	181.5 ± 65.3	61.1 ± 19.4	12.1 ± 2.6
RW	203.3 ± 129.1	77.2 ± 26.3	16.8 ± 2.4*

[a] Values represent mean ± SD. HDL, high-density lipoprotein; PFC, pair-fed control; EtOH, ethanol; RW, red wine. *, $P < 0.05$ vs EtOH. $N = 6$ in each group; Newman−Keuls post hoc test.

lipid profiles are shown in **Table 3**. No differences in triglycerides and total cholesterol were observed among groups, but HDL-cholesterol was increased by RW treatment when compared to EtOH-treated rats.

Oxidative Stress Markers. Oxidative damage to lipids and DNA in hepatic tissue is shown in **Figure 2**. Regarding lipid peroxidation, it was found that MDA levels were not affected by chronic EtOH treatment. However, prolonged RW ingestion markedly decreased MDA equivalents in comparison to the PFC and EtOH groups ($P < 0.001$ for both). It must be emphasized that RW-treated rats presented only vestigial levels of this lipid peroxidation product. Concerning oxidative damage to DNA, analyses revealed that 8-OHdG levels were increased in the liver of EtOH-treated animals when compared to RW-treated and PFC animals ($P < 0.05$ for both). In contrast, no significant differences were found between PFC and RW groups.

The effect of treatments on hepatic glutathione levels can be seen in **Table 4**. Chronic consumption of both EtOH and RW resulted in a decrease of GSX as well as of GSH levels in liver homogenates as compared to PFC animals ($P < 0.01$ and $P < 0.001$, respectively). No differences were found between EtOH and RW rats relative to the two latter parameters. EtOH treatment also resulted in a significant reduction of GSSG levels in comparison to the remaining treatments ($P < 0.05$ for both), whereas RW did not significantly change GSSG in relation to PFC group.

Results concerning glutathione-related enzymes are illustrated in **Figure 3**. The effect of EtOH on GR was opposite that of RW, the first having increased ($P < 0.001$) and the latter having decreased ($P < 0.01$) GR activity relative to controls. We also found that the activities of GST and Se-GPX were significantly enhanced by prolonged RW intake ($P < 0.01$ and $P < 0.001$, respectively) and were not affected by EtOH exposure.

The activities of CAT and total SOD in the liver of PFC, EtOH, and RW rats can be observed in **Figure 4**. CAT activity was significantly increased after chronic EtOH ingestion when compared to the other two experimental groups ($P < 0.001$),

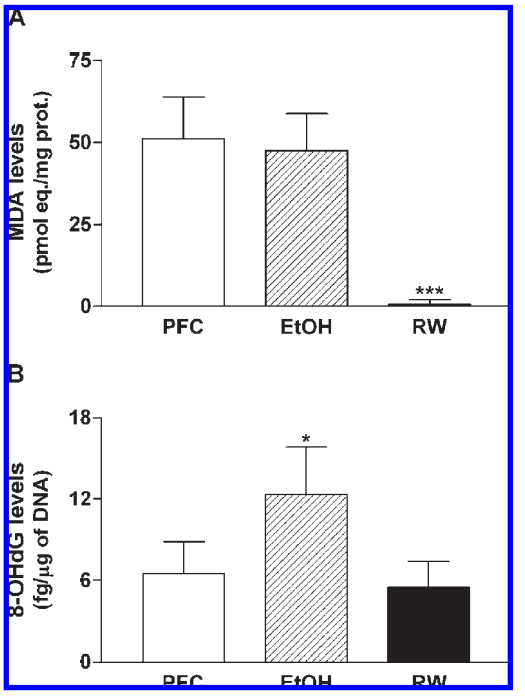

Figure 2. Malondialdehyde (MDA) (**A**) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) (**B**) levels in hepatic tissue of pair-fed control (PFC), ethanol (EtOH), and red wine (RW) rats. Columns represent means, and vertical bars correspond to one SD. *, $P < 0.05$ vs PFC and RW; ***, $P < 0.001$ vs PFC and EtOH; $N = 6$ in each group; Newman−Keuls post hoc test.

Table 4. Concentration of Hepatic Glutathione Levels[a]

group	GSX	GSH	GSSG	GSH/GSSG
PFC	37.02 ± 4.28	35.97 ± 3.98	0.48 ± 0.11	77.03 ± 13.45
EtOH	28.22 ± 3.27**	27.53 ± 3.30**	0.34 ± 0.07*	75.52 ± 14.26
RW	23.86 ± 5.57***	22.78 ± 5.46***	0.54 ± 0.12	43.03 ± 9.70†††

[a] Values are expressed as mean ± SD. PFC, pair-fed control; EtOH, ethanol; RW, red wine; GSX, total glutathione; GSH, reduced glutathione; GSSG, oxidized glutathione. Units: GSX, GSH and GSSG, nmol/mg protein. *, $P < 0.05$ vs PFC and RW; **, $P < 0.01$ vs PFC; ***, $P < 0.001$ vs PFC; †††, $P < 0.001$ vs PFC and EtOH; $N = 6$ in each group; Newman−Keuls post hoc test.

which presented very similar CAT activity values. SOD activity also increased following EtOH ingestion when compared to RW-treated and PFC animals ($P < 0.01$ and $P < 0.05$, respectively).

