Diminution of tissue lipid peroxidation in rats is related to the in vitro antioxidant capacity of wine

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Abstract

Wine polyphenols could reinforce the endogenous antioxidant system, thereby diminishing oxidative damage. Studies in chronic models to understand the relationship between the bioavailability of polyphenols and their biological effects are still lacking. The aim of the present study was to prove the hypothesis that the antioxidant capacity of wines in vitro is positively correlated with the antioxidant capacity of plasma and negatively correlated with tissue lipid peroxidation, after chronic wine consumption. Adult rats received: water (control group), wine having variable phenolic content, ethanol (12.5% v/v) or alcohol-free red wine, for 4 weeks. The antioxidant capacity of wines in vitro and that of plasma induced in vivo were assessed through the reduction of ferric iron (FRAP, ferric reducing ability of plasma). Lipid peroxidation (production of thiobarbituric acid reactive substances, TBARS), and the activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), were determined in kidney, liver and lung. The phenolic content of wines was positively correlated with their FRAP values in vitro ($r = 0.407$, $p < 0.002$). Also, the relationship between wine FRAP in vitro to its respective plasma value in vivo showed a positive correlation ($r = 0.433$, $p < 0.005$). Phenolic concentration of wine did not influence the activity of CAT, SOD and GSH-Px of the three organs studied, but it was negatively correlated with their production of TBARS ($r = -0.852$, -0.891 and -0.790 for kidney, liver and lung, respectively, $p < 0.001$). The present data provide evidence that the antioxidant capacity of wine in vitro implicates a homologous effect in vivo, thus helping to modulate tissue lipid peroxidation.

Keywords: FRAP; Wine; Lipid peroxidation; Polyphenols; Antioxidant enzymes

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Introduction

Epidemiological studies have demonstrated that regular consumption of moderate amounts of wine reduces the incidence of death from atherosclerosis and coronary artery disease (Kannel and Ellioson, 1996). This effect may be partly related to the ability of ethanol to increase the plasma concentration of the cardio-protective high-density lipoprotein (HDL) (Gaziano et al., 1993). In addition, chronic exposure to ethanol induces upregulation of antioxidant enzymes of rat liver and kidney (Roig et al., 1999), but beneficial effects of red wine surpass those merely attributable to alcohol (Grønbaeck et al., 1995). This enhanced protection may be due to the presence of polyphenols, strong antioxidant compounds particularly abundant in red wine (McDonald et al., 1998). These compounds are synthesized in the epidermal cells and in the seed of the grape, what explains why the white wine that is not made in the presence of these components shows low levels of polyphenols (Jamroz and Beltowski, 2001) in contrast with red wine that uses the skin of the grape in the process of fermentation. Polyphenols exert a large array of biological actions, such as free radical-scavenging, metal chelation and enzyme modulation (Pietta et al., 1998), as well as reduction of the susceptibility of low density lipoproteins (LDL) to oxidation both in vitro (Bertelli et al., 1996) and in vivo (Nigdikar et al., 1998). Also, polyphenols may participate in the regulation of vascular tone (Adriantsitohaina, 1999) or in the inhibition of platelet aggregation (Keevil et al., 2000). Clinical studies have shown a significant rise in serum antioxidant capacity after consumption of red wine (Whitehead et al., 1995; Durak et al., 1999), an effect also reported in rats (Rodrigo et al., 2002) which could reinforce the systemic endogenous antioxidant defense system. Also, attempts to characterize the antioxidant effect attributable to the non-alcoholic components of red wine have been reported. In this regard, reduction of the oxidation susceptibility of LDL in a dose-dependent manner was recently reported for pyraltin, a concentrate of wine polyphenols (Van Golde et al., 2004). Accordingly, the in vitro antioxidant capacity of red wine was suggested as a parameter accounting for the quality of wine and the interest in studying the relative capacity of wines to influence plasma FRAP was strengthened (Jamroz and Beltowski, 2001). Taken together, these data attribute the health benefits of wines to their antioxidant capacity, but studies correlating this parameter with the magnitude of the biological response in vivo are still lacking. The aim of the present study was to determine the relationship between the antioxidant capacity of wine in vitro and its biological effects in the rat related to the response of the antioxidant system in vivo.

