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Resveratrol, a red wine polyphenol, attenuates ethanol-induced oxidative stress in rat liver

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Abstract

The involvement of oxidative stress in the pathogenesis of alcoholic diseases in the liver has been repeatedly confirmed. Resveratrol, a natural phytoalexin present in grape skin and red wine possesses a variety of biological activities including antioxidant. This study was conducted to evaluate whether resveratrol has a preventive effect on the main indicators of hepatic oxidative status as an expression of the cellular damage caused by free radicals, and on antioxidant defence mechanism during chronic ethanol treatment. Wistar rats were treated daily with 35% ethanol solution (3 g/kg/day i.p.) during 6 weeks and fed basal diet or basal diet containing 5 g/kg resveratrol. Control rats were treated with i.p. saline and fed basal diet. Experimentally, chronic ethanol administration leads to hepatotoxicity as monitored by the increase in the level of hepatic marker enzymes and the appearance of fatty change, necrosis, fibrosis and inflammation in liver sections. Ethanol also enhanced the formation of MDA in the liver indicating an increase in lipid peroxidation, a major end-point of oxidative damage, and caused drastic alterations in antioxidant defence systems. Particularly the activities of hepatic superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were found reduced by ethanol treatment while glutathione reductase (GR) activity was unchanged. Dietary supplementation with resveratrol during ethanol treatment inhibited hepatic lipid peroxidation and ameliorated SOD, GPx and CAT activities in the liver. Conclusively, we can suggest that resveratrol could have a beneficial effect in inhibiting the oxidative damage induced by chronic ethanol administration, which was proved by the experiments that we conducted on rats.

Keywords: Oxidative stress; Liver; Chronic ethanol; Resveratrol; Antioxidant

Introduction

Considerable experimental and clinical evidence has contributed to support a key role of oxidative stress in the pathophysiological processes of liver injury related to excessive alcohol consumption (Cahill et al., 2002; Castilla et al., 2004). The metabolism of ethanol gives rise to the generation of excess amounts of reactive oxygen species (ROS) and has a detrimental effect on cellular antioxidant defence system (Navasumrit et al., 2000; Ozaras et al., 2003). Thus, numerous interventions have been put forward to counteract the vulnerability of the liver to oxidative challenges during alcohol consumption by reinforcing the endogenous antioxidant defence system (Koch et al., 2000; Ozaras et al., 2003).

Resveratrol (3, 4′, 5-trihydroxystilbene) is a natural phytoalexin synthesized in a wide variety of plant species including grapes as a response to environmental stress or fungal infection. It constitutes one of the polyphenolic compounds of red wine and is responsible for the beneficial effect of regular wine consumption at moderate amounts (Frémont, 2000). The positive effects of resveratrol in biological systems are wide-ranging as a cancer chemoprevention agent (Wolter et al., 2004), a powerful anti-inflammatory factor (Donnelly et al., 2004) and an

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antioxidant agent (Cai et al., 2003). Several investigations have cited the possible role and protective effects of resveratrol against certain forms of oxidant damage, through a hydrogen-electron donation from its hydroxyl groups (Lüpez-Velez et al., 2003). The consequences are a capacity to scavenge ROS, a protective effect against DNA damage and lipid peroxidation in cell membrane (Leonard et al., 2003). Accordingly, this study was designed to evaluate the possible role of resveratrol, when given as a supplement diet, in protecting liver from oxidative stress during chronic ethanol exposure.

Materials and methods

Chemicals

Resanex, a partially purified red wine extract titrated at 85% resveratrol and 15% proanthocyanidin, was purchased from Selmedica heathcare (Korea). 2-thiobarbituric acid (TBA) was obtained from Sigma Chemicals Co. (Germany). Absolute ethanol (99.5%) was purchased from Carlo Erba reagent (France). All other chemicals used were of analytical grade.

