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Moderate ethanol ingestion, redox status, and cardiovascular system in the rat

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Abstract

Moderate intake of alcoholic beverages decreases the incidence of cardiovascular pathologies, but it is in dispute if cardioprotective effects are due to ethanol, to polyphenolic compounds present in beverages or to a combination of both. In humans, effects of high, moderate, and low doses of alcoholic beverages are widely studied, but effects of pure alcohol remain unclear. On the other hand, experiments with laboratory animals are centered on high toxicological doses of ethanol but not on low doses. In the present study, we have aimed to mimic in the rat the pattern of alcohol intake in Mediterranean population. Alcohol ingestion is spread along the day and not always related to solid food consumption. We tried to define the beneficial and harmful effects of pure ethanol ingestion without polyphenol's influence. Experimental rats were given 1% ethanol in their drinking water for 30 days, resulting in a daily ingestion of 0.27 mL of ethanol/rat/d. Ethanol ingestion did not cause deleterious effects on the general status of the animals, but it decreased cholesterol, triglycerides, and catecholamine stores' rate of utilization in peripheral sympathetic system. Moreover, ethanol lowered pulmonary arterial pressure and did not alter systemic arterial pressure. In the liver, the reduced glutathione/oxidized glutathione ratio was augmented and lipid peroxide, superoxide dismutase, and glutathione peroxidase activities were decreased. However, catalase activity was unaltered. Liver cytochrome P4502E1 distribution and protein level and activity were unchanged by ethanol ingestion. Data indicate a lack of harmful effects and underscore a set of potentially beneficial effects of this dose of ethanol.

Keywords: Oxygen reactive species (ROS); Antioxidant enzymes; Cardiovascular system; Pulmonary arterial hypertension; Cholesterol; Cytochrome P4502E1

Introduction

Ethanol is an important component of wine and has constituted as an important voluntary component of human diet for thousands of years, and yet, in spite of the stirring of the field produced by the formulation of the French paradox (Ferrieres, 2004), there are not conclusive doctrines on the effects of moderate alcohol ingestion in human health in the epidemiological and observational studies (Blackhurst and Marais, 2005; Goldberg et al., 2003; Govoni et al., 1994). This status reveals that there are many variables associated to human drinking (e.g., amount, type of beverage, form or cadence of ethanol ingestion, and other lifestyle and uncontrolled psychosocial variables). The margin of ethanol ingestion considered in studies is very wide, with moderate ingestion having an upper limit of around 40 g/d for adult men (Govoni et al., 1994; Hines et al., 2001) and a high toxicological ingestion with undefined limits; some authors had tried to elucidate what might be considered as physiological or toxicological effects of ethanol (Bonnefont-Rousselot et al., 2001; McDonough, 2003). The type of beverage is another variable of interest. Thus, in epidemiological studies, the beneficial effects of moderate ethanol ingestion are usually (De Gaetano et al., 2003; Di Castelnuovo et al., 2002; Gronbaek et al., 1995), but not always (Klatsky et al., 1997; Mukamal et al., 2003; Rimm et al., 1996), best evidenced if the beverage is rich in polyphenols and other antioxidant compounds (e.g., red wine and dark beer; see Li and Mukamal, 2004). However, the poor bioavailability and metabolism of the antioxidants present in beverages renders difficulty in the acceptance of physiologically relevant effects (Li and Mukamal, 2004; Williamson and Manach, 2005). In this regard, Rimm et al. (1996) concluded that

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alcohol consumption can be linked directly to an individual's risk of coronary heart disease, providing strong evidence that all alcoholic drinks are linked with lower risk of coronary heart disease. Similar conclusions have been attained by Klatsky et al. (1997) and Li and Mukamal (2004). The cadence of drinking adds complexity in evaluating the effects of ethanol in epidemiological studies. With the same amount of ingestion per week, there are clear differences; beneficial effects are best evidenced in steady daily moderate drinkers versus weekend-oriented drinkers (Ferrieres, 2004; Govoni et al., 1994). Furthermore, it is conceivable that for the same amount of ethanol ingested per diem, peak plasma ethanol concentrations must be quite different in meals-related drinkers (Mediterranean cultures) versus happy hour-type drinkers.

However, experimental studies in animal models have been oriented toward the high toxicological doses (McDonough, 2003), with very few studies using physiologically relevant doses of ethanol and with limited parameters monitored (Arola et al., 1997).

Our aim in the present study has been to administer ethanol to rats in the amount and cadence that can correspond to the most common drinking behavior in Mediterranean countries: two to three glasses of wine with 11% of ethanol distributed around the day (200–300 mL of wine/d or 18–27 g of ethanol/d). To achieve this level of ingestion and a comparable drinking cadence in rats, we determined in pilot experiments that adding 1% ethanol (vol/vol) to the drinking water resulted in ad libitum daily intake of 0.22 g of ethanol/rat/d. As it is well known, rats metabolize ethanol threefold faster than human, 300 mg/kg body weight/h in rats (Mikata et al., 1963) versus 100 mg/kg body weight/h in humans (Wartburg, 1967). Consequently, this 1% ethanol administration in rats is equivalent to 18 g in a standard human male, a value well within the recommendation for cardiovascular protection (Costanzo et al., 2010). Our data evidenced a total absence of harmful effects and not only that, an amelioration of certain parameters that lend support to the notion that moderate and spaced alcohol drinking might have beneficial health effects.

Materials and methods

Experimental animals

Male adult Wistar rats had free access to standard rat solid diet (A04; Panlab SL, Barcelona, Spain) and drinking water until sacrifice. Rats were weighed at the start and at the end of the experiments. For a period of 30 days, the rats received drinking water with 1% ethanol (experimental group) or regular tap water (control group); both beverages were freshly prepared once a day with a daily measurement of water ingestion. We did not use nasogastric intubation because it is not a voluntary consumption of drink. Experimental protocols were performed in anesthetized rats (we used sodium pentobarbital 60 mg/kg body weight or ketamine 133 mg/kg in the experiments of blood pressure recordings). The Institutional Committee of the University of Valladolid for Animal Care and Use approved the protocols.