Materials and methods

Wine samples

The wines used in the present study were forty-six samples of different types of Chilean wine, with a 12.5% ethanol concentration (Table 1). Total phenolic compounds were quantified following a spectrophotometric procedure at 726 nm (Reyes and Cisneros-Zevallos, 2003), with minor modifications. Total phenolic compounds were expressed as milligrams of chlorogenic acid (CGA)/mL from a standard curve.

Table 1
Samples of Chilean wines studied

Name, wine	Harvest	Valley
Urmeneta, Sauvignon Blanc*	2001	Central
Pionero, Merlot*	2001	Central
Pionero, Semillón*	1998	Central
Sta Emiliana, Merlot*	2002	Central
Antares, Cabernet Sauvignon*	2001	Central
Sta Carolina, Cabernet Sauvignon*	2001	Central
Sunrise, Merlot*	2002	Central
120 Tres Medallas, Chardonay*	2002	Central
Errázuriz, Cabernet Sauvignon:		
Errázuriz, very early harvest	2002	Central
Errázuriz, early harvest	2002	Central
Errázuriz, late harvest	2002	Central
Errázuriz, normal harvest	2002	Central
Errázuriz, without defollation	2002	Central
Errázuriz, normal defollated	2002	Central
Errázuriz, excessively defollated	2002	Central
Los Sarmientos de Tarapaca, Merlot*	2001	Central
Concha y Toro, Exportación*	2001	Central
Concha y Toro, Cabernet Sauvignon*	2002	Central
Carta Vieja, Cabernet Sauvignon*	2002	Maule
León de Tarapacá, Cabernet Sauvignon*	2001	Maipo
El Almendral, Vino dulce añejo*	2001	Maipo
Ventisquero, Syrah	2001	Maipo
Canepa, Reservada vino tinto*	2001	Maipo
Planella, Resevada vino tinto*	2001	Maipo
Hijuelas de Quillón, Vino tinto*	2001	Maipo
Doña Gabriela, Sauvignon Blanc	2000	Limarí
León de Tarapacá, Sauvignon Blanc	2001	Maipo
Sta Emiliana, Rosé*	2002	Central

* Two samples: early and late harvest per year.

Animals

One hundred and fifty four male Wistar rats weighing 200 ± 12 g, from the Departamento de Nutrición, Facultad de Medicina Universidad de Chile, were randomly assigned to one of four groups and each was allowed free access to a standard pellet diet (Champion SA, Santiago, Chile) and one of the following beverages, as the sole drinking fluid, for a period of four weeks: water (control group, n = 20), wine having 12.5% ethanol and different phenolic concentration (wine group, n = 114), aqueous solution of ethanol 12.5%, v/v (ethanol group, n = 10) and alcohol-free red wine (alcohol-free red wine group, n = 10). Red wine dealcoholization was performed on the red wine sample having the highest phenolic concentration (12.82 mg CGA/mL) by evaporation at 25 °C for 4 h, with vacuum, progressively and gradually, up to -3Mpa (Serafini et al., 1998). The animals were individually housed in wire cages and maintained at 25 °C in an animal room with a 12 h-light/dark cycle. The daily fluid intake was measured with graduated Richter tubes. The management of rats was carried out according to internationally

accepted ethical guidelines. The study protocol was approved by the Comité de Bioética, Facultad de Medicina, Universidad de Chile.

Antioxidant capacity of wine samples in vitro

Antioxidant capacity was measured according to the FRAP method, which was initially developed to assay plasma antioxidant capacity, but which can also be used to analyze other fluids. This method measures the ability of the antioxidants contained in a sample to reduce ferric-tripyridyltriazine (Fe^{+3}-TPTZ) to a ferrous form (Fe^{+2}) that absorbs light at 594 nm. The solution to be analyzed was first diluted 10–200 fold with deionized water to fit within the linearity range. The working FRAP reagent was prepared ex tempore by mixing 10 volumes of 300 mmol/l acetate buffer, pH 3.6, with 1 volume of 10 mmol/TPTZ (2,4,6-tripyridyl-s-triazine) (Sigma-Aldrich) in 40 mM HCl and with 1 volume of 20 mM $FeCl_3$. Then, 3 mL of a working FRAP reagent was warmed to 37 °C and this absorbency was read against water at 594 nm (reagent blank). Subsequently, 100 μl of sample and 300 μl of deionized water was added to the FRAP reagent and the absorbency was monitored for 8 minutes. However, because there was little increase in absorbency between 4 and 8 minutes of incubation, it was necessary to use its value after 4 min for further calculations. The sample was incubated at 37 °C throughout the monitoring period. The reagent blank and sample (the absorbency of the appropriately diluted sample without FRAP reagent) were subtracted from the final absorbency at 4 min and the result was compared with the standard curve prepared using different concentrations in the range of 100–1000 mM $FeSO_4$. The linearity of the relationship between Fe^{2+} concentration and absorbency was very good within this concentration range ($r^2 = 0.98$). The results were corrected for dilution and expressed in μM Fe^{2+}.