Inflammation Markers. No significant differences were detected in mean liver ALP activity (nmol of *p*-NP produced/min/mg of protein) among PFC (2.26 ± 0.40), EtOH (1.94 ± 0.46), and RW (1.98 ± 0.43) rats. Also, taking into consideration both the presence of the inhibitor and the three groups of animals, there were no differences regarding levamisole and L-Phe effects upon hepatic ALP activity (approximately to 50 and 10%, respectively). Likewise, NF-κB activity was not significantly different when the three groups of rats were compared (100.0 ± 15.9, 92.4 ± 11.7, and 100.0 ± 8.8% of control in PFC and EtOH- and RW-treated rats, respectively).

Morphological Observations. Qualitative data concerning hepatic morphology can be seen in **Figure 5**. There were no apparent morphological differences between the livers from PFC and RW-treated animals (**Figure 5A**,**C**). However, in the livers of EtOH-treated rats (**Figure 5B**) there were images of fibrosis in some portal spaces and increased lipid accumulation. No fibrosis or significant lipid deposits were observed in PFC and RW-treated rats.

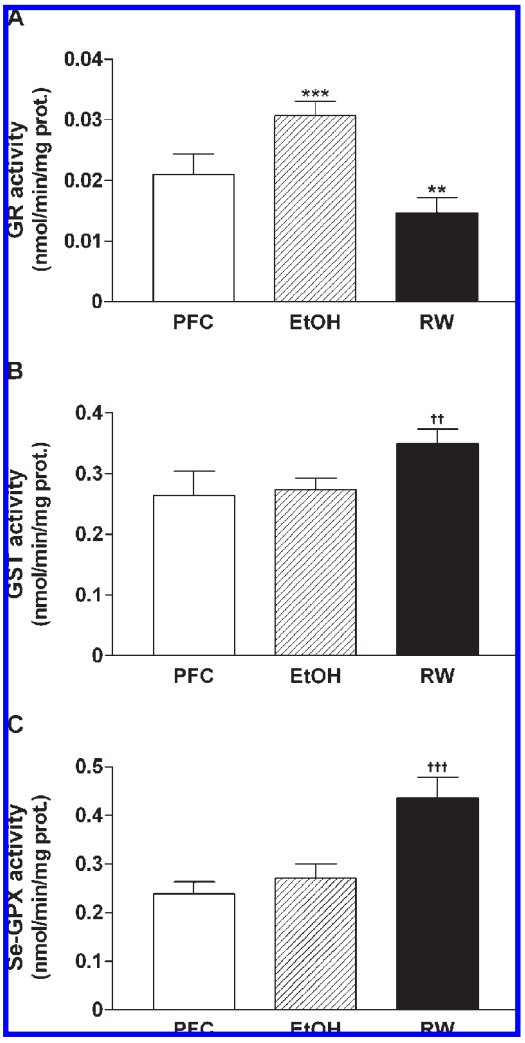

Figure 3. Glutathione-related enzyme activities in the liver homogenates of pair-fed control (PFC), ethanol (EtOH), and red wine (RW) rats. Glutathione reductase (GR) (**A**), glutathione *S*-transferase (GST) (**B**), and selenium-dependent glutathione peroxidase (Se-GPX) (**C**) activities were significantly affected by chronic RW consumption. Columns represent means, and vertical bars correspond to one SD. **, $P < 0.01$ vs PFC; ***, $P < 0.001$ vs PFC and RW; ††, $P < 0.01$ vs PFC and EtOH; †††, $P < 0.001$ vs PFC and EtOH; $N = 6$ in each group; Newman−Keuls post hoc test.