Blood and tissue removal

Blood was obtained by direct cardiac puncture after an ample thoracotomy. The plasma was obtained after 2,000*g*, 15 min centrifugation of whole blood at 4°C and was assayed immediately or stored at −80°C.

Livers were quickly excised from rats, washed in ice-cold saline, immediately immersed in liquid nitrogen to freeze, and stored at −80°C.

Plasma ethanol levels

Ethanol analysis was performed in plasma obtained from EDTA collected blood using the enzymatic micromethod of Brink et al. (1954). The low plasma levels of ethanol forced the introduction of modifications in the method consisting the reduction of the assay volume to 2 mL, whereas keeping the plasma sample in 100 μL. With these modifications, the lowest detected ethanol levels were below 0.1 mM or 0.59 μg/100 μL.

Basic hematology, blood biochemistry, and blood lipids determination

Complete EDTA–blood was used for cellular blood counting in an Advia® Flow Cytometer (Bayer AG, Leverkusen, Germany), whereas heparinized plasma was used to determine glucose, hepatic enzymes, and lipids in a modular turbidimeter analyzer (Roche Diagnostics, F. Hoffmann-La Roche Ltd., Basel, Switzerland).

Determination of plasma nitrites and nitrates

Plasma was obtained as described previously and was assayed immediately or stored at −80°C. A method (Granger et al., 1996) based in Griess reaction was used to measure nitrites and nitrates.

Determination of endothelial nitric oxide synthase in the lung

The endothelial nitric oxide synthase (eNOS) protein present in lung homogenates was determined by Western blot. Lung protein samples (50 μg of protein determined by Lowry) were separated by *sodium* dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (MiniProtan®, Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, MA). PVDF membranes were probed with mouse monoclonal or rabbit antibodies (Becton, Dickinson and Company [BD]; New Jersey, NJ) against eNOS and inducible nitric oxide synthase (iNOS) (diluted at 1:400 or 1:500). As second antibodies, we used anti-mouse or anti-rabbit IgG-horseradish

peroxidase both from BD at a dilution of 1:6,000 or 1:4,000. Membranes were probed with the enhanced chemiluminescence (ECL) PLUS kit (Santa Cruz Biotechnology Inc., California, CA).

Catecholamines and serotonin determination in plasma and serotonin in platelets

Citrated blood was centrifuged to obtain the platelet-rich plasma (PRP). PRP was again centrifuged and supernatant plasma was withdrawn into tubes containing 60 mg/mL sodium metabisulfite and frozen at −80°C until plasma catecholamines (CA) and plasma serotonin (5-HT) analysis was performed. Pellet (platelet concentrate) was resuspended in prewarmed external medium and divided into two aliquots. One aliquot was used to perform a platelet count and the other was centrifuged to obtain a pellet (washed platelet concentrate), which was resuspended in a mixture of ultrapure water: 10% metaphosphoric acid (1:1). After incubation (0°C for 10 min) and centrifugation, supernatant was frozen at −80°C until analysis of 5-HT in platelets. For CA and 5-HT plasma analyses, an aliquot of supernatant plasma (500 μL) was adsorbed in Waters Oasis-hydrophilic−lipophilic balance cartridges (Waters, Milford, MA), eluted with 0.5 mL of citric acid (26.7 mM) and 2.5% methanol (pH = 2.9) for *high-performance liquid chromatography with electrochemical detection (*HPLC−ED) analysis, as described by Raggi et al. (1999). For 5-HT analysis in platelets, an aliquot of supernatant coming from washed platelet concentrate (usually 100 μL) was injected into the HPLC system for 5-HT separation and quantification. Chromatographic conditions and quantification procedures have previously been described (Vicario et al., 2000), except for the mobile phase that consisted a mixture of sodium dihydrogen phosphate (150 mM) and methanol (6%) (pH 3.6).

Determination of CA content and ^{3}H-CA synthesis rate in renal artery

Renal artery (RA) were dissected out from the rats and immediately placed in a lucite chamber containing ice-cold saline and dissected free of connective tissue. Segments approximately of 1 cm long were used. The rate of synthesis of CA was measured by incubating segments of RA in 600 μL of Tyrode solution containing the CA precursor ^{3}H-tyrosine and enzyme cofactors as previously described (Vicario et al., 2000). RAs were weighed and homogenized (perchloric acid 0.1 N/EDTA 0.1 mM, 300 μL). The homogenates were centrifuged and aliquots (50 μL) of the supernatants were directly injected in the HPLC system. The mobile phase was the same used for plasma CA. Fractions of the column effluent were collected at 1 mL/min for 30−35 min and counted in Liquid Scintillation to determine the ^{3}H-CA synthesis rate. Amounts of ^{3}H-CA and ^{3}H-tyrosine were calculated by adding the disintegrations per minute (dpm) present in the correspondent fractions after subtraction of the blanks (dpm present in the fractions between peaks). Identification and quantification of CA content were done against external standards.

Measurement of systemic arterial pressure and pulmonary arterial pressure

Ketamine anaesthetized rats were tracheostomized and ventilated with room air (Hardard Rodent Ventilator 683; South Natick, Massachusetts, MA) at a frequency of 40 cycles/min and a positive expiratory pressure of 2 cm H_2O. Arterial pressure (AP) was recorded in the carotid artery and pulmonary arterial pressure (PAP) was recorded in the pulmonary artery, both with a catheters connected to a pressure transducer to record systemic or systolic and diastolic pressures. Both transducers were connected to an acquisition card (Power Lab 16SP; ADI Instruments, Castle Hill, Australia) that sent the signal to the computer for instant to instant monitoring of pressures and stored for offline analysis. Transducer calibration was made with a mercury manometer.

Determination of plasma endothelin 1

Plasma was assayed immediately with the ELISA assay of Biomedica (Biomedica Medizinprodukte, Vienna, Austria) or stored at −80°C.