Animals

The study was performed in males, adult Wistar albino rats, 200–230 g purchased from SIPHAT (Tunis, Tunisia). Before any experience, all animals were kept one week under the same laboratory conditions of temperature (22±2 °C), relative humidity (70±4%) and a 12 h light/dark cycle and received a nutritionally standard diet (SICO, Sfax, Tunisia) and tap water. All experiments were carried out with the approval of local animal use committee. Procedures involving the animals and their care conformed to the institutional guidelines, in compliance with national and international laws and Guidelines for the USA of Animals in Biomedical Research (Giles, 1987).

Experimental design

After an acclimation period, rats were randomly divided into three groups of 12 animals each. The first group served as untreated controls and received a daily intraperitoneal (i.p.) injection of 0.9% (w/v) NaCl. The second group was given a daily dose of 3 g/kg body mass of 35% ethanol solution by i.p. injection for the whole experimental period, lasting 6 weeks. The third group was given the same dose of ethanol but allowed basal diet supplemented with resveratrol at the dose of 5 g/kg. Briefly, diet (~15–20 g/animal/day) was freshly prepared from the powder and resveratrol was added just before mixing with a blender. Food intake from control, ethanol and ethanol/resveratrol rats was daily recorded. At the end of the experimental period, the animals were sacrificed by decapitation. Previous studies have been conducted with rats using different ways of ethanol administration and different time of exposure. 3 g/kg b.m. was chosen because this dose produces moderate toxicity (Ogilvie et al., 1998) and a blood alcohol peak within 15 min of administration via i.p route which remain high for at least 3 h (Ogilvie et al., 1997). Alcohol was diluted to 35% to prevent peritoneal irritation. Resveratrol intake was monitored by total food intake. Resveratrol dose used in this study is 250 mg/kg BW/day which is roughly equivalent to the optimal dose preconized in rats (300 mg/kg BW/day) Crowell et al., 2004).

Preparation of serum and tissue extract

Blood was taken and let stand for 30 min at room temperature. Serum was obtained by centrifugation at 300 ×*g* for 10 min and stored at −80 °C in aliquots until the analysis. Livers were excised immediately, washed with ice-cold physiologic saline solution (0.9%), blotted dry and weighed. Tissues were homogenized for 30 s in 10 volume of ice-cold 10 mM phosphate buffered saline (PBS, pH 7.4) containing 1.15% KCl. Homogenates were centrifuged at 800 ×*g* to remove cell debris and nuclei; the supernatants were centrifuged at 10,000 ×*g* for 10 min. Portions of the post-mitochondrial supernatant were removed for malondialdehyde (MDA) measurement. The remaining supernatant was pipetted into a clean centrifuge tubes and centrifuged further at 105,000 ×*g* for 45 min using a 50 Ti rotor in a Beckman model L90 ultracentrifuge. Post-mitochondrial and cytosolic fractions were stored at −80 °C in aliquots until analysis within one week. The whole procedure was conducted at 0–4 °C.

Biochemical indicators of liver function

Serum aspartate aminotransferase (AST) (E.C. 2. 6. 1. 1.), alanine aminotransferase (ALT) (E.C. 2. 6. 1. 2.), alkaline phosphatase (ALP) (E.C.3. 1. 3. 1), γ-glutamyltransferase (GGT) and total bilirubin activities were determined using commercially available diagnostic kits supplied by Randox laboratories (Ardmore, Northern Ireland, UK).

Liver histology

Histological assessment was used to complete the study of liver damage. A piece of liver tissue was fixed in 10% formalin for 24 h, routinely processed and embedded in paraffin. Sections of 5–6 μm thick were cut and stained with hematoxylin and eosine (H and E).

Lipid peroxidation

The lipid peroxidation product in hepatic post-mitochondrial fraction was determined according to the spectroscopic technique of Draper and Hadley (1990) based on the formation of the thiobarbituric reactive substances (TBARS) and expressed as the extent of malondialdehyde (MDA) production. An extinction coefficient of $1.56\times10^{5}M^{-1}cm^{-1}$ was used.