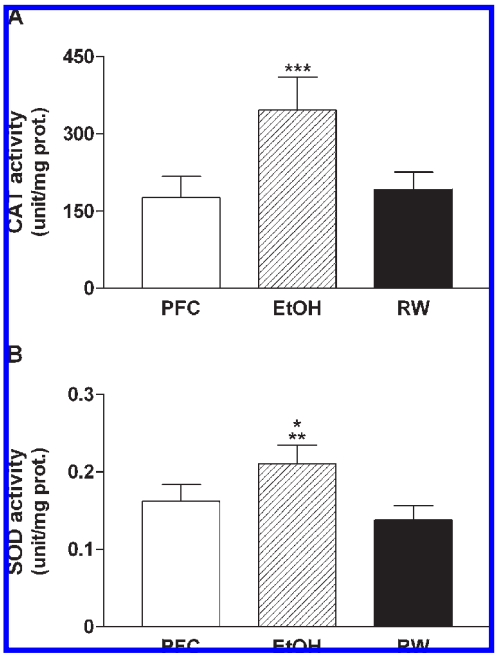

Figure 4. Catalase (CAT) (**A**) and total superoxide dismutase (SOD) (**B**) activities in the liver of pair-fed control (PFC), ethanol (EtOH), and red wine (RW) rats. Columns represent means, and vertical bars correspond to one SD. *, $P < 0.05$ vs PFC; **, $P < 0.01$ vs RW; ***, $P < 0.001$ vs PFC and RW; $N = 6$ in each group; Newman−Keuls post hoc test.

DISCUSSION

The large number of studies showing the deleterious effects of ethanol consumption to hepatic functions (*39*−*41*) is being currently confronted with growing evidence based on epidemiological studies showing the beneficial effects of red wine to overall health (*1*, *42*). Moderate red wine ingestion is being linked to improved metabolic and inflammatory profiles and redox status in several organs, including the liver (*20*, *22*, *43*, *44*). However, long-term studies combining an evaluation of both oxidative stress and inflammatory markers are scarce. In fact, to our knowledge this is the first experimental study of 6 months of ethanol or red wine ad libitum consumption that combines an evaluation of MDA, 8-OHdG, glutathione, SOD, CAT, GST, Se-GPX, GR, NF-κB, ALP, and liver morphology. Taking into consideration the significant energy supplied by ethanol, the control animals were pair-fed, adding energy to the diet in the form of sucrose. Therefore, when the data obtained in treated groups are analyzed in comparison to these PFC, three issues should be kept in mind: (1) the different amount of liquid ingestion in PFC when compared to the other groups, probably related to the osmotic effect of sucrose; (2) the similarity of MDA levels found in PFC and EtOH groups; and (3) the similarity of glutathione-related enzyme activities between PFC and EtOH groups. Facing this parallelism, the results observed in RW-treated rats are really impressive as oxidative stress marker alterations accompanying PFC or EtOH treatments were not visible in the RW-treated animals.

We found no increase in TBARS in the EtOH group, as already described (*14*, *16*, *19*). Together with the unchanged levels of TBARS, we observed a reduction in total, reduced, and oxidized glutathione contents in the EtOH group when compared to the PFC group. Indeed, the role of glutathione as a free radical scavenger (*45*−*47*) may help explain both the decrease of GSH and the absence of alterations in TBARS. Thus, the observed increase in GR activity would be determinant for the regeneration of the cellular pool of GSH from GSSG and in the maintenance of the GSH/GSSG ratio. No other changes were observed in the enzymes using glutathione in these EtOH-treated animals.

Several other mechanisms resulting in a decrease in glutathione as a consequence of ethanol intake have been proposed: (a) depletion of GSH levels via the generation of oxidants (*13*); (b) impairment of glutathione synthesis (*17*); (c) alteration of cysteine availability; (d) inactivation/reduced activity of methionine adenosyltransferase and methionine synthase; (e) increased GSH efflux (*18*); and (f) direct binding of glutathione to acetaldehyde (*48*). However, in certain experimental conditions, ethanol has been demonstrated to increase liver GSH as a result of rebound GSH synthesis due to oxidative stress and/or to an efficient glutathione recycling (*14*, *19*, *20*).

The stimulation of hepatic antioxidant enzymes or molecules as a protective mechanism against continuous oxidative insults, including those caused by ethanol-induced free radicals, has already been reported (*14*, *20*, *21*). Accordingly, CAT and SOD were increased in EtOH-treated rats. CAT, an enzyme related to

