



Hepatocyte growth factor, vascular endothelial growth factor, glial cell-derived neurotrophic factor and nerve growth factor are differentially affected by early chronic ethanol or red wine intake

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ABSTRACT

Ethanol intake during pregnancy and lactation induces severe changes in brain and liver throughout mechanisms involving growth factors. These are signaling molecules regulating survival, differentiation, maintenance and connectivity of brain and liver cells. Ethanol is an element of red wine which contains also compounds with antioxidant properties. Aim of the study was to investigate differences in hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), glial cell-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) in brain areas and liver by ELISA of 1-month-old male mice exposed perinatally to ethanol at 11 vol.% or to red wine at same ethanol concentration. Ethanol was administered before and during pregnancy up to pups' weaning. Ethanol *per se* elevated HGF in liver and cortex, potentiated liver VEGF, reduced GDNF in the liver and decreased NGF content in hippocampus and cortex in the offspring. We did not find changes in HGF or NGF due to red wine exposure. However, we revealed elevation in VEGF levels in liver and reduced GDNF in the cortex of animals exposed to red wine but the VEGF liver increase was more marked in animals exposed to ethanol only compared to the red wine group. In conclusion the present findings in the mouse show differences in ethanol-induced toxicity when ethanol is administered alone or in red wine that may be related to compounds with antioxidant properties present in the red wine.

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1. Introduction

Growth factors are signaling molecules that are able to influence survival, differentiation, maintenance and connectivity of different cells, including brain and liver cells (Allen and Dawbarn, 2006; Levi-Montalcini, 1987; W. Sun et al., 2002). Prominent growth factors playing critical roles in the physiopathology of brain and liver cells are nerve growth factor (NGF) and hepatocyte growth factor (HGF). Vascular endothelial growth factor (VEGF) and glial cell-derived neurotrophic factor (GDNF) have also been found to be produced by and acting upon brain and liver cells (Burke, 2006; Maharaj et al., 2006). Prenatal ethanol intake is known to cause a plethora of behavioral, biochemical and structural effects in postnatal life in

the liver and brain (Addolorato et al., 1997; Niccols, 2007). However, the mechanisms throughout which these deleterious effects occur, as well as the effects induced in the constitutive concentration of these important signaling molecules are not known. It has been shown that acute and chronic ethanol treatment enhances the production of reactive oxygen species, lowers cellular antioxidant levels, and increases oxidative stress in many tissues, especially the liver and the brain. Ethanol-induced oxidative stress plays a major role in the mechanisms by which ethanol produces liver and brain injury (Dey and Cederbaum, 2006). Using a mouse model of early ethanol administration (Fiore et al., 2009) the aim of the present study was to investigate differences in toxicity due to early (prenatal and postnatal up to weaning) chronic ethanol or red wine consumption (at same ethanol concentration, 11%) on the levels of HGF, VEGF, NGF and GDNF in the liver, and brain.

2. Materials and methods

2.1. The mouse model of ethanol and red wine administration

CD-1 outbred female mice were housed singularly in Plexiglas cages (33 cm × 13 cm × 14 cm) under standardized conditions with pellet food (enriched

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standard diet purchased from Mucedola, Settimo Milanese, Italy). A 12L:12D lighting regime was used. To fully mimic chronic ethanol exposure the liquid administration started 60 days before pregnancy as previously shown (Fiore et al., 2009). Dams were divided in three groups: ethanol, red wine and sucrose ($n=6$ for each group). Animals of the ethanol group received *ad libitum*, as only source of liquid, ethanol (11 vol.%) dissolved in water (Carneiro et al., 2005; Dubois et al., 2006). Ethanol used for the preparation of the drinking solutions was obtained from Merck (Darmstadt, Germany) and was of analytical grade. Red wine *ad libitum* (11 vol.%, red wine from different grapes of different regions of center Italy) was the only source of liquid in the red wine group. The sucrose group received sucrose dissolved in water at equivalent caloric intake of the ethanol group and was used as control group. This treatment schedule ended at the time of pups weaning. This method of liquid administration was chosen to limit stress due to handling to pregnant mice. Fluid intake was measured regularly and the amounts consumed were calculated (Fiore et al., 2009). All groups received pellet food *ad libitum* as above. Mating took place over a period of 2 days. The day of plug detection was designated as gestational day 0. At birth each litter was reduced to four males and four females when possible to maintain a uniform sex ratio as previously shown (Cirulli et al., 1997). Pups remained with their own mother. Only male mice were used. All efforts were made to minimize and reduce animal suffering and for limiting the number of animals used. All animal experiments were carried out following the procedure described by the guidelines of the European Community Council Directive of 24 November 1986 (86/609/EEC).