Antioxidant status

Analyses of antioxidant enzyme activities were performed using a BioRad UV-Visible spectrophotometer with a "kinetics" program.

Superoxide dismutase (SOD) (E.C.1.15.1.1) level in cytosolic fraction was estimated using Ransod kit supplied by Rondox laboratories (Ardmore, Northern Ireland, UK). Xanthine and xanthine oxidase were used to generate superoxide anion, which reacts with 2-(4-indophenyl)-3-(4-nitro-phenyl)-5-phenyl tetrazolium chloride (INT) to form a red formazan dye. Changes in the absorbance were determined at 505 nm during the first 3 min of the reaction. Enzyme activities in the samples were calculated from a standard curve. One enzyme unit of SOD is defined as the amount, which inhibits the INT reaction by 50%. Specific activities are defined as units/mg protein.

Glutathione peroxidase (GPx) (E.C. 1. 11. 1. 9) level was assayed by the method of Paglia and Valentine (1967). After the addition of cumene hydroperoxide, the conversion of NADPH to NAD^+ by glutathione reductase and generated oxidised glutathione was continuously monitored spectrophotometrically at 340 nm for 2 min. One unit of GPx activity was expressed as the amount of enzyme catalysing the oxidation of 1 nmol NADPH/min/mg protein.

Glutathione reductase (GR) (E.C. 1. 6. 4. 2) activity was also determined using Randox commercial kit by monitoring the oxidation of NADPH in the presence of GSSG. GR was measured at 340 nm for 5 min. One unit of activity is equal to the micromoles NADPH oxidized/min/mg protein.

Catalase (CAT) (E.C. 1. 11. 1. 6) activity was assayed at 20 °C according to the method of Aebi (1984) slightly modified. Hydrogen peroxide (H_2O_2) disappearance was monitored kinetically at 240 nm. The molar extinction coefficient of 43.6 M cm-1 was used to determine CAT activity. One unit of activity is equal to the μmoles of H_2O_2 degraded/min/mg protein.

Protein assay

Protein concentrations in the post-mitochondrial and the cytosol fractions of the liver were determined by the method of Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

All results are expressed as mean±standard deviation. Statistical significance of the difference between group means was performed by one-way ANOVA followed by Student's *t* test. Differences with $p<0.05$ were considered to be significant.

Table 1
Growth parameters in rats after chronic ethanol administration nourished with or without resveratrol supplemented diet

Parameter	Control	Ethanol	Ethanol/resveratrol
Weight gain (%)	27.74±3.67	9.41±1.71^{aa}	17.99±0.76^{a}
Food intake (g/100g bw/24-h)	6.73±0.18	5.72±0.05^{aa}	6.26±0.23
Relative liver weight (%)	3.86±0.10	4.26±0.08^{aa}	4.20±0.12^{a}

Data represent mean±SD of 12 individual values.
$^{a}p<0.05$ or $^{aa}p<0.01$ when compared with controls.

Table 2
Biochemical indicators of liver function in serum of rats after chronic ethanol administration nourished with or without resveratrol supplemented diet

Parameter	Control	Ethanol	Ethanol/resveratrol
AST (U/L)	106.46±9.44	151.43±16.28^{aa}	116.27±5.15^{b}
ALT (U/L)	40.40±2.22	70.57±12.97^{aa}	38.30±1.68^{bb}
ALP (U/L)	155.21±11.85	243.45±36.23^{aa}	174.90±18.69
GGT (U/L)	27.29±2.22	45.71±2.51^{a}	27.55±2.65^{b}
Bilirubin (μmol/L)	4.32±0.53	3.70±0.36	3.86±0.88

AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, GGT: gamma glutamyltransferase.
Data represent mean±SD of 12 individual values.
$^{a}p<0.05$ or $^{aa}p<0.01$ when compared with controls.
$^{b}p<0.05$ or $^{bb}p<0.01$ when compared with ethanol.