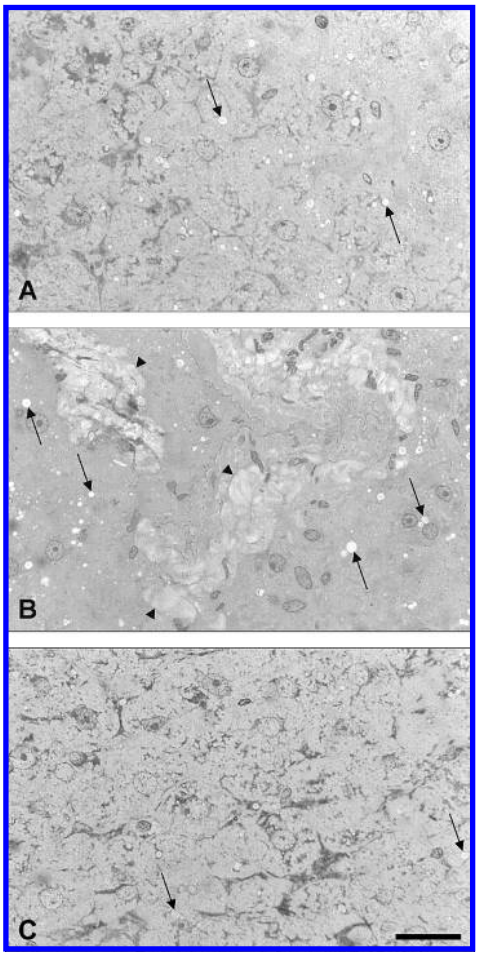

Figure 5. Micrographs of hepatic tissue obtained from pair-fed control (**A**), ethanol (**B**), and red wine (**C**) rats. The hepatocytes in **B** presented abundant lipid deposits (arrows), and portal spaces present fibrosis (arrowheads). In **A** and **C** the lipid deposition in hepatocytes was smaller and fibrosis in portal spaces was not visible. Scale bar, 10 μm.

ethanol metabolism, has been previously shown to be consistently increased after ethanol intake in a variety of experimental paradigms (*14*, *20*, *21*). The relevant role of SOD in protection against ethanol-induced free radicals has been demonstrated with SOD-1-knockout mice (*49*) and rats expressing recombinant adenovirus containing manganese-dependent SOD (*50*).

Oxidative damage to DNA, evaluated using 8-OHdG levels, was not prevented in EtOH animals when compared to the PFC group. In fact, it has been reported that ethanol intake is often associated with impaired utilization and/or increased deposition of iron in the liver (*15*). Consequently, hydroxyl radical production would be more likely to occur in this cellular location due to increased iron deposition in DNA/DNA-binding proteins, leading to enhanced 8-OHdG formation (*51*). However, direct DNA damage resulting from the acetaldehyde generated during ethanol metabolism, and the subsequent formation of adducts with DNA, and inhibition of DNA repair systems may also take place (*48*).

Morphological observations of liver sections revealed lipid accumulation and fibrosis in EtOH-treated rats that were not accompanied by inflammation or ALP activity alterations. ALP activity is normally employed as a marker for hepatic lesions (*52*). The progression of alcoholic liver injury is characterized by the initial appearance of fatty liver, followed by inflammation, necrosis, and apoptosis, culminating in fibrosis and, in some cases, cirrhosis (*12*, *53*). Interactions between lipid accumulation and activation of inflammatory cells, in the liver, are likely involved in exacerbating the early response of this organ to ethanol, leading to injury during long-term exposures (*12*).

It is known that chronic ethanol consumption results in hepatic lipid accumulation due to utilization of ethanol as the preferred fuel instead of fat. Additionally, ethanol oxidation induces alterations in the NAD^+/NADH ratio that promote lipogenesis through enhanced formation of acylglycerols (*11*−*13*). Oxidative stress could also be involved in the regulation of lipid metabolism by ethanol through regulatory molecules such as PPAR-α or sterol regulatory element-binding protein 1 activation (*54*). Also, alterations in the redox status, added to acetaldehyde, stimulate collagen synthesis (*11*, *12*). Activation of NF-κB and activator protein-1 in Kupffer cells can trigger the transcriptional up-regulation of genes encoding fibrogenic cytokines stimulating hepatic stellate cells (*15*).

The fact that this is a long-term study may justify the absence of alterations in ALP and NF-κB activities. We verified that, although there are structural changes in the liver, no alterations were found in these inflammatory markers. As in the initial stages of ethanol exposure its metabolism via alcohol dehydrogenase results in a shift of the hepatocyte redox state and accumulation of fatty acids, it is plausible to expect alterations of ALP and NF-κB. However, due to possible adaptation to the chronic presence of ethanol, the redox state of the hepatocyte probably changed to a new steady state (*12*). In other words, we can hypothesize that the earlier stages of the inflammatory process may have been resolved and homeostatic mechanisms may have shifted the balance to a new point. The existence of this new balance is consistent with the adaptations in enzyme activities and the unchanged levels of lipid peroxidation products and GSH/GSSG ratio observed. Nevertheless, it is important to stress that liver fibrosis can also be present in alcoholic liver diseases without accompanying inflammation, and both acetaldehyde and transforming growth factor have been described to be involved in this process (*12*, *13*, *55*).