Determination of angiotensin-converting enzyme

Angiotensin-converting enzyme (ACE) levels were determined with a colorimetric and enzymatic assay kit following the supplier instructions (Trinity Biotech Plc, Co Wicklow, Ireland).

Measurement of reduced and oxidized glutathione

Reduced and oxidized glutathione (GSH and GSSG) was determined by Griffith method as recently described in detail (Gonzalez et al., 2004).

Measurement of lipid peroxides

Livers were homogenized in phosphate buffer (PB) 20 mM (pH = 7.4, wt/vol = 0.2). The homogenates were centrifuged and we added to 1 mL of supernatant 10 μL of 0.5 M butylated hydroxytoluene in acetonitrile to prevent the oxidation of the sample, and lipid peroxides (LPOs) levels were determined immediately according to the instructions given by the supplier of the assay kit (Bioxytech LPO-586; OXIS Health Products, Portland, OR).

Measurement of glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was assayed as described (Paglia and Valentine, 1967). The assay measures the disappearance of NADPH because of a coupled reaction; firstly GPx oxidizes GSH to GSSG in the presence of peroxide and secondly, GSSG is back reduced to GSH

by glutathione reductase using NADPH as donor of reduction equivalents. The activity of GPx in the sample (Δabsorbance/min) was calculated on the basis of the molar extinction coefficient for NADPH (6220 UA/mol). One unit of activity is equal to one micromole NADPH oxidized min^{-1} mg^{-1} protein.

Catalase activity in the liver

Livers were homogenized in cold 50 mM PB (pH = 7 and wt/vol = 0.05–0.10) containing 1 mM EDTA and 1% Triton X-100. The homogenates were centrifuged and the supernatants were used to measure catalase (CAT) activity immediately or stored at −80°C until the assay. The CAT activity was determined by using a titrimetric determination based on the CAT assay kit from Sigma (CAT100; Sigma–Aldrich, St. Louis, MO). One unit of CAT activity equals the micromole of hydrogen peroxide (H_2O_2) degraded per minute per milligram of protein at pH = 7 at 25°C at a substrate concentration of 50 mM of H_2O_2.

Superoxide dismutase activity in the liver

Livers were homogenized in cold 0.25 M sucrose buffer (pH = 7.4 and wt/vol = 0.1) containing 10 mM Tris and 1 mM EDTA. The homogenates were centrifuged and the supernatant was used to measure superoxide dismutase (SOD) activity immediately or stored at −80°C. The SOD activity was determined with an indirect method (SOD determination kit; Fluka, Madrid, Spain). SOD is expressed as activity units, one unit being the amount of enzyme capable of inhibiting the reduction of cytochrome c by 50% in a coupled system with xanthine oxidase at pH 7.8 and 25°C in a 3 mL reaction mixture.

Preparation of hepatic microsomal fraction and measurement of CYP4502E1 activity

A fragment of cold saline washed liver was used to obtain microsomal fractions following the procedure as previously described (Dávila-Borja et al., 2007). Microsomal fractions were stored at −80°C. Protein concentration in the microsomal fractions was determined according to Lowry. CYP2E1 activity was measured by the rate of oxidation of p-nitrophenol to p-nitrocatecol in the presence of NADPH (Reinke and Moyer, 1985). The reaction was performed with 1 mg of microsomal protein for 10 min at 37°C. The results are expressed as nanomole/minute/milligram of protein.

Western blot of CYP4502E1 in hepatic microsomal fraction

Microsomal protein samples (approximately 6 μg protein) were separated by 10% SDS-PAGE and transferred to PVDF membranes. PVDF membranes were probed with rabbit monoclonal anti-CYP4502E1 (Stressgen; Assays Designs; 1:1,000) and with secondary antibody (goat anti-rabbit IgG; Santa Cruz Biotechnology Inc., California, CA; 1:5,000). Proteins were detected using an ECL kit (Santa Cruz Biotechnology Inc., California, CA). Relative densities from CYP2E1 bands were determined using β-actin as reference.

Hepatic CYP4502E1 immunocytochemistry

Two control and two experimental rats were perfused by gravity (1 m column) through the left ventricle with 4% (vol/vol) paraformaldehyde in 0.1 M PB (pH = 7.40) at 4°C. Livers were removed and postfixed (1 h, 4% paraformaldehyde in PB) and transferred to 30% (wt/vol) sucrose in PB for cryoprotection. After embedding in Shandon Criomatrix (Thermo), the tissues were frozen at −20°C. Sections of 10 μm thickness were obtained in a Shandon Cryotome E (Thermo Electron Corporation) and collected in glass slides coated with 3-aminopropyltriethoxysilane (Sigma, Spain). Sections processing and incubation with antibodies has been described previously (Sudheer et al., 2007). Antibodies used were rabbit anti-CYP4502E1 (Stressgen; Assays designs) and goat anti-rabbit IgG Alexa 594 (Molecular Probes, Invitrogen), both a dilution of 1:1,000. Negative controls were similarly incubated but in the absence of primary antibody. Sections were photographed by fluorescence microscopy with appropriated filters, using a Zeiss Axioscop 2 (*mot plus*) microscope equipped with a digital camera (CoolSnap cf) and analyzed with Metamorph 6.3 software.

Statistics

All data are expressed as the mean ± standard error of the mean. Statistical analysis were performed by two tails Student's *t*-test for unpaired data. Values of $P < .05$ were considered to indicate statistical significance.

Results

Animals

At the onset of the experiments, all rats had nearly identical body weight (270–310 g). Body weight gain during the 30 days of the experiment was also comparable and there was not any statistically significant difference between groups (Table 1). The amount of water drunk by control and experimental rats was also quite similar, 24.07 ± 2.17 and 27.10 ± 1.03 mL/d, respectively. Mean level of ethanol ingested was 0.27 ± 0.01 mL/rat/d and levels of ethanol in plasma oscillated between 0.3 and 0.50 mM with an average of 0.41 ± 0.03 mM (Table 1). Red cell count, hematocrit, leukocyte, and platelet numbers were not different in control and experimental group (Table 1). Glycemia and insulin levels were not statistically different between both groups, although ethanol group showed a tendency to have higher insulin levels than controls (6.0 ± 0.8 vs. 4.2 ± 0.4 ng/mL; $P = .066$).