Results

Growth performance and liver weight

At the end of the experiment, control and ethanol treated rats with or without resveratrol gained weight (Table 1). The mean body weight gain of ethanol-treated rats was 9.41±1.71% against 27.74±3.67% in control rats ($p<0.01$). Dietary supplementation of resveratrol to ethanol-treated rats tends to ameliorate growth performance, and body weight gain was 17.99±0.76% ($p<0.05$ when compared to control). We found throughout the 42-day experiment that food intake was lower ($p<0.01$) in the first group of ethanol-treated rats and normal in the second group supplemented with resveratrol (Table 1). As at the end of treatment all resveratrol supplemented diet was absorbed, resveratrol intake was considered to be 250 mg/kg b.m/day which is safety (Crowell et al., 2004).

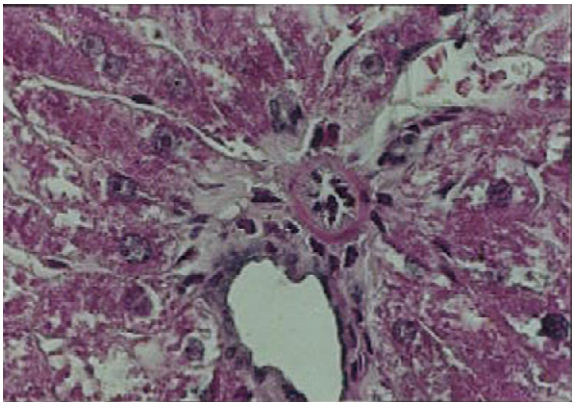

Fig. 1. Normal liver histologic aspect from a control rat H and E, stain, ×1000. It is composed of hexagonal or pentagonal lobules with central veins and peripheral hepatic triads or tetrads embedded in connective tissue. Hepatocytes are arranged in trabecules running radiantly from the central vein and are separated by sinusoids containing Kupffer cells. They are regular and contain a large spheroid nucleus with a distinctly marked nucleolus and peripheral chromatin distribution. Some cells have tow nuclei each.

The relative liver weight of the ethanol-treated group at the end of the experiment was greater ($p<0.01$) than that of the control group. Liver weight of ethanol-treated rats having resveratrol-supplemented diet was also higher but with a lower rate ($p<0.05$) (Table 1).