The red wine used in the study (Vignetta, Caldirola, Italy) contains more than 800 total polyphenols expressed as gallic acid equivalents in mg/l of red wine. In particular, anthocyanins values expressed as cyanidin chloride equivalents in mg/l of red wine were more than 160 (Fiore et al., 2009).

2.2. HGF, VEGF, GDNF and NGF determination

Growth factors were analyzed in the liver, frontal cortex and hippocampus of 1-month-old mice with ELISA kits following the indication provided by the manufacturer. Animals were sacrificed by CO₂ exposure and the brain and liver were quickly removed. Brain areas were dissected according to the Mouse Brain Atlas (Franklin and Paxinos, 1997). The tissues were homogenized with ultrasonication in extraction buffer (0.01 M Tris–HCl buffer, pH 7.4, containing 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA 2 mM PMSF, 50 mM leupeptin, 100 µg/ml pepstatin, and 100 µg/ml aprotinin) and centrifuged at 4 °C for 10 min, 13,000 rpm and supernatants were recovered (EDTA, ethylenediamine-tetraacetic acid; EGTA, ethyleneglycol-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride). HGF evaluation was carried out with the ELISA kit “mouse HGF DuoSet ELISA development system catalog number DY2207” by R&D (Minneapolis, MN, USA). VEGF evaluation was carried out with the ELISA kit “VEGF DuoSet ELISA development system catalog number DY493” by R&D (Minneapolis, MN, USA). GDNF evaluation was carried out with ELISA kits “GDNF Emax ImmunoAssay System number G7621” by Promega (Madison, WI, USA). NGF was carried out with the ELISA kit “NGF Emaxtm ImmunoAssay System number G7631” and “BDNF Emaxtm ImmunoAssay System number G6891” by Promega (Madison, WI, USA). Data are represented as pg/µg total proteins and all assays were performed in duplicate (Di Fausto et al., 2007; Fiore et al., 2008).

2.3. Statistical analysis

Data were analyzed by ANOVA with ethanol, red wine and sucrose as main factors. A difference of 0.05 or less was considered statistically significant. Post hoc comparisons were performed using the Tukey's HSD test.

3. Results

3.1. The mouse model of early ethanol/red wine exposure

General data on the mouse model have been previously shown (Fiore et al., 2009) and revealed no differences between groups of dams in pregnancy duration, neither in pups delivery, pups mortality and sex ratio. Ethanol and red wine groups consumed less liquid compared to controls, however, ethanol and red wine groups consumed equivalent amounts of liquid corresponding to the same caloric intake since the red wine used in the present study had 605 kcal/l (from the indication provided by the wine producer, www.caldirola.it), energetic value deriving only from the alcohol content of the wine. Data also showed that adult animals exposed to only ethanol had disrupted levels of both NGF and BDNF in the hippocampus and other brain areas associated with impaired ChAT immunopositivity in the septum and Nuclei Basalis and with altered cognition and emotional behavior. However, mice exposed to red wine had no change in the behavior or in ChAT immunopositiv-

ity but a decrease in hippocampal BDNF and a mild NGF decrease in the cortex. Dams blood ethanol levels (Fiore et al., 2009) were comparable between the red wine and the ethanol groups measured at gestational day 15. The blood ethanol levels (Fiore et al., 2009) of 7-day-old offspring were also analogous between the two groups.