Preparation of samples for the biochemical analyses

After the treatment blood samples were obtained from the carotid artery under anesthesia with sodium pentobarbital (40 mg/kg, ip), approximately an hour after the last fluid consumption. The samples were collected in plastic tubes with 5% EDTA and served to measure the ferric reducing ability of plasma (FRAP) (Benzie and Strain, 1996), expressing the antioxidant capacity of plasma in μM. Blood ethanol levels at the time of sacrifice were determined by an enzymatic micromethod (Brink et al., 1954). Then, the livers, kidneys and lungs, all were perfused with buffer Tris 0.01M pH 7.0, prior to extraction and homogenization in the same solution. The organs were blotted on filter paper, weighed, and minced in ice-cold 0.25 M sucrose and homogenized. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined in plasma samples by a spectrophotometric assay using a commercial kit (Sigma).

Antioxidant enzymes

Homogenates of the tissues in 0.25 M sucrose were used for the determination of superoxide dismutase (SOD) activity; and 1.15% KCl-0.010 M Tris pH 7.40 buffer was used to determine the activities of both catalase (CAT) and glutathione peroxidase (GSH-Px). The SOD assay is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11btetrahydro-3,9,10-trihydroxybenzo(c)-fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbency at 525 nm (Nebot et al., 1993). One SOD unit (U) is defined as the activity that doubles the autoxidation

background. The results were expressed as U/mg protein. CAT activity was assayed from the kinetic of breakdown of hydrogen peroxide at 240 nm (Aebi, 1974) by the supernatant of 2,400 g; and expressed on the basis of the rate constant of the first order reaction (k) /mg protein. Soluble GSH-Px activity was measured in the cytosolic fraction (supernatant of 100,000 g) by a spectrophotometric method based on the reduction of glutathione disulfide coupled to the NADPH oxidation by glutathione reductase (Flohé and Gunzier, 1984). One GSH-Px unit is defined as the activity that oxidizes 1 μmol NADPH per minute. The activity of GSH-Px was expressed as U/mg protein.

Lipid peroxidation

The assay for products of lipid peroxides was performed spectrophotometrically at 532 nm by the thiobarbituric acid reaction at pH 3.5, followed by solvent extraction with a mixture of n-butanol/pyridine (15/1, v/v) (Ohkawa et al., 1979). Tetramethoxy-propane was used as the external standard and lipid peroxidation was expressed as thiobarbituric acid reactive substances (TBARS)/mg protein.

Materials

The reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany) and Riedel-de Häen (Germany), and were of the highest commercial grade available.

Statistical analyses

The results were expressed as means ± SEM. For study of correlation Spearman's test was applied. The analysis of the activity of antioxidant enzymes was performed by one-way analysis of variance (ANOVA) plus the Bonferroni's test. The differences were considered statistically significant at $p < 0.05$.

Results

Body weight gain, fluid intake and blood ethanol levels

Body weight gain (g/kg/day) and fluid intake (mL/kg/day), for control, ethanol, red wine and alcohol-free red wine groups, and blood ethanol levels for ethanol and red wine groups, during the experimental feeding period (28 d), are shown in Table 2. There were no significant differences in fluid intake and

Table 2
Body weight gain, fluid intake and blood ethanol levels

	Control (n = 20)	Wine (n = 10)	Ethanol (n = 10)	Alcohol-free red wine (n = 10)
Body weight gain (g/kg/day)	10.1 ± 0.3	9.7 ± 0.4	9.3 ± 0.5	10.2 ± 0.6
Fluid intake (mL/kg/day)	9.90 ± 0.3	11.50 ± 0.4	10.4 ± 0.5	9.6 ± 0.8
Ethanol intake (g/kg/day)	–	4.5 ± 0.3	4.1 ± 0.2	–
Ethanol levels (mM)	–	1.13 ± 0.09	1.05 ± 0.11	–

Values are means ± SEM, number of rats in parenthesis.