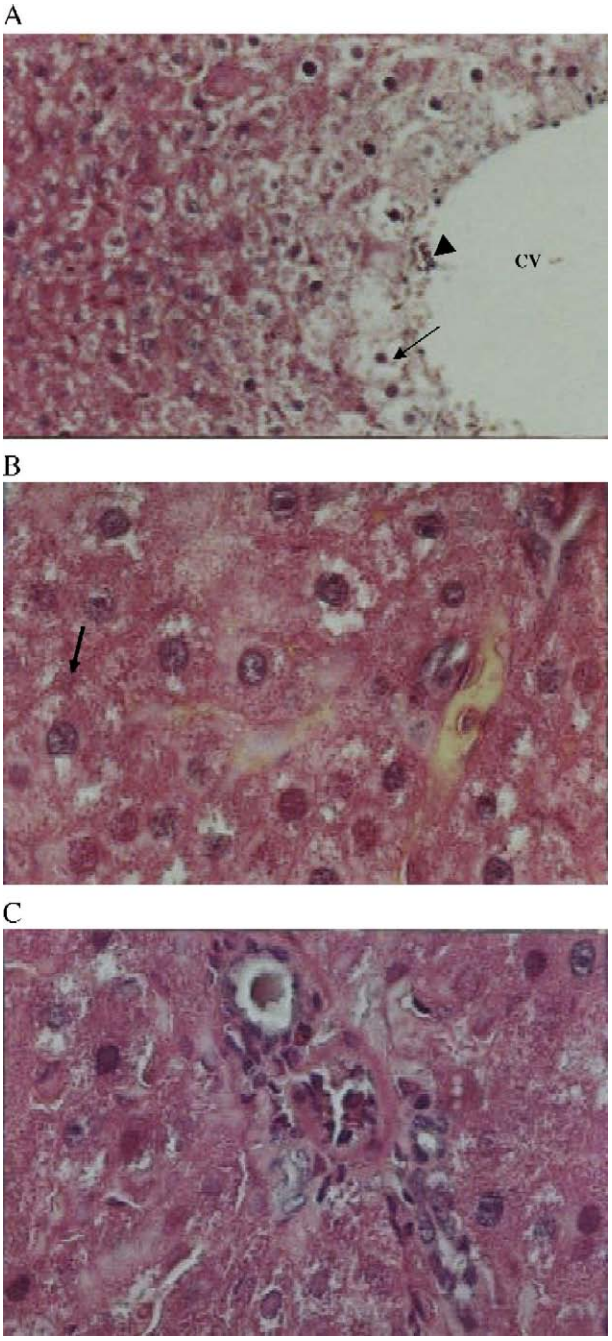

Fig. 2. Liver from an ethanol-treated rat H and E, stain, (A×400) and (B, C×1000). Panel A: The radial arrangement of the hepatic plates is disrupted. Cell sizes are enlarged, nuclear chromatin is more compact, slightly smaller nucleoli are not conspicuous. Necrosis of hepatocytes is observed near a central vein (CV) — nuclei are contracted, pycnotic with condensed chromatin, cytoplasm is strongly acidophilic (thin arrow). Diffusion of some erythrocytes into the CV (small arrow). Panel B: The cytoplasm of hepatocytes is light, foamy and filled with empty vacuole-like spaces. Note the presence of collagen and pericellular fibrosis (large arrow). Panel C: Some sinusoids were overfilled with erythrocytes and the walls of most sinusoids showed inflammatory cells.

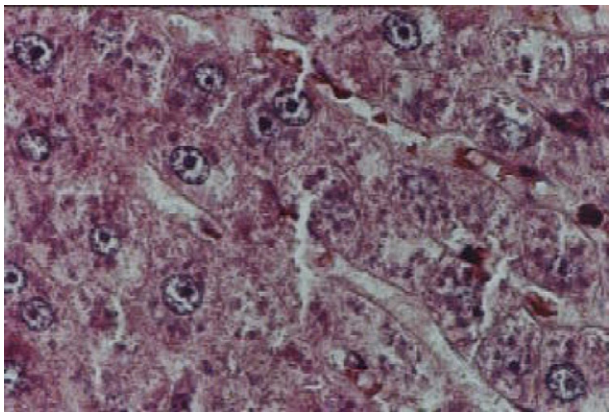

Fig. 3. Liver from an ethanol-treated rat fed resveratrol supplemented diet H and E, stain, (×1000). No abnormalities were observed in hepatic lobules, except for a light infiltration of inflammatory cells. Steatosis and necrosis were nearly absent.

Biochemical indicators of liver function

Ethanol administration affected biochemical markers of liver function. As shown in Table 2, serum AST, ALT, ALP, GGT levels but not bilirubin were enhanced ($p<0.05$) in chronically ethanol-treated rats nourished with basal diet. When diet was supplemented with resveratrol, the activities of these enzymes were practically restored and reached the normal values of control rats.

Histological assessment of the liver

The liver of control rats showed a normal structure (Fig. 1), which was influenced by the administration of ethanol with severe fatty accumulation, inflammation, fibrosis and necrosis of hepatocytes (Fig. 2A, B and C). In contrast, the histological examination of tissue sections from rats exposed to ethanol injection and nourished the resveratrol supplemented diet showed an improvement of liver morphology except for mild inflammation. Necrotic cells and fibrosis are nearly absent (Fig. 3).