Table 1
Ethanol ingestion, body weight evolution, ethanol plasma levels, and basic hematology in control and ethanol groups

Treatment parameter	Control 0–30 d ($n = 15$)	Ethanol 0–30 d ($n = 15$)
Body weight gain (g)	52.39 ± 3.88	49.97 ± 3.84
Fluid intake (mL/d)	24.07 ± 2.17	27.10 ± 1.03
Ethanol ingestion (mL/d)	—	0.27 ± 0.01
Plasma ethanol (mM)	—	0.41 ± 0.03 $n = 9$
Erythrocytes ($\times 10^6/\mu L$)	7.48 ± 0.09	7.59 ± 0.10
Hematocrit (%)	42.60 ± 0.35	42.90 ± 0.40
Leucocytes ($\times 10^3/\mu L$)	3.20 ± 0.24	2.91 ± 0.38
Platelets ($\times 10^3/\mu L$)	712.9 ± 8.3	701.7 ± 24.8

Total plasma proteins and transaminases levels

Being the liver one of the early targets for ethanol damage, we have measured, in addition to total plasma proteins whose decrease is a slow onset marker of liver alteration, plasma aspartate and alanine aminotransferases as early indicators of hepatic damage. All parameters were within normal values and no differences were observed between control and ethanol-drinking rats (Fig. 1 left).

Plasma cholesterol, HDL, and triglycerides

Because plasma lipids are critical markers for cardiovascular disease and therefore targets for almost any nutritional (e.g., ethanol) or pharmacological agent capable of altering vascular disease risk, it was of great importance to assess if moderate ethanol ingestion per se changed the lipid profile. We determined total plasma cholesterol, HDL, and triglycerides (Fig. 1 right). We found that cholesterol and triglyceride levels experienced a small but significant decrease after 30 days of ethanol ingestion, representing an 11 and a 19% in cholesterol and triglycerides, respectively.

Systemic AP and vasoactive markers

Mean AP for control (119 ± 11 mm Hg; $n = 8$) and ethanol group (112 ± 20 mm Hg; $n = 8$, respectively) were not statistically different. The mean heart rate observed in the groups of animals oscillated between 300 and 380 beats/min, and there was not any statistically significant difference among them (data not shown).

Fig. 2A shows plasma CA levels in control and ethanol-drinking rats. Control norepinephrine (NE) and epinephrine levels were 34.03 ± 3.30 and 48.07 ± 4.94 pmol/mL, respectively. Both CA showed minor statistically nonsignificant variations in the experimental group. Content and synthesis rate of NE in RA were markedly decreased (Fig. 2B, C) in the experimental group ($P < .01$). No significant differences were observed in dopamine content of RA, but its synthesis rate decreased significantly ($P < .01$).

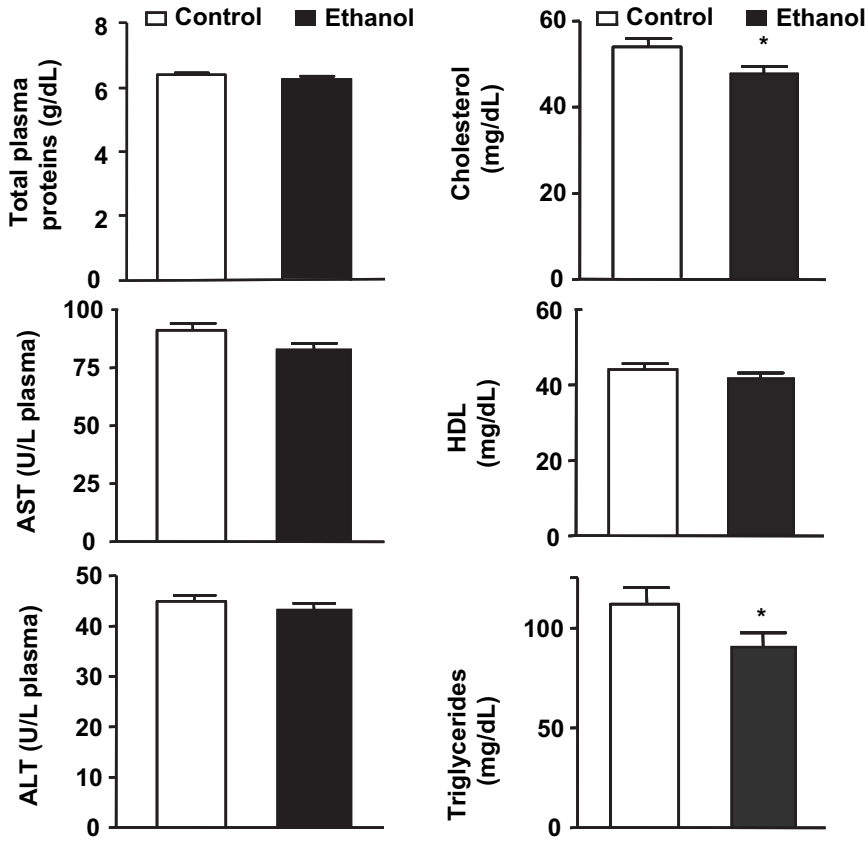

Fig. 1. Effects of low ethanol ingestion on total plasma protein, aspartate, alanine aminotransferases, total cholesterol, HDL, and triglycerides in plasma. Data are means ± standard error of the mean of 11–18 individual values. *$P < .05$ in ethanol 1% group versus controls.