3.2. HGF in the liver, cortex and hippocampus

Fig. 1 shows the results on the ELISA for HGF in the liver, cortex and hippocampus of mice exposed to ethanol only and to red wine. In the liver and in the cortex, animals exposed to ethanol only had highest values when compared to the red wine group or to controls ($p < 0.05$ in the ANOVA and post hoc). No differences between groups were scored for the hippocampus.

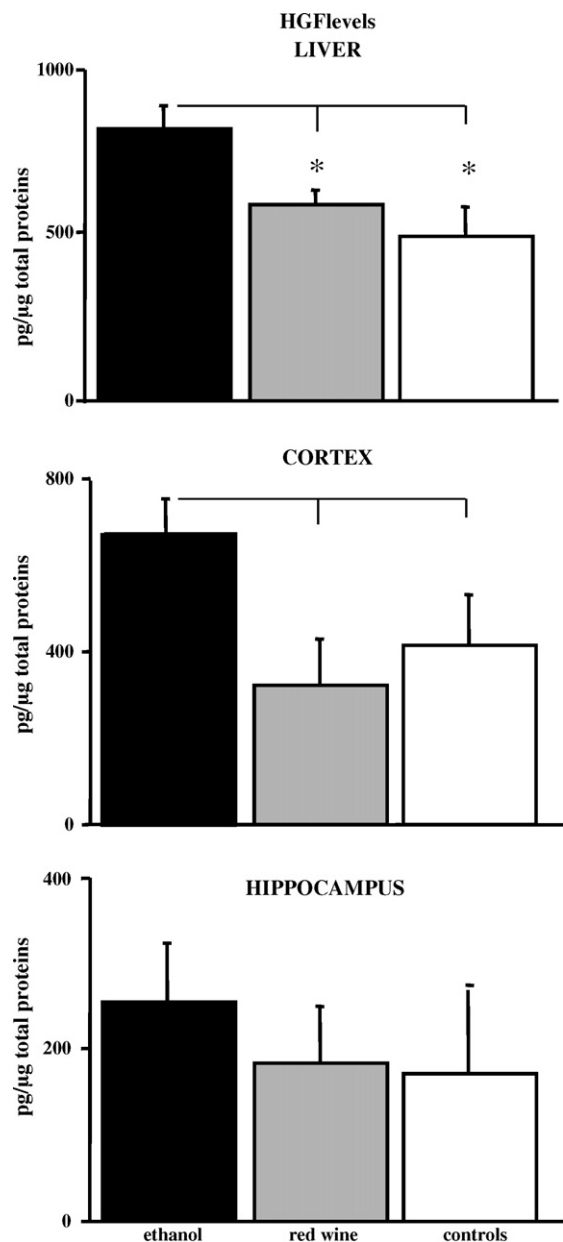


Fig. 1. HGF levels expressed as pg/µg total proteins in the liver, frontal cortex and hippocampus of 1-month-old mice exposed to ethanol, red wine and sucrose as controls. Asterisks indicate significant differences between groups ($*p < 0.05$). The vertical lines in the figure indicate pooled S.E.M. derived from appropriate error mean square in the ANOVA.