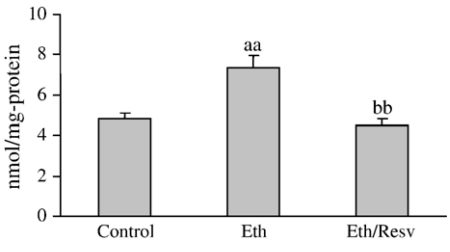

Fig. 4. Hepatic MDA in rats after chronic ethanol administration for 6 weeks nourished with or without resveratrol supplemented diet. Eth: ethanol-treated group, Eth/Resv: ethanol-treated group nourished with resveratrol supplemented diet. Data represent mean±SD of 12 individual values. a: $p<0.05$ or aa: $p<0.01$ when compared with controls. b: $p<0.05$ or bb: $p<0.01$ when compared with ethanol.

Table 3
Hepatic antioxidant enzymes in rats after chronic ethanol administration nourished with or without resveratrol supplemented diet

Parameter	Control	Ethanol	Ethanol/resveratrol
SOD (U/mg-protein)	32.93±5.57	16.79±2.07^{aa}	30.58±6.30^{b}
GR (U/mg-protein)	0.18±0.01	0.19±0.01	0.21±0.02
GPx (U/mg-protein)	1.17±0.11	0.88±0.05^{a}	1.18±0.12^{b}
CAT (U/mg-protein)	226.93±16.23	152.58±20.48^{aa}	212.74±29.07^{b}

SOD: superoxide dismutase, GR: glutathione reductase, GPx: glutathione peroxidase, CAT: catalase.
Data represent mean±SD of 12 individual values.
$^{a}p<0.05$ or $^{aa}p<0.01$ when compared with controls.
$^{b}p<0.05$ or $^{bb}p<0.01$ when compared with ethanol.

Lipid peroxidation of the liver

Chronic administration of ethanol led to an increase in hepatic MDA level indicating an enhancement in the lipid peroxidation potential of the liver ($p<0.01$) (Fig. 4). Although this increase was more than the double, resveratrol supplementation to ethanol-treated rats showed an efficiency to attenuate MDA formation in the liver.

Activities of liver antioxidant enzymes

Table 3 summarizes the activities of hepatic antioxidant enzymes. Rats treated chronically with ethanol exhibited a marked decrease in the activities of SOD ($p<0.01$), GPx ($p<0.05$) and CAT ($p<0.01$) whereas GR activity was not affected. Conversely, alcoholic-rats fed resveratrol-enriched diet showed a spectacular restoration of hepatic SOD, GPx and CAT activities which attain control values ($p<0.05$ when compared to ethanol group).

Discussion

In the first protocol of this study, it was obvious that chronic ethanol administration produced toxicity in rats, as monitored by weight loss and decreased food intake. It also induced hepatotoxicity. This is clearly evident by morphological alterations reflected by inflammation, necrosis, fibrosis, fatty and collagen accumulation, accompanied by substantial augmentation in the levels of transaminases, ALP and GGT. Furthermore, ethanol led to an increase in the lipid peroxidation potential of the liver and a decrease in the activities of hepatic antioxidant enzymes reflecting an oxidant stress in the liver. Serum GGT within its normal range may be also considered as a marker of oxidative stress (Lim et al., 2004) since cellular GGT has a central role in glutathione homeostasis by initiating the breakdown of extracellular glutathione (GSH)—the critical antioxidant defence for the cell (Kugelman et al., 1994).