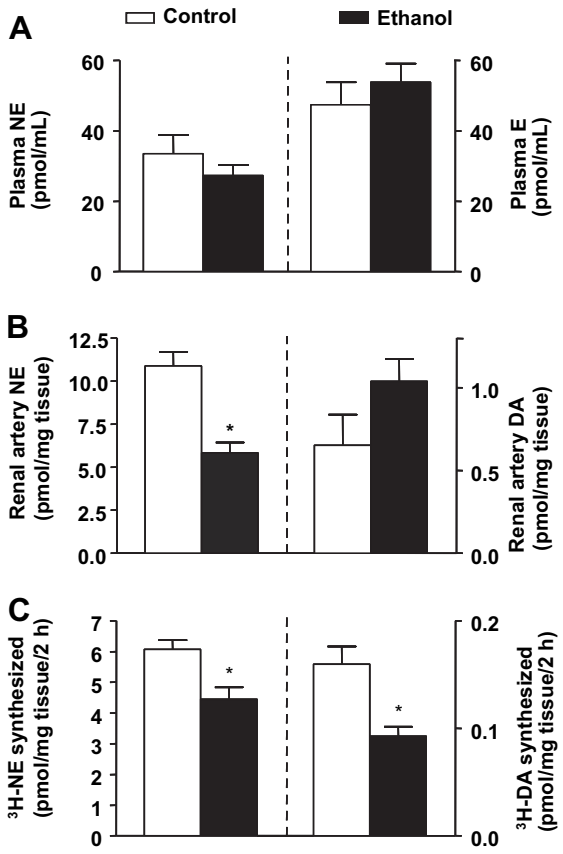

Fig. 2. Effects of low ethanol ingestion on catecholamines levels in plasma and catecholamines content and synthesis rate in renal artery. (A) Norepinephrine (NE) and epinephrine levels in plasma in control and 1% ethanol group. Data are means ± standard error of the mean (S.E.M.) of 11–14 individual data in all the cases. (B) NE and dopamine (DA) levels in renal artery in control and 1% ethanol group. Data are means ± S.E.M. of 10–15 individual values in all the cases. (C) Synthesis rate of ^{3}H-NE and ^{3}H-DA in renal artery in control and 1% ethanol group. Data are means ± S.E.M. of 10 individual data in all the cases. *$P < .01$ in ethanol 1% group versus controls.

Plasma endothelin-1 and ACE levels in control rats were 2.08 ± 0.22 fmol/mL ($n = 26$) and 182.5 ± 4.74 U/L ($n = 30$), respectively, and ethanol ingestion did not alter these levels.

PAP, lung eNOS, and plasma nitrites and nitrates levels

Mean PAP (mPAP; Fig. 3A) in a control and ethanol-drinking groups were 16.60 ± 0.50 mm Hg and 14.10 ± 0.61 mm Hg, respectively ($n = 8$; $P < .05$). Right ventricular to whole heart weight ratios were not different (data not shown).

Platelet 5-HT levels (Fig. 3B) were 7.18 ± 0.29 in control rats and 5.67 ± 0.69 pmol/10^6 platelets ($n = 8$ in both cases; $P < .05$).

We performed Western blots for eNOS and iNOS in lung tissue of the same animals and we found no statistically significant change in the expression of eNOS or iNOS with ethanol (data not shown). We also determined the levels of nitrates and nitrites in plasma and have not found differences among control and ethanol-drinking rats (Fig. 3D).

Effects of ethanol ingestion on GSH and GSSG levels in liver

Absolute GSH and GSSG levels (Fig. 4A) and GSH/GSSG ratios in the liver (Fig. 4B) showed minor but significant differences between control and experimental group, with a decrease in GSSG and maintenance of GSH in experimental ethanol-drinking rats tissue and increase in GSH/GSSG ratios. Figure 4C shows LPO levels in the liver in control and experimental animals. Ethanol ingestion caused a very significant decrease in LPO levels ($P < .001$).

Effects of ethanol ingestion on the activities of antioxidant enzymes

Figure 5 shows the activities of SOD encountered in liver in control and experimental rats. Ethanol ingestion caused a decrease in liver SOD (22.5%; $P < .05$) and GPx (18%; $P < .05$) activities. However, CAT activity was not modified by ethanol ingestion.

Effects of ethanol ingestion on CYP4502E1

Figure 6 shows the immunocytochemical distribution, relative CYP4502E1 protein levels, and enzymatic activity in control and ethanol-drinking rats. In the top two low-magnification images (Fig. 6A), the classical distribution of CYP4502E1 around the central veins can be appreciated (Ingelman-Sundberg et al., 1988). Neither the distribution nor the apparent intensity of the reaction was changed by ethanol ingestion (remaining images in Fig. 6A). Consistent with the images, the amount of CYP4502E1 protein relative to β-actin was identical in the liver of control and ethanol-drinking rats (Fig. 6B). Figure 6B shows that ethanol ingestion for 30 days did not modify liver microsomal CYP4502E1 activity.

Discussion

The present work represents the most ample study performed on the effects of low doses of ethanol ingestion on laboratory animals. Overall, we have monitored a great number of parameters in control and in experimental rats that have been ingesting ethanol in drinking water at rates of 0.27 mL/rat/d (0.22 g/rat/d), which would be equivalent to 65 mL/standard human male/d. For discussion, results are grouped in three categories. Firstly, we shall consider parameters defining the general status of the animals (body weight gain, basic hematological parameters, blood glucose, insulin levels, and transaminases and lipid profile levels in plasma). Secondly, we will discuss parameters related to systemic and pulmonary pressure, and thirdly, we will consider parameters related to redox status.