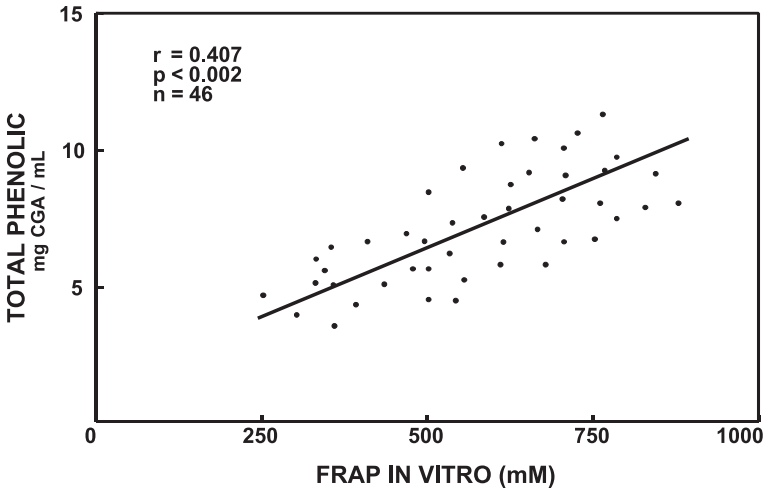

Fig. 1. The relationship between wine FRAP in vitro and its total phenolics content. CGA, chlorogenic acid. The correlation was established through Spearman's test ($r = 0.407$; $p < 0.002$; $n = 46$). Results are means of two rats each in duplicate.

body weight gain between the groups. Blood ethanol levels of ethanol group were comparable to those of red wine group.

Phenolic concentration and antioxidant capacity in vitro of wine samples

Fig. 1 shows the relationship between FRAP in vitro and total phenolics, reaching a high correlation ($r = 0.407$; $p < 0.002$; $n = 46$). The FRAP values of wines ranged from 258 to 895 mM Fe^{2+} and the maximum phenolic content was 12.82 mg CGA/mL.

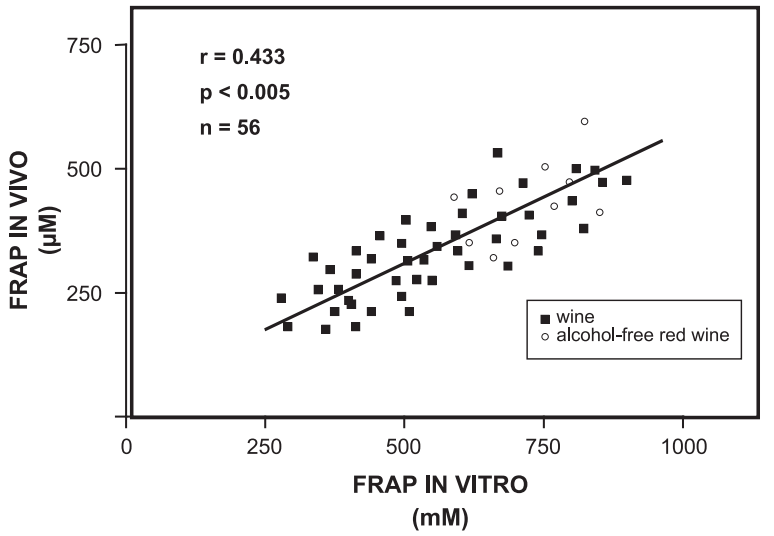

Fig. 2. The relationship between wine and alcohol free red wine FRAP values in vitro and the respective plasma value obtained in vivo by its chronic administration. The correlation was established through Spearman's test ($r = 0.433$; $p < 0.005$; $n = 56$). Results are means of two rats each in duplicate.

Relationship between the antioxidant capacity in vitro and in vivo

The relationship between the parameter FRAP in vitro, directly measured in the wines and alcohol-free red wine samples, versus their respective plasma values obtained in vivo by chronic administration to rats is shown in Fig. 2. These two parameters were positively correlated ($r = 0.433$; $p < 0.005$; $n = 56$).