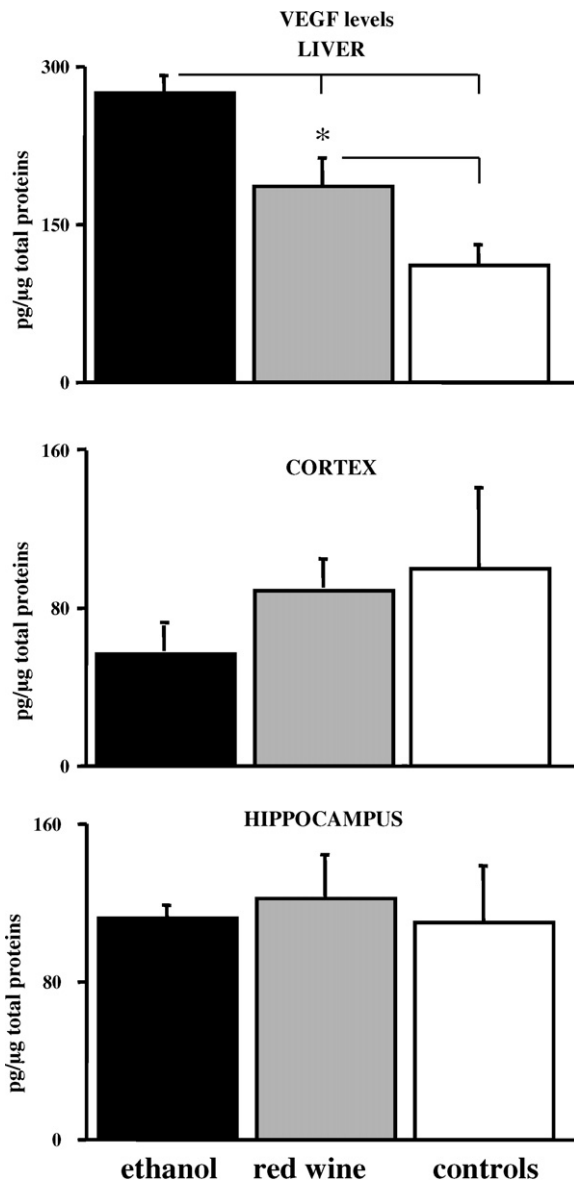


Fig. 2. VEGF levels expressed as pg/μg total proteins in the liver, frontal cortex and hippocampus of 1-month-old mice exposed to ethanol, red wine and sucrose as controls. Asterisks indicate significant differences between groups ($*p < 0.05$). The vertical lines in the figure indicate pooled S.E.M. derived from appropriate error mean square in the ANOVA.

3.3. VEGF in the liver, cortex and hippocampus

Data on VEGF are shown in Fig. 2. In the liver, ethanol administration induced an elevation in VEGF in both ethanol group and red wine group ($p < 0.01$ in the ANOVA). However, the liver of mice exposed to ethanol only revealed highest levels of VEGF when compared to the red wine group ($p < 0.05$ in post hoc) and to controls ($p < 0.01$ in post hoc comparison). No significant changes in VEGF were found in the cortex and hippocampus.

3.4. GDNF in the liver, cortex and hippocampus

GDNF was affected by ethanol administration and these findings are shown in Fig. 3. In the liver, GDNF was reduced in animals exposed to ethanol only ($p < 0.05$ in the ANOVA and post hoc) when compared to the other groups. In the cortex no changes were found for the animals exposed to ethanol only, however, low GDNF values