The most interesting findings of the present study were observed in RW-treated rats as we failed to observe the changes in lipid peroxidation, CAT, SOD, and liver morphology that were present in EtOH-treated animals. More importantly, the levels of 8-OHdG were not increased and MDA was fairly reduced by RW treatment in comparison to PFC. Red wine is a rich source of polyphenols well-known for their antioxidant properties (*1*), possessing a plethora of other biological activities, namely, anti-inflammatory properties and the ability to interfere with lipid metabolism and absorption (*56*). Moreover, the iron-chelating and radical-scavenging properties of polyphenols present in red wine have been reported to protect against oxidative damage of DNA and lipids (*57*−*59*) and may be implicated in the improvement of hepatic oxidative status of RW-treated rats. Therefore, the reduced TBARS and the unaltered 8-OHdG, SOD, and CAT seen in this group of animals confirm the assumption of the protective role mediated by polyphenols. Additionally, the nonalcoholic components of red wine attenuate the ethanol-induced enhancement of CYP2E1 activity, contributing to a decrease in oxidative stress (*22*).

The activation of GST and Se-GPX by RW treatment may have contributed to the marked reduction of MDA levels observed in these animals in comparison to the other groups. The absence of such an effect in the EtOH group reinforces the modulation of these enzymes by the polyphenols present in RW (*45*). Also, the observed increase in GST and Se-GPX activities along with the decrease in GR activity can justify the decrease in hepatic GSX and GSH without alterations in GSSG levels producing the reduction in the GSH/GSSG ratio observed only in RW-treated animals. Hepatic conjugation of red wine

polyphenols with GSH (*47*) could additionally contribute to the lower hepatic content of GSH found in RW-treated rats.

The absence of morphological alterations in the liver of RW-treated animals further supports the protective role of nonalcoholic components of RW from ethanol-induced deleterious events. As the few available studies regarding the modulation by polyphenols of collagen and lipid hepatic accumulation reveal a decrease or prevention of the disturbing events (*60*−*62*), we can hypothesize that there were no transient changes in the inflammatory markers NF-κB and ALP in the initial periods of ethanol consumption. With regard to lipid accumulation, polyphenols have been described to possess a strong anti-hyperlipidemic effect through AMP-activated protein kinase, pointing to a novel mechanism leading to a decrease in lipid accumulation. However, we must bear in mind that wine consumption can induce cirrhosis, most particularly when associated with deficient nutrient intake (*10*).

The present results show noticeable improvements of several hepatic parameters following prolonged red wine consumption in contrast with the animals with similar ethanol intake. This striking difference may be related to the nonalcoholic components of red wine and highlights the paramount importance of considering the biological matrix when evaluating the effects of alcoholic beverage intake. Interestingly, a protective effect of modest wine drinking against nonalcoholic fatty liver disease in humans has recently been published (*63*).

ABBREVIATIONS USED

8-OHdG, 8-hydroxy-2′-deoxyguanosine; ALP, alkaline phosphatase; BSA, bovine serum albumin; bw, body weight; CAT, catalase; DTNB, 5,5-dithiobis(2-nitrobenzoic) acid; EtOH, ethanol; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase; GSX, total glutathione; L-Phe, L-phenylalanine; MDA, malondialdehyde; NBT, nitroblue tetrazolium; NF-κB, nuclear factor-kappa B; PFC, pair-fed control; *p*-NP, *p*-nitrophenol; *p*-NPP, *p*-nitrophenylphosphate; RW, red wine; Se-GPX, selenium-dependent glutathione peroxidase; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

ACKNOWLEDGMENT

We gratefully acknowledge Professor Manuel Paula-Barbosa from the Department of Anatomy, Faculty of Medicine, University of Porto, Portugal, for carefully reading the manuscript. We thank Professor Victor de Freitas for the quantification of RW polyphenols and Professor Delminda Neves for the measurement of plasma lipid profiles. The wine used in this work was a gift from "Quinta do Vale Meão," Douro, Portugal.

LITERATURE CITED

(1) German, J. B.; Walzem, R. L. The health benefits of wine. *Annu. Rev. Nutr.* **2000**, *20*, 561–593.

(2) Woodward, M.; Tunstall-Pedoe, H. Alcohol consumption, diet, coronary risk factors, and prevalent coronary heart disease in men and women in the Scottish heart health study. *J. Epidemiol. Community Health* **1995**, *49*, 354–362.

(3) Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523–1526.

(4) Gronbaek, M.; Becker, U.; Johansen, D.; Tonnesen, H.; Jensen, G.; Sorensen, T. I. Population based cohort study of the association between alcohol intake and cancer of the upper digestive tract. *Br. Med. J.* **1998**, *317*, 844–847.

(5) Prescott, E.; Gronbaek, M.; Becker, U.; Sorensen, T. I. Alcohol intake and the risk of lung cancer: influence of type of alcoholic beverage. *Am. J. Epidemiol.* **1999**, *149*, 463–470.

(6) Solfrizzi, V.; D'Introno, A.; Colacicco, A. M.; Capurso, C.; Del Parigi, A.; Baldassarre, G.; Scapicchio, P.; Scafato, E.; Amodio, M.; Capurso, A.; Panza, F. Alcohol consumption, mild cognitive impairment, and progression to dementia. *Neurology* **2007**, *68*, 1790–1799.