Antioxidant enzymes

The effect of wine (12.5% ethanol, v/v) consumption on three antioxidant enzymes is shown in Table 3. Despite the fact that these wines have different phenolic concentrations (3.58; 7.26 and 12.82 mg CGA/mL, for Sauvignon Blank, Rosé and Cabernet Sauvignon, respectively), there were no significant differences in the activity of CAT, SOD or GSH-Px in the kidney, liver and lung between the groups.

Relationship between tissue lipid peroxidation and plasma antioxidant capacity

The relationship between the results obtained for lipid peroxidation (nmol TBARS/mg protein) and FRAP in different organs (kidney, liver and lung) for control (n = 20), red wine of different phenolic concentration (n = 46), ethanol (n = 10) and alcohol-free red wine (n = 10) groups are shown in Fig. 3. The TBARS levels in kidney were lesser than those found in lung and liver. It can be seen that FRAP

Table 3
Effect of phenolic content of red wine on the activity of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) of kidney, liver and lung of the rat

	CAT k/mg ptotein	SOD U/mg protein	GSH-Px U/mg protein
Kidney			
Control	0.43 ± 0.01	13.45 ± 0.63	0.095 ± 0.002
S. B.[a,*]	0.41 ± 0.02	13.01 ± 0.76	0.093 ± 0.002
R.[b]	0.45 ± 0.03	14.01 ± 0.54	0.098 ± 0.001
C. S.[c]	0.51 ± 0.01	15.58 ± 0.66	0.099 ± 0.003
Liver			
Control	0.27 ± 0.01	10.45 ± 0.75	0.082 ± 0.003
S. B.[a]	0.25 ± 0.02	10.01 ± 0.76	0.081 ± 0.004
R.[b]	0.31 ± 0.03	11.08 ± 0.68	0.088 ± 0.007
C. S.[c]	0.33 ± 0.01	11.58 ± 0.77	0.092 ± 0.006
Lung			
Control	0.075 ± 0.08	7.71 ± 0.69	0.069 ± 0.016
S. B.[a]	0.077 ± 0.02	6.55 ± 0.56	0.071 ± 0.011
R.[a]	0.079 ± 0.06	7.78 ± 0.81	0.078 ± 0.009
C. S.[c]	0.081 ± 0.05	8.08 ± 0.67	0.082 ± 0.004

Values are means ± SEM (n = 6 rats for each group).
S.B. = Sauvignon Blank; R = Rosé; C.S. = Cabernet Sauvignon.
[a] 3.58 mg CGA/mL.
[b] 7.26 mg CGA/mL.
[c] 12.82 mg CGA/mL (CGA: chlorogenic acid).
* Ethanol concentration for every wine was 12.5% (v/v).