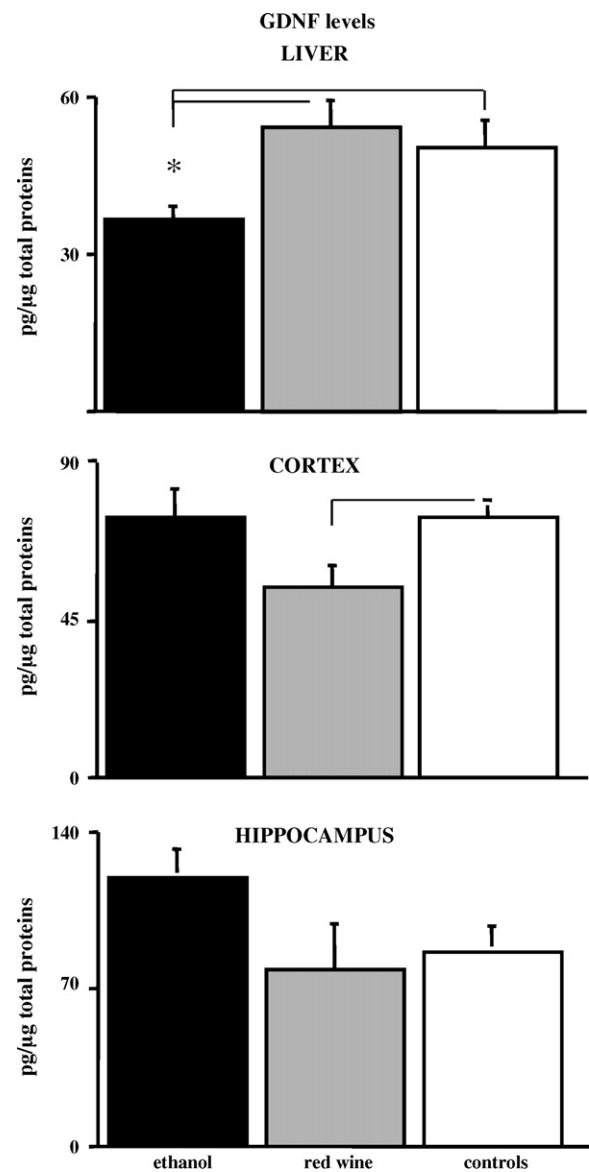


Fig. 3. GDNF levels expressed as pg/μg total proteins in the liver, frontal cortex and hippocampus of 1-month-old mice exposed to ethanol, red wine and sucrose as controls. Asterisks indicate significant differences between groups ($*p < 0.05$). The vertical lines in the figure indicate pooled S.E.M. derived from appropriate error mean square in the ANOVA.

($p < 0.05$ in the ANOVA) were observed in the red wine group when compared to the other groups ($ps < 0.05$ in post hoc). In the hippocampus, no significant differences between groups were found.

3.5. NGF in the liver, cortex and hippocampus

Data on NGF are shown in Fig. 4. In the liver no changes were found due to ethanol or red wine intake. In the cortex and hippocampus, low levels of NGF were found in the ethanol group ($ps < 0.05$ in the ANOVA) compared to both the remaining groups for the cortex ($ps < 0.05$ in post hoc) or to controls for the hippocampus ($p < 0.05$ in post hoc).

4. Discussion

Two major questions were addressed in the present study. The first one was to investigate whether four important growth factors present in the brain and in the liver are similarly or differently

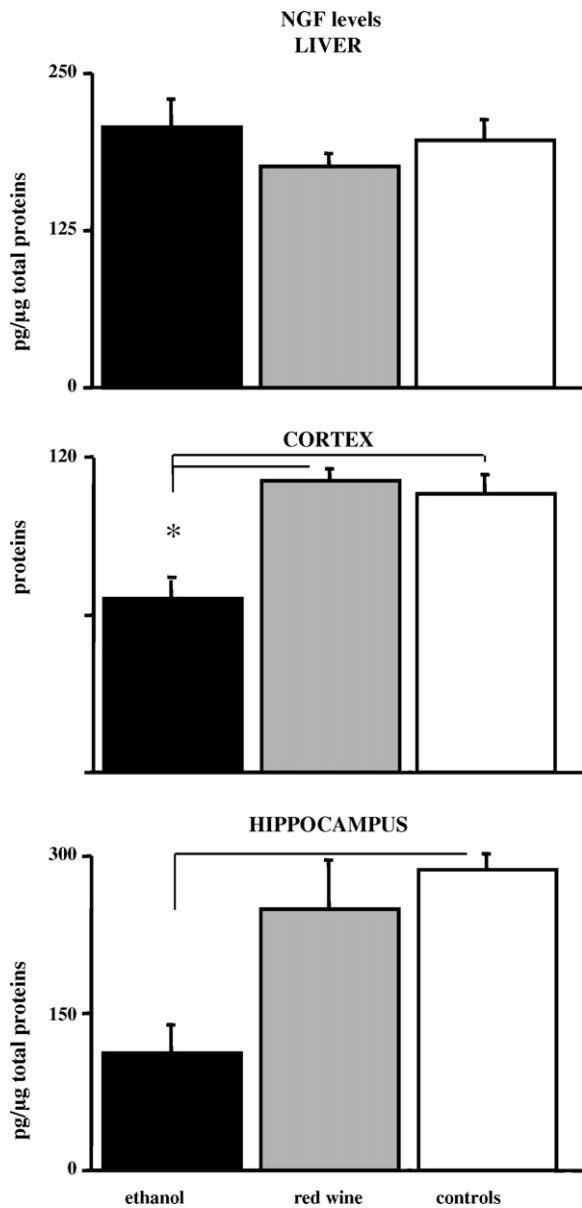


Fig. 4. NGF levels expressed as pg/μg total proteins in the liver, frontal cortex and hippocampus of 1-month-old mice exposed to ethanol, red wine and sucrose as controls. Asterisks indicate significant differences between groups ($*p < 0.05$). The vertical lines in the figure indicate pooled S.E.M. derived from appropriate error mean square in the ANOVA.