(7) Assunção, M.; Santos-Marques, M. J.; de Freitas, V.; Carvalho, F.; Andrade, J. P.; Lukoyanov, N. V.; Paula-Barbosa, M. M. Red wine antioxidants protect hippocampal neurons against ethanol-induced damage: a biochemical, morphological and behavioral study. *Neuroscience* **2007**, *146*, 1581–1592.

(8) de Freitas, V.; da Silva Porto, P.; Assunção, M.; Cadete-Leite, A.; Andrade, J. P.; Paula-Barbosa, M. M. Flavonoids from grape seeds prevent increased alcohol-induced neuronal lipofuscin formation. *Alcohol Alcohol.* **2004**, *39*, 303–311.

(9) Soleas, G. J.; Diamandis, E. P.; Goldberg, D. M. Wine as a biological fluid: history, production, and role in disease prevention. *J. Clin. Lab. Anal.* **1997**, *11*, 287–313.

(10) Lieber, C. S. Alcohol: its metabolism and interaction with nutrients. *Annu. Rev. Nutr.* **2000**, *20*, 395–430.

(11) Lieber, C. S. Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. *Alcohol* **2004**, *34*, 9–19.

(12) Nagy, L. E. Molecular aspects of alcohol metabolism: transcription factors involved in early ethanol-induced liver injury. *Annu. Rev. Nutr.* **2004**, *24*, 55–78.

(13) Das, S. K.; Vasudevan, D. M. Alcohol-induced oxidative stress. *Life Sci.* **2007**, *81*, 177–187.

(14) Oh, S. I.; Kim, C. I.; Chun, H. J.; Park, S. C. Chronic ethanol consumption affects glutathione status in rat liver. *J. Nutr.* **1998**, *128*, 758–763.

(15) Albano, E. Alcohol, oxidative stress and free radical damage. *Proc. Nutr. Soc.* **2006**, *65*, 278–290.

(16) Azzalis, L. A.; Junqueira, V. B.; Simon, K.; Giavarotti, L.; Silva, M. A.; Kogake, M.; Simizu, K.; Barros, S. B.; Fraga, C.; Porta, E. A. Prooxidant and antioxidant hepatic factors in rats chronically fed an ethanol regimen and treated with an acute dose of lindane. *Free Radical Biol. Med.* **1995**, *19*, 147–159.

(17) Lauterburg, B. H.; Davies, S.; Mitchell, J. R. Ethanol suppresses hepatic glutathione synthesis in rats in vivo. *J. Pharmacol. Exp. Ther.* **1984**, *230*, 7–11.

(18) Lu, S. C.; Huang, Z. Z.; Yang, J. M.; Tsukamoto, H. Effect of ethanol and high-fat feeding on hepatic γ-glutamylcysteine synthetase subunit expression in the rat. *Hepatology (Baltimore, MD)* **1999**, *30*, 209–214.

(19) Kawase, T.; Kato, S.; Lieber, C. S. Lipid peroxidation and antioxidant defense systems in rat liver after chronic ethanol feeding. *Hepatology (Baltimore, MD)* **1989**, *10*, 815–821.

(20) Roig, R.; Cascon, E.; Arola, L.; Blade, C.; Salvado, M. J. Moderate red wine consumption protects the rat against oxidation in vivo. *Life Sci.* **1999**, *64*, 1517–1524.

(21) Bailey, S. M.; Patel, V. B.; Young, T. A.; Asayama, K.; Cunningham, C. C. Chronic ethanol consumption alters the glutathione/glutathione peroxidase-1 system and protein oxidation status in rat liver. *Alcohol.: Clin. Exp. Res.* **2001**, *25*, 726–733.

(22) Orellana, M.; Varela, N.; Guajardo, V.; Araya, J.; Rodrigo, R. Modulation of rat liver cytochrome P450 activity by prolonged red wine consumption. *Comp. Biochem. Physiol.*−*Part C: Toxicol. Pharmacol.* **2002**, *131*, 161–166.

(23) Assunção, M.; Santos-Marques, M. J.; de Freitas, V.; Paula-Barbosa, M. M.; Carvalho, F. Modulation of rat cerebellum oxidative status by prolonged red wine consumption. *Addiction Biol.* **2008**, *13*, 337–344.

(24) Santos-Marques, M. J.; Carvalho, F.; Sousa, C.; Remião, F.; Vitorino, R.; Amado, F.; Ferreira, R.; Duarte, J. A.; Bastos, M. L. Cytotoxicity and cell signalling induced by continuous mild hyperthermia in freshly isolated mouse hepatocytes. *Toxicology* **2006**, *224*, 210–218.

(25) Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. *Methods Enzymol.* **1978**, *52*, 302–310.