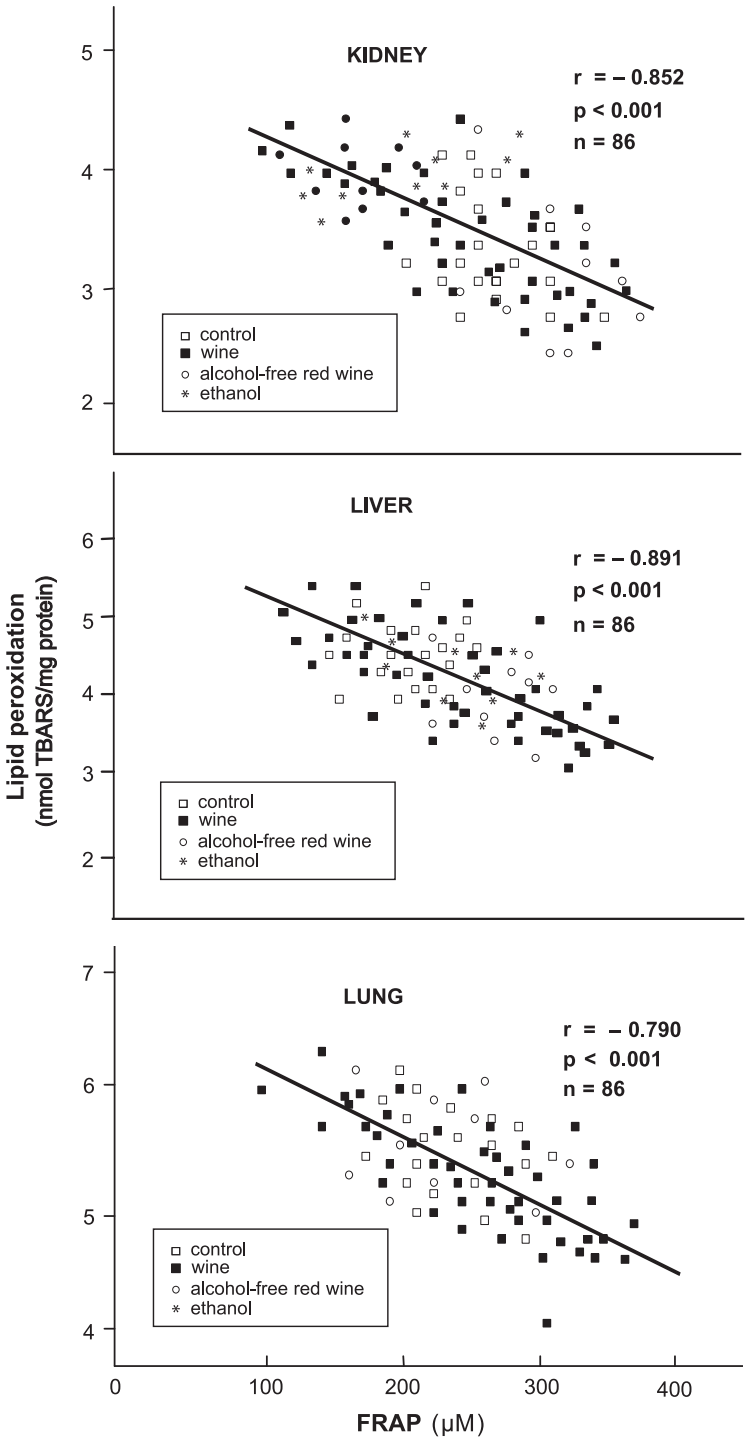

Fig. 3. Effect of antioxidant capacity of wine in vitro on lipid peroxidation of kidney, liver and lung of the rat. The correlation was established through Spearman's test.

Table 4
Effects of wine, ethanol and alcohol-free red wine on serum liver enzymes and lipid peroxidation in kidney, liver and lung

	Contro (n = 20)	Wine (n = 10)	Ethanol (n = 10)	Alcohol-free red wine (n = 10)
ALT (U/L)	27.3 ± 3.2	28.0 ± 4.0	34.4 ± 3.7*	26.0 ± 2.9
AST (U/L)	56.2 ± 2.4	55.8 ± 4.2	75.1 ± 4.0*	55.3 ± 3.8
TBARS (nmol/mg prot)				
Kidney	3.81 ± 0.31	3.65 ± 0.36	3.96 ± 0.40	3.72 ± 0.37
Liver	4.73 ± 0.50	4.38 ± 0.29	4.72 ± 0.31	4.48 ± 0.40
Lung	5.84 ± 0.50	5.38 ± 0.45	6.30 ± 0.52	5.45 ± 0.33

Values are means ± SEM, number of rats in parenthesis. ALT, alanine aminotransferase. AST, aspartate aminotransferase. U, units of activity. TBARS, thiobarbituric acid reactant substances.

* $p < 0.05$ vs control.

was negatively correlated with the tissue levels of TBARS obtained for the three organs ($r = -0.852$, -0.891 and -0.790, for kidney, liver and lung, respectively).

Lipid peroxidation and serum liver enzymes

A comparison of lipid peroxidation found in the wine sample showing the highest phenolic content with the average of control, ethanol and alcohol-free red wine groups, together with serum liver enzymes for the same groups appears in Table 4. Serum ALT and AST (U/L) of ethanol group were 40 and 60% higher, but no significant differences were found in the wine and alcohol-free red wine groups, respectively, compared with control values ($p < 0.05$). Lipid peroxidation of kidney, liver and lung was not changed by red wine and alcohol-free red wine consumption, but following ethanol consumption these three organs showed 30, 42 and 25% increases, respectively ($p < 0.05$) (Table 4).