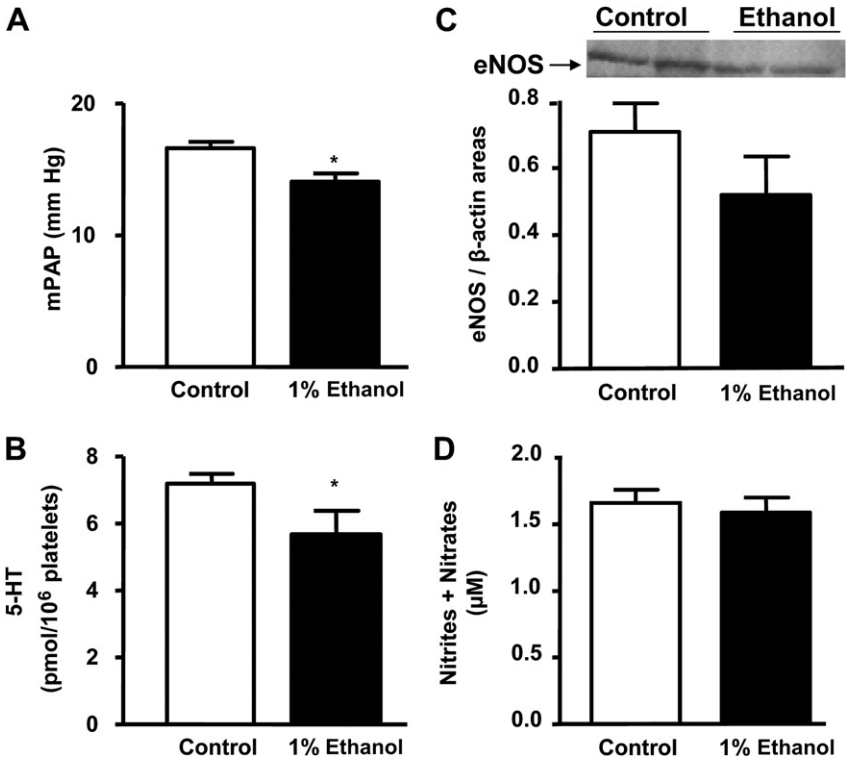

Fig. 3. Effects of low ethanol ingestion on pulmonary arterial blood pressure, platelet serotonin content, lung nitric oxide synthase, and plasma nitrites/nitrates. (A) Mean pulmonary arterial blood pressure in the three groups of animals. Data are means ± standard error of the mean (S.E.M.) of 17 individual values for controls and 8–10 for 1% ethanol group. (B) Platelet serotonin content in control and 1% ethanol group. Data are means ± S.E.M. of 8 individual values. (C and D) Lung nitric oxide synthase and plasma nitrites/nitrates. Means ± S.E.M. of 17 individual values for controls and 10 individual values for the experimental group. $*P < .05$ ethanol 1% group versus controls.

General status of the animals

At the outset of the discussion, we want to state that the amount of alcohol and the cadence of its ingestion, which mimic the most common pattern of alcohol ingestion in Spain, did not produce in our animals any change that can be qualified as pathologic, either reflecting organ/tissue damage or being potentially pathogenic. On the contrary, we consider that all modifications observed in the ethanol-drinking rats can be considered beneficial, that is, promoters or enhancers of well-being.

The rate of weight gain was unaffected by ethanol; ethanol group should compensate the ethanol calories ingested by reducing their intake of diet, so that total energy intake was similar in both groups, control and ethanol, as was observed by Cornier et al. (2002). No significant changes were observed in basic hematology after ethanol intake for 30 days. Our hematological findings contrast with the alterations encountered in heavy alcohol consumption: anemia, a decrease in white cell count and tendency to suffer infections and thrombocytopenia with prolongation in bleeding time (Ballard, 1997).

Our findings in the rat indicate, in agreement with human studies, that low-ethanol ingestion does not produce deleterious effects in glucose balance (Van de Wiel, 2004)

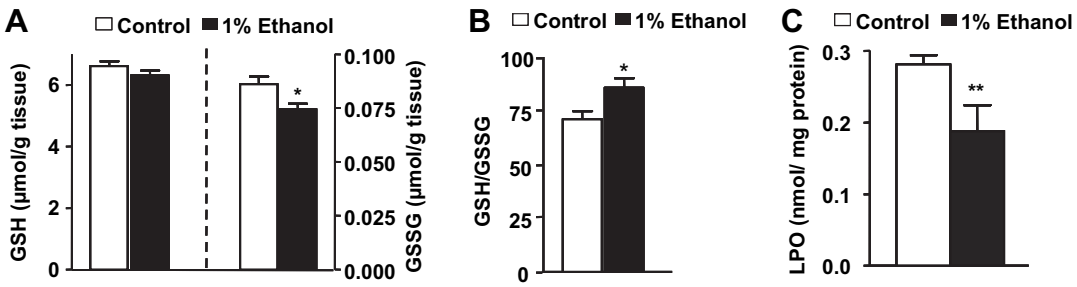

Fig. 4. Effects of low ethanol ingestion on reduced glutathione (GSH) and oxidized glutathione (GSSG) and GSH/GSSG ratios and lipid peroxide levels in liver. Data are means ± standard error of the mean of 20–22 individual values in control conditions and 10–12 for the experimental groups. $*P < .05$ and $**P < .001$ for ethanol 1% group versus controls.

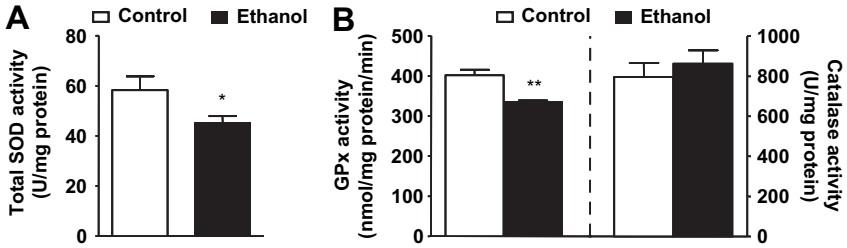

Fig. 5. Effects of low ethanol ingestion on liver activity of superoxide dismutase (SOD) total, glutathione peroxidase (GPx), and catalase. Data are means ± standard error of the mean of 11–22 individual values in GPx activity and 7–9 individual values for SOD and catalase. *$P < .05$ and **$P < .01$ for ethanol 1% group versus controls.

or in insulin sensitivity in contrast with some authors who have established that low-ethanol consumption increased insulin sensitivity (Furuya et al., 2003). Liver function, as assessed by total plasma protein and transaminases levels, was not impaired by moderate alcohol ingestion. Our findings are comparable with those reported by Arola et al. (1997) in the rat.