(26) Balu, M.; Sangeetha, P.; Murali, G.; Panneerselvam, C. Modulatory role of grape seed extract on age-related oxidative DNA damage in central nervous system of rats. *Brain Res. Bull.* **2006**, *68*, 469–473.

(27) Simonetti, P.; Ciappellano, S.; Gardana, C.; Bramati, L.; Pietta, P. Procyanidins from *Vitis vinifera* seeds: in vivo effects on oxidative stress. *J. Agric. Food Chem.* **2002**, *50*, 6217–6221.
(28) Warholm, M.; Guthenberg, C.; von Bahr, C.; Mannervik, B. Glutathione transferases from human liver. *Methods Enzymol.* **1985**, *113*, 499–504.
(29) Flohé, L.; Günzler, W. A. Assays of glutathione peroxidase. *Methods Enzymol.* **1984**, *105*, 114–121.
(30) Carlberg, I.; Mannervik, B. Glutathione reductase. *Methods Enzymol.* **1985**, *113*, 484–490.
(31) Aebi, H. Catalase in vitro. *Methods Enzymol.* **1984**, *105*, 121–126.
(32) Flohé, L.; Ötting, F. Superoxide dismutase assays. *Methods Enzymol.* **1984**, *105*, 93–104.
(33) Martins, M. J.; Negrão, M. R.; Hipólito-Reis, C. Alkaline phosphatase from rat liver and kidney is differentially modulated. *Clin. Biochem.* **2001**, *34*, 463–468.
(34) Martins, M. J.; Negrão, M. R.; Hipólito-Reis, C.; Azevedo, I. Physiologic concentrations of bile salts inhibit rat hepatic alkaline phosphatase but not the intestinal isoenzyme. *Clin. Biochem.* **2000**, *33*, 611–617.
(35) Martins, M. J.; Negrão, M. R.; Hipólito-Reis, C.; Azevedo, I. Arginine and a polyarginine peptide inhibit alkaline phosphatase activity: possible consequences for cellular transport systems. *Clin. Biochem.* **2001**, *34*, 435–437.
(36) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(37) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
(38) Sarmento, A.; Soares-da-Silva, P.; Teixeira, A. A.; Azevedo, I. Effects of denervation induced by 6-hydroxydopamine on cell nucleus activity of arterial and cardiac cells of the dog. *J. Auton. Pharmacol.* **1987**, *7*, 119–126.
(39) French, S. W. Ethanol and hepatocellular injury. *Clin. Lab. Med.* **1996**, *16*, 289–306.
(40) Hoek, J. B.; Pastorino, J. G. Ethanol, oxidative stress, and cytokine-induced liver cell injury. *Alcohol* **2002**, *27*, 63–68.
(41) Gheorghiu, M.; Bara, C.; Pasarica, D.; Brasoveanu, L.; Bleotu, C.; Toparceanu, F.; Trandafir, T.; Diaconu, C. C. Ethanol-induced dysfunction of hepatocytes and leukocytes in patients without liver failure. *Roum. Arch. Microbiol. Immunol.* **2004**, *63*, 5–33.
(42) Ruf, J. C. Overview of epidemiological studies on wine, health and mortality. *Drugs Exp. Clin. Res.* **2003**, *29*, 173–179.
(43) Svegliati-Baroni, G.; Jezequel, A. M.; Orlandi, F. Wine: risk factors for liver disease and antifibrotic compounds. *Drugs Exp. Clin. Res.* **1999**, *25*, 143–145.
(44) Rodrigo, R.; Castillo, R.; Carrasco, R.; Huerta, P.; Moreno, M. Diminution of tissue lipid peroxidation in rats is related to the in vitro antioxidant capacity of wine. *Life Sci.* **2005**, *76*, 889–900.
(45) Masella, R.; Di Benedetto, R.; Vari, R.; Filesi, C.; Giovannini, C. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. *J. Nutr. Biochem.* **2005**, *16*, 577–586.
(46) Pompella, A.; Visvikis, A.; Paolicchi, A.; De Tata, V.; Casini, A. F. The changing faces of glutathione, a cellular protagonist. *Biochem. Pharmacol.* **2003**, *66*, 1499–1503.
(47) Spencer, J. P.; Abd-el-Mohsen, M. M.; Rice-Evans, C. Cellular uptake and metabolism of flavonoids and their metabolites: implications for their bioactivity. *Arch. Biochem. Biophys.* **2004**, *423*, 148–161.
(48) Seitz, H. K.; Stickel, F. Risk factors and mechanisms of hepatocarcinogenesis with special emphasis on alcohol and oxidative stress. *Biol. Chem.* **2006**, *387*, 349–360.