Discussion

The present data demonstrate that the antioxidant capacity of wines in vitro correlates positively with the antioxidant defenses induced both in plasma and in some organs of the rat by chronic exposure. In this respect, the evidence of a protective effect of wine, particularly of red wine, could be attributed to the antioxidant actions of polyphenols (Pietta et al., 1998; Rodrigo and Rivera, 2002). This family of compounds includes substances such as anthocyanins, resveratrol, galic acid, catechin, myricetin, quercetin, and others, likely responsible for an enhancement of the antioxidant capacity of plasma (Ferrali et al., 1997; Durak et al., 1999), and for the upregulation of the antioxidant enzymes (Roig et al., 1999; Rodrigo et al., 2002). Although the pharmacokinetics and bioavailability of these compounds are poorly understood, they have been found in human plasma and urine after wine consumption (German and Walzem, 2000).

Our results, which showed a more marked elevation of plasma FRAP in the rat for red wines having higher phenolic concentration, are in agreement with similar data reported in human subjects under acute exposure to red wine (Whitehead et al., 1995; Maxwell et al., 1994), in whom this effect was also accompanied by an intracellular increase of the antioxidant capacity in erythrocytes (Durak et al., 1999). Therefore, it should be expected a prevention against lipid peroxidation by red wine in vivo, derived from

its antioxidant properties, at least partly explained by its abundance in polyphenols, which may prevent cellular peroxidation reactions and lessen atherosclerotic complications through inhibition of LDL oxidation. However, studies about the influence of chronic wine consumption on plasma levels of FRAP have not been carried out previously. For the first time, the present data demonstrate a high correlation between the parameter FRAP in vitro and in vivo, after chronic exposure to wine (Fig. 2), an effect which is also dependent upon the phenolic contents (Fig. 1). Interestingly, ethanol-induced toxic effects, assessed by increased serum transaminases and increased tissue lipid peroxidation, do not accompany the beneficial effects of wine (Table 4), thus suggesting a protection by the non-alcoholic components. Moreover, It could be expected that the wine-induced increase of antioxidant capacity of plasma contribute to the enhancement of the antioxidant defense systems of organs like kidney, liver and lung, due to their well-known high perfusion rate. Accordingly, the results here communicated clearly demonstrate a diminution of tissue lipid peroxidation in these organs, since TBARS production was negatively correlated with plasma values of FRAP (Fig. 3).

The lack of modulation of the antioxidant enzymes by wine polyphenols could highlight the role of the antioxidant capacity of plasma in the prevention of tissue lipid peroxidation. Consequently, lipid peroxidation in the three studied organs would be a function of wine phenolic concentration (Figs. 1 and 3). Nevertheless, the presence of ethanol is known to be responsible for the upregulation of CAT and GSH-Px (Rodrigo et al., 2002). Thus, our data support the hypothesis that, apart from the ethanol effect on the enzymatic antioxidant system, in vitro values of wine FRAP could serve to assess its potential biological benefit as antioxidant agent in vivo, thereby protecting the organs against the oxidative challenges caused by ROS. This finding gives rise to an empirical way of inferring the bioavailability of wine antioxidants and contributes to assess the effect of wine on antioxidant defenses in vivo. In addition, these data contribute to validate FRAP assessment as a useful parameter suitable to estimate the quality of wines in terms of their protective effect against the oxidative challenges. On this line, this study provides new biochemical evidence supporting the contribution of wine polyphenols to diminish the risk or progression of chronic diseases. Nevertheless, further studies in humans are required to confirm this view.

However, alternative mechanisms exerted by wine polyphenols on other antioxidant systems should not be discarded, such as protection of other organs against the oxidative damage. For example, it has been recently reported that the inhibition by leptin of two antioxidant enzymes contained in plasma lipoproteins (paraoxonase 1 and platelet activating factor-acetylhydrolase) could play a role in the atherogenesis of hyperleptinemic obese individuals (Beltwoski et al., 2003). The modulation by wine polyphenols of these antioxidant enzymes, as well as of other ROS producing enzymes, needs to be explored to get a better understanding of wine antioxidant effect.

In summary, the present data provide evidence that the measurement of the antioxidant capacity of red wine in vitro implicates a homologous effect in vivo, thus contributing to diminish lipid peroxidation in kidney, liver and lung in a dose-dependent manner. The implementation of this method could be applied to other sources of antioxidants of the diet.

Acknowledgements

This research was supported by the Fondo Nacional de Ciencia y Tecnología (FONDECYT, grant 1990784). The technical assistance of Diego Soto is also acknowledged.

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