Our results are in agreement with similar data reported in different experimental models of ethanol exposure in rats (Ozaras et al., 2003; Jurczuk et al., 2004; Husain et al., 2005) and confirm the pathogenic role of oxidative stress in the liver. Several mechanisms by which ethanol could promote oxidative stress have been suggested (Lieber, 2000) including increased generation of ROS at the microsomal level, especially through the involvement of cytochrome P450IIE1, which is induced by administration of a large ethanol dose or by chronic ethanol ingestion (Nordmann, 1994; Albano et al., 1999; Navasumrit et al., 2000; Sun and Sun, 2001). The ROS in turn have the capability of initiating membrane lipid peroxidation and affect the antioxidant system (Nordmannn et al., 1992). By-products of lipid peroxidation have been shown to promote collagen production (Parola et al., 1993; Tsukamoto et al., 1995) and to form adducts with proteins (Xu et al., 1998). They have been suggested to be initiators of inflammatory response and fibrosis and therefore suspected to play an important role in alcoholic liver disease (Ingelman-Sunderberg et al., 1993).

In view of the first results, we try in the second protocol of our study to investigate the ability of a natural antioxidant from red wine, resveratrol, to inhibit ethanol-induced liver damage when given as a supplement diet to the chronically ethanol-treated rats. We have previously demonstrated that resveratrol appears as a good candidate in the prevention of ethanol-induced lipid peroxidation in the liver and other organs as heart, brain and testis (Kasdallah-Grissa et al., 2006). In the present study we showed that resveratrol diminished hepatic tissue injury as monitoring by normal liver architecture except for mild inflammation. Consistent with the improved morphology, treatment with this dietary regimen was associated with a corresponding reduction in levels of transaminases, ALP, GGT indicating a protective role of resveratrol against ethanol toxicity in the liver. Furthermore, liver activities of antioxidant enzymes, namely, SOD, GPx and CAT were visibly ameliorated by resveratrol supplemented diet during ethanol administration. A part of these results are in agreement with those of (Sun et al., 1999) who showed that dietary supplementation of grape polyphenols prevented ethanol-induced changes in hepatic morphology.

The antioxidant activity of resveratrol has been repeatedly confirmed against the peroxidation of low-density lipoprotein (LDL) (Frémont et al., 1999), liver microsomes (Cai et al., 2003) and neuron cells (Chanvitayapongs et al., 1997; Sun et al., 1997) beside its ability to improve the activity of antioxidant enzymes in erythrocytes (Young et al., 2000); it consequently protects cell death even during ethanol intoxication (Chanvitayapongs et al., 1997; Sun et al., 1997). It is thought that because it contains highly hydrophilic and lipophilic properties and it can provide more effective protection than other well-known antioxidants, such as vitamin E and C (Chanvitayapongs et al., 1997; Murcia and Martinez-Tome, 2001).

The antioxidant mechanism of resveratrol may be related to its ability to detoxify superoxide and nitrite anion produced by neutrophils and macrophages (Leiro et al., 2002; Cavallaro et al., 2003; Shigematsu et al., 2003), trapping both peroxyl radical and/or hydroxyl radical and reducing α-tocopheroxyl radical to regenerate the endogenous tocopherol (Cai et al., 2003). The capacity of resveratrol to scavenge ROS might be attributed to a hydrogen-electron donation from its hydroxyl groups (Lüpez-Velez et al., 2003).

The hepatoprotective activity of resveratrol against fibrogenesis has been explained by the capacity of this polyphenol to inhibit the activation of stellate cells by disrupting signal

transduction pathway and cell cycle protein expression. Stellate cells have been shown to play a critical role in the development of liver fibrosis and were enhanced by oxidative stress (Kawada et al., 1998). Anti-inflammatory effects of resveratrol are also well demonstrated by its ability to inhibit the production of nitric oxide (Kawada et al., 1998; Flesch et al., 1998) and tumor necrosis factor α (TNF-α) (Kawada et al., 1998; Bi et al., 2005).

In conclusion, the present study gives evidence of hepatic damage caused by chronic ethanol exposure and shows the capability of resveratrol to prevent this toxicity by inhibiting the peroxidation of lipids and improving the activity of antioxidant enzymes. However, the accurate mechanism is not clear so far to propose the potential therapeutic use of resveratrol in preventing the liver from ethanol-induced oxidative damage and further studies are needed.

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