affected by chronic consumption of ethanol. The rationale of this experimental approach is based on a number of well established evidences showing that HGF, VEGF, NGF and GDNF may play a crucial action on survival, differentiation and function of specific tissues, namely brain and liver, known to be severely affected by ethanol (Aloe, 2006; Lalani et al., 2005; Tahara et al., 1999). We found a HGF potentiation in the liver and cortex and a liver VEGF elevation in the ethanol group. We have also shown an ethanol-induced GDNF decrease in the liver and a decreased NGF content in the hippocampus and cortex in mice exposed to ethanol only. It should be noted that we performed the study with an additional control group exposed to water only (Fiore et al., 2009) that was not included in the statistic since it had similar results to the sucrose group.

HGF regulates cell growth, cell motility, and morphogenesis, is secreted by mesenchymal cells and acts as a multi-functional cytokine on cells of mainly epithelial origin. Its ability to stimulate

mitogenesis, cell motility, and matrix invasion gives it a central role in angiogenesis, tumorigenesis, and tissue regeneration. As for HGF and liver, HGF expression is up-regulated in various forms of liver injury including experimental alcoholic liver disease. HGF following ethanol intoxication in the liver was found to be produced by Kupffer cells and endothelial cells whereas its receptor c-Met was seen only in hepatocytes. This occurred in the presence of ethanol-induced necrosis and inflammation suggesting that HGF may be a protection factor against liver injury or may contribute to accelerate the regenerative process (Lalani et al., 2005). Concurrently, HGF elevation was observed in the cortex of mice exposed to ethanol only and it has been hypothesized that HGF is involved in the development and maintenance of cortical neurons during differentiation, motogenesis, neuritogenesis and neuronal survival (W. Sun et al., 2002). We also found that chronic ethanol exposure enhanced the presence of VEGF in the liver suggestive of endothelial and vascular cell response. VEGF is a mitogen that specifically acts on endothelial cells and has various effects, including mediating increased vascular permeability, inducing angiogenesis, vasculogenesis, endothelial cell growth, promoting cell migration, and inhibiting apoptosis, thus it is possible that chronic ethanol intake promotes liver neo-vascularization and concurs to the liver tumorigenesis induced by ethanol since ethanol intake can lead to development of liver neoplasia (Gu et al., 2005; Gu et al., 2001). VEGF has been extensively investigated recently in various hepatic diseases such as primary and secondary hepatocellular carcinoma, liver cirrhosis, hepatitis and even benign tumors in liver (Furuse, 2008; Pang and Poon, 2007; Park et al., 2000). The protein of VEGF has an inclination to increase in acute and chronic hepatitis and tends to decrease in cirrhosis both in tissue expression and circulating levels (Bardag-Gorce et al., 2002; Shi et al., 2001).

An additional novel finding of the present study indicates that ethanol may affect GDNF levels in the liver with a decrease compared to controls or red wine exposed animals. The functional significance of this new finding is not known but it could be associated with the altered liver dopamine content due to ethanol intoxication (Burke, 2006; George et al., 2007). Indeed, the recombinant form of this protein was shown to promote the survival and differentiation of dopaminergic neurons in culture, and was able to prevent apoptosis of motor neurons induced by axotomy (McAlhany et al., 2000). In addition, an excessive alcohol consumption may be blocked by GDNF delivery in the ventral tegmental area in the rat (Carnicella et al., 2009).