(49) Kessova, I. G.; Ho, Y. S.; Thung, S.; Cederbaum, A. I. Alcohol-induced liver injury in mice lacking Cu, Zn-superoxide dismutase. *Hepatology (Baltimore, MD)* **2003**, *38*, 1136–1145.
(50) Wheeler, M. D.; Nakagami, M.; Bradford, B. U.; Uesugi, T.; Mason, R. P.; Connor, H. D.; Dikalova, A.; Kadiiska, M.; Thurman, R. G. Overexpression of manganese superoxide dismutase prevents alcohol-induced liver injury in the rat. *J. Biol. Chem.* **2001**, *276*, 36664–36672.
(51) Halliwell, B.; Gutteridge, J. M. *Free Radicals in Biology and Medicine*, 4th ed.; Oxford University Press: Oxford, U.K., 2007.
(52) Van Hoof, V. O.; De Broe, M. E. Interpretation and clinical significance of alkaline phosphatase isoenzyme patterns. *Crit. Rev. Clin. Lab. Sci.* **1994**, *31*, 197–293.
(53) Becker, U.; Deis, A.; Sorensen, T. I.; Gronbaek, M.; Borch-Johnsen, K.; Muller, C. F.; Schnohr, P.; Jensen, G. Prediction of risk of liver disease by alcohol intake, sex, and age: a prospective population study. *Hepatology (Baltimore, MD)* **1996**, *23*, 1025–1029.
(54) You, M.; Crabb, D. W. Recent advances in alcoholic liver disease II. Minireview: molecular mechanisms of alcoholic fatty liver. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **2004**, *287*, G1–G6.
(55) Kato, J.; Sato, Y.; Inui, N.; Nakano, Y.; Takimoto, R.; Takada, K.; Kobune, M.; Kuroiwa, G.; Miyake, S.; Kohgo, Y.; Niitsu, Y. Ethanol induces transforming growth factor-α expression in hepatocytes, leading to stimulation of collagen synthesis by hepatic stellate cells. *Alcohol.: Clin. Exp. Res.* **2003**, *27*, 58S–63S.
(56) Nijveldt, R. J.; van Nood, E.; van Hoorn, D. E.; Boelens, P. G.; van Norren, K.; van Leeuwen, P. A. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **2001**, *74*, 418–425.
(57) Lodovici, M.; Guglielmi, F.; Casalini, C.; Meoni, M.; Cheynier, V.; Dolara, P. Antioxidant and radical scavenging properties in vitro of polyphenolic extracts from red wine. *Eur. J. Nutr.* **2001**, *40*, 74–77.
(58) Guo, M.; Perez, C.; Wei, Y.; Rapoza, E.; Su, G.; Bou-Abdallah, F.; Chasteen, N. D. Iron-binding properties of plant phenolics and cranberry's bio-effects. *Dalton Trans.* **2007**, *43*, 4951–4961.
(59) Faustino, R. S.; Clark, T. A.; Sobrattee, S.; Czubryl, M. P.; Pierce, G. N. Differential antioxidant properties of red wine in water soluble and lipid soluble peroxyl radical generating systems. *Mol. Cell. Biochem.* **2004**, *263*, 211–215.
(60) McKim, S. E.; Konno, A.; Gabele, E.; Uesugi, T.; Froh, M.; Sies, H.; Thurman, R. G.; Arteel, G. E. Cocoa extract protects against early alcohol-induced liver injury in the rat. *Arch. Biochem. Biophys.* **2002**, *406*, 40–46.
(61) Zang, M.; Xu, S.; Maitland-Toolan, K. A.; Zuccollo, A.; Hou, X.; Jiang, B.; Wierzbicki, M.; Verbeuren, T. J.; Cohen, R. A. Polyphenols stimulate AMP-activated protein kinase, lower lipids, and inhibit accelerated atherosclerosis in diabetic LDL receptor-deficient mice. *Diabetes* **2006**, *55*, 2180–2191.
(62) Kaviarasan, S.; Viswanathan, P.; Anuradha, C. V. Fenugreek seed (*Trigonella foenum graecum*) polyphenols inhibit ethanol-induced collagen and lipid accumulation in rat liver. *Cell Biol. Toxicol.* **2007**, *23*, 373–383.
(63) Dunn, W.; Xu, R.; Schwimmer, J. B. Modest wine drinking and decreased prevalence of suspected nonalcoholic fatty liver disease. *Hepatology (Baltimore, MD)* **2008**, *47*, 1947–1954.

Received February 18, 2009. Revised manuscript received May 25, 2009. Accepted June 11, 2009. This work received financial support from Fundação para a Ciência e a Tecnologia (FCT−Centro de Morfologia Experimental (CME), U121/94, and Centro de Farmacologia e Biopatologia Química (CFBQ), U38, POCTI, Feder, Programa Comunitário de Apoio, SFRH/BD/19497/2004 and SFRH/BPD/40110/2007.