The picture on plasma lipid parameters found in our experimental animals is fully positive. Total plasma cholesterol and triglycerides are lower and HDL levels remain unaltered in ethanol-drinking rats. Control values for the three parameters are within the lower range of those reported by other authors (Artiss et al., 2006). There is a tight correlation of high levels of these three parameters with the risk of cardiovascular disease, then, we can state that moderate alcohol ingestion per se would be protective for the rat cardiovascular system through lipids fall, solving the disputes encountered in the literature (see Introduction section) on whether alcohol or the antioxidant compounds present in red wine and other alcoholic beverages are the beneficial components of beverages.

Systemic and pulmonary pressure and related parameters

Moderate ethanol consumption for 30 days did not modify the APs. Thus, hypertension, considered as a major risk factor for cardiovascular disease, is not encountered in our animals. In addition, we could suggest that the general sympathetic tone is lower in animals after 30 days of alcohol ingestion. We have measured CA levels in plasma and in the sympathetic nerve endings/fibers located in the blood vessels as exemplified by the RA and plasma NE levels, which represent an important factor in determining the tone of the vascular smooth muscle cells, that showed a tendency to decrease at 30 days of alcohol ingestion, although it was not significant. This decrease was accompanied by reduced levels of NE in the RA. Additionally, we found a decrease in the rate of CA synthesis in the RA after 30 days of ethanol intake. On the whole, the general sympathetic tone was low in animals after 30 days of alcohol ingestion, and this decreased activity would represent a factor that would oppose to any increase in blood pressure (Biaggioni, 2003).

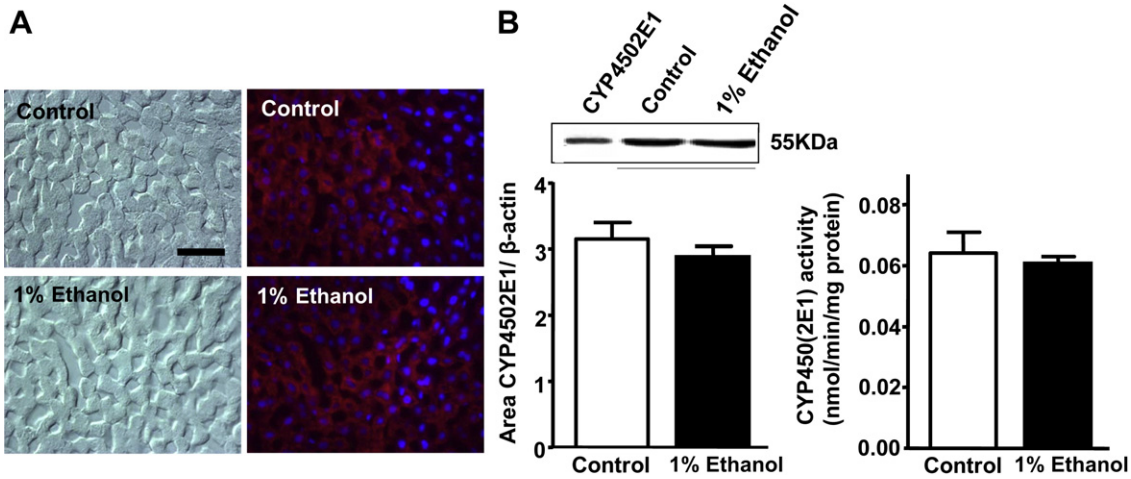

Fig. 6. Effects of ethanol ingestion on CYP4502E1 immunocytochemical distribution, Western blot analysis and its enzymatic activity in liver microsomal fraction. (A) It is shown in pairs of bright field and anti-CYP4502E1 stained (in red) histological liver sections, which have their nuclei stained in blue (Dapi). The upper pair of low-magnification images (bar = 200 μm) is aimed to evidence the normal centrilobular expression of CYP4502E1 in a control rat. The remaining pairs of higher magnification images (bar = 50 μm) are from a control and ethanol-drinking rats. In the two cases, the pictures show areas of transition from the periphery toward the center of the lobule. (B) At the left, it shows the relative density of CYP4502E1 to β-actin bands in liver microsomal fractions of the two different groups of animals. At the right, it shows the CYP4502E1-dependent enzymatic activity of liver microsomal fraction of control and 30 days ethanol-dinking rats. Data are means ± standard error of the mean of 10 individual values.

On the other hand, moderate alcohol ingestion produced a decreased statistically significant change in PAP at 30 days. This decrease directed us to search for nitric oxide synthase expression in lung tissue because nitric oxide (NO) is a major regulator of pulmonary artery resistances; and contrary to expectations, there was a tendency to decrease in eNOS protein and iNOS was not induced. Plasma nitrites and nitrates were nearly identical in both groups suggesting that NO synthesis and degradation in the entire organism is not altered. Our findings are at variance with Fitzpatrick et al. (1993) and Flesch et al. (1998) who found that red wine or equivalent doses of quercetin produced endothelium-dependent NO-mediated relaxation of aortic and coronary rings, whereas ethanol or white wine did not. Venkov et al. (1999) working with isolated bovine aortic endothelial cells reported that pure ethanol (0.1%; 2.1 mM) produced a rapid and long-lasting increase in eNOS expression. Both reports were obtained in in vitro preparations of systemic vessels and not in whole animal exposed to ethanol.

Next, we directed our search toward 5-HT, more specifically to platelet 5-HT. There are many studies showing that platelets are the main store for 5-HT existing in equilibrium between plasma and platelet levels of this biogenic amine intimately related to pulmonary arterial hypertension (MacLean, 1999). Our data indicate that platelet 5-HT levels decreased after 30 days of ethanol ingestion suggesting that plasma 5-HT levels should decrease but this reduction was below detection in our HPLC system. This platelet 5-HT levels drop is coincident with a decrease in mPAP. Our interpretation of these two findings is that they could be causally linked and thereby can explain the decrease in PAP observed.