NGF levels were also affected by early ethanol administration with a decrease in the hippocampus and cortex, data in line with studies on chronic exposure to ethanol in the rat (Miller and Mooney, 2004). It has also been shown that aged rats chronically exposed to ethanol had a marked but transient elevation in brain NGF levels (Gericke et al., 2006) and temporary neurotrophins elevation has been found in rats prenatally exposed to ethanol for a few days (Heaton et al., 2000). Evidences reported recently indicate that HGF is present in the CNS and similarly to NGF possesses neurotrophic activity (Koyama et al., 2003; Niimura et al., 2006; Sofroniew et al., 2001). However, whether NGF and HGF have overlapping or distinct effects on brain and liver cells is not known. Our findings suggest that following chronic ethanol intake, NGF-producing and HGF-producing cells are differently affected. While the mechanisms implicated in the marked alteration of these proteins have not been yet delineated, it is known by previous studies that NGF or HGF administration may have neuroprotective, hepatoprotective and vascular-protective effects (Chao et al., 2006; Kaido and Imamura, 2001; Niimura et al., 2006).

The second question addressed in this study was to examine the effects induced by chronic red wine intake since recent works consider red wine administration with positive nutritional properties for the presence of chemicals with protective characteristics

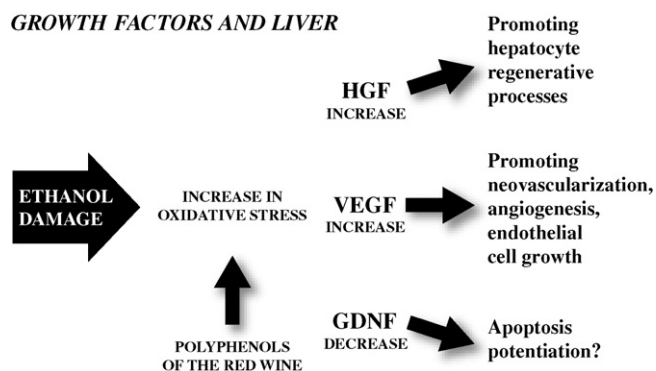


Fig. 5. Schematic representation of HGF, VEGF and GDNF mouse liver response following early ethanol damage. The elevation in oxidative stress may be counteracted by the polyphenols content of the red wine.

(Mattivi et al., 2002; Opie and Lecour, 2007). Quite interestingly, in the present experiment we found no changes in HGF or NGF due to red wine exposure, but elevated VEGF levels in the liver and reduced GDNF in the cortex. However, the VEGF liver increase was much more marked in animals exposed to ethanol only compared to the red wine group suggestive of differences in liver toxicity levels between the ethanol only group and the red wine group. The data obtained show also that red wine consumption may affect the presence of GDNF in the CNS. At the present time it is not known the functional significance or the physiological consequences of this finding. In the rat it has been shown that postnatal ethanol-induced reduction of GDNF mRNA in cortex but not in the hippocampus or no GDNF changes at all (Okamoto et al., 2006; Tsuji et al., 2008). However, the GDNF changes we found in the cortex of mice exposed to red wine could be related to compounds present in the red wine and not to ethanol *per se* or to a combination of both (Cho et al., 2008; Dulak, 2005). Data to support this hypothesis illustrate a role played by red wine ingestion in dopamine turnover (de la Torre et al., 2006) or protection following dopaminergic damage (Blanchet et al., 2008; Gursoy and Buyukuyal, 2008).

Our mouse study reveals also marked differences in ethanol-induced toxicity when ethanol is administered as ethanol only or combined to red wine. These differences in brain and liver toxicity could be related to compounds with putative antioxidant properties present in the red wine (see Fig. 5) as the polyphenols (Das and Das, 2007; Sang et al., 2005; A.Y. Sun et al., 2002) or to the role played by polyphenols in modulating cell functions. Indeed polyphenols target many components of intracellular signaling pathways including pro-inflammatory mediators, regulators of cell survival and apoptosis, and tumor angiogenic and metastatic switches by modulating a distinct set of upstream kinases, transcription factors and their regulators (Kundu and Surh, 2008; Shankar et al., 2007). In addition, an altered presence of growth factors in the liver due to ethanol may represent also an early harmful event for the brain. In conclusion these data show marked differences in ethanol-induced toxicity when ethanol is administered alone or in the red wine that may be related to the antioxidant properties of the red wine.

Conflict of interest

None declared.

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