Parameters related to redox status

Our data indicate that a moderate doses of ethanol neither produces sustained oxidative damage nor induces the ethanol-metabolizing CYP4502E1 and ameliorate the redox status of the animals. Therefore, we suggest that a part of the antioxidant effects of alcoholic beverages is because of ethanol itself. Our findings contrast with those obtained for some authors who show that ethanol ingestion causes a clear oxidative damage, both in experimental animals and humans (Kovacic and Cooksy, 2005; Lieber, 1997; McDonough, 2003; Wu and Cederbaum, 2003). These experiments were carried out with high doses of ethanol in all cases.

A plausible explanation for the differential dose-dependent effects of ethanol can be attained if the basic properties of ethanol metabolism are taken into account. First step of ethanol metabolism is an oxidation that can be carried out by three different enzymes; alcohol dehydrogenase (ADH), the microsomal cytochrome P-450-dependent ethanol-oxidizing system, and CAT (Dinu et al., 2005; Ponnappa and Rubin, 2000; Riveros-Rosas et al., 1997), all produce acetaldehyde as the initial catabolite. Acetaldehyde accumulation produce an ample fan of noxious effects (Brooks and Theruvathu, 2005; Lieber, 1997); for this reason the imbalance between the acetaldehyde generating capacity and mitochondrial ability to oxidize it via aldehyde dehydrogenase is so important.

Liver ADHs (there are several isoforms) have Km for ethanol in the range of 0.1−1 mM, the microsomal cytochrome P-450-dependent ethanol-oxidizing system has Km in the range of 16−24 mM, and the CAT Km for ethanol is not known (Lands, 1998). According to those Km, we expected that the main pathway to metabolize alcohol in low-moderate ingestion should be ADH producing a small quantity of acetaldehyde that does not achieve to be harmful and therefore the highly uncoupled microsomal ethanol oxidizing system would have not or have a minimal participation in the first step of ethanol metabolism. This could be the situation in our animals. Our findings on CYP4502E1 distribution, protein levels, and activity support this conclusion.

When high doses of ethanol are ingested, the microsomal CYP450 system become important producing high quantity of acetaldehyde that exceed the mitochondrial ability to oxidize it and cause oxidative stress. This explains a clear oxidative damage showed by others authors.

Glutathione is the main cellular antioxidant and one of the parameters more important to evaluate oxidative damage. The levels of GSH and GSSG found in liver of our control animals are comparable to those reported by other authors (Halliwell and Gutteridge, 2007). In experimental animals, i.e., after 30 days of ethanol ingestion, there was not indication oxidative damage; on the contrary, in comparison to controls, ethanol-ingesting animals exhibited a significant decrease in GSSG levels and a significant increase in GSH/GSSG ratios, suggesting an antioxidant effect of ethanol in the liver.

Free radicals can react with membrane phospholipids or plasma lipoproteins producing LPO; the LPO measurement is another parameter to evaluate oxidative damage. It is difficult to compare absolute LPO levels found in our experiments with data from the literature because the oxidative breakdown of membrane fatty acids yields a great variety of byproducts in different proportions and different methods determine different groups of these byproducts (Halliwell and Gutteridge, 2007; Punchard and Kelly, 1996). It should be stated that high levels of ethanol ingestion cause an increase of lipid peroxidation in rat liver, kidney, brain, and heart (Das and Vasudevan, 2007). However, what is important is that, intraexperimentally we have observed a significant decrease in LPO in the liver of experimental animals after 30 days of ethanol exposure, indicating that low doses of ethanol protects from the ongoing basal damage of cell membranes lipids and presumably oxidation of plasma lipoproteins. These findings are very relevant because oxidized lipids are at the core of the atherogenic process (Hofnagel et al., 2007).

Control activities of the three reactive oxygen species (ROS)-deposing enzymes assayed (SOD, CAT, and GPx) found in our study are comparable with previously reported values (Heap et al., 1995; Sudheer et al., 2007). The effects of ethanol ingestion on the activity of these enzymes would

suggest, in agreement with the rest of the data, that the production of ROS has diminished in experimental animals, and hence the downregulation of SOD and GPx.

Although in the present study, we have not addressed the mechanisms involved in the redox protection provided by moderate ingestion of ethanol, we consider that an induction of high-affinity aldehyde dehydrogenase II by ethanol can satisfactorily explain our findings. This enzyme is induced by moderate ethanol ingestion (Kimura et al., 2009) and it can oxidize acetaldehyde as well as 4-hydroxynonenal and other lipid-derived aldehydes (Halliwell and Gutteridge, 2007). Therefore, increased activity of aldehyde dehydrogenase II would directly account for the observed decrease in LPOs. In addition, it would also explain the increase in GSH because spontaneous glutathione transferase catalyzed conjugation with GSH is an alternative pathway to dispose lipid-derived aldehydes. Consistent with our interpretation, several recent studies have demonstrated that the cardioprotection afforded by moderate ethanol consumption is mediated by activation of aldehyde dehydrogenase II and subsequent decrease of 4-hydroxynonenal protein adducts (Churchill et al., 2009). Our interpretation is also consistent with recent findings of Ren (2007) and Guo et al. (in press) on mice overexpressing aldehyde dehydrogenase II; these mice, in comparison with their correspondent wild-type animals, showed reduced hepatic apoptosis and cardiac damage induced by high-ethanol ingestion and a parallel decrease in LPOs.

In conclusion, our study demonstrates that the ingestion of low doses of ethanol in the rat in a continuous time frame, which reasonably mimics the most common pattern of drinking in Spain, has not had any deleterious effect; on the contrary, it produces a decrease in plasma lipids, a decrease in the turnover of NE in sympathetic endings, a decrease in PAP, a tendency to diminish the production of ROS, a decrease in LPO, and hence the downregulation of SOD and GPx. That would indicate that it is beneficial to ingest moderate doses of alcohol spaced along the day.

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