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Basic nutritional investigation

Olive polyphenol effects in a mouse model of chronic ethanol addiction

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ABSTRACT

Objectives: Alcohol addiction elicits oxidative imbalance and it is well known that polyphenols possess antioxidant properties. We investigated whether or not polyphenols could confer a protective potential against alcohol-induced oxidative stress.
Methods: We administered (*per os*) for two months 20 mg/kg of olive polyphenols containing mostly hydroxytyrosol in alcoholic adult male mice. Hydroxytyrosol metabolites as hydroxytyrosol sulfate 1 and hydroxytyrosol sulfate 2 were found in the serum of mice administered with polyphenols with the highest amount in animals treated with both polyphenols and alcohol. Oxidative stress was evaluated by FORT (free oxygen radical test) and FORD (free oxygen radical defense) tests.
Results: Alcoholic mice showed a worse oxidative status than nonalcoholic mice (higher FORT and lower FORD) but polyphenol supplementation partially counteracted the alcohol pro-oxidant effects, as evidenced by FORT.
Conclusions: A better understanding of the antioxidant protection provided by polyphenols might be of primary interest for drug discovery and dietary-based prevention of the damage associated with chronic alcohol abuse.

Introduction

In recent years, considerable attention has been devoted to lifestyle and diet as key strategies to improve health and to fight chronic diseases. As for nutrition and diet, plant polyphenols, which are bioactive molecules present in our diet, have been the subject of extensive investigation [1]. Polyphenols are a class of organic compounds characterized by the presence of multiple phenolic units. Among the most important polyphenols tannins are found in all families of vegetables, comprising up to 50% of the dry weight of leaves. There are a number of food sources of polyphenols, such as fruits and vegetables, red wine, green and black tea, coffee, chocolate, olive leaves, extra virgin olive oil, and olives [2] and they are well known to elicit crucial biological effects including antioxidant, anticancer and antiinflammatory properties [3]. Indeed, animal models and clinical studies indicate that polyphenols may have protective effects in various pathologic states including inflammation, apoptosis, mitochondrial dysfunction and, particularly relevant, oxidative stress and reactive oxygen species (ROS) production [4]. In this regard olive oil is considered an important constituent of the traditional Mediterranean diet and many of the health advantages of this diet have been attributed to olive oil's polyphenols [5,6].

Oxidative imbalance is one of the most important mechanisms leading to alcohol-induced toxicity. The metabolism of ethanol is closely linked to ROS generation and oxidative stress. An important area of research in the study of alcohol and alcoholism is the understanding of the role of ROS in ethanol-induced disease for its importance in the development of innovative therapeutics to prevent ROS action and the toxic effects of ethanol [7].

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Alcohol induces oxidative stress by direct action, increasing the production of oxidant chemical species, and/or by indirect action, reducing the antioxidant capacity of the cells. The consequences are alterations of lipids and proteins and epigenetic modifications of DNA that is particularly vulnerable to the effects of oxidative stress.

Occasional exposure to alcohol is sufficient to induce an imbalance in the intracellular redox state and produce tissue damage. Some target tissues of ethanol intoxication are the brain and the liver, which show a high metabolic rate involving a large amount of oxygen consumption mainly required to regenerate the adenosine triphosphate (ATP) necessary to maintain the intracellular ion homeostasis and to allow cell survival [8]. However, the elevated oxygen consumption makes the tissue more susceptible to generate oxidative stress and ROS hyperproduction [8].

In this study, we examined the causal relationship between polyphenols supplementation and oxidative stress in a mouse model of chronic alcohol addiction, which is known to be characterized by oxidative imbalance. We aimed at investigating whether or not polyphenols may confer a protective potential against alcohol-associated damage by providing antioxidant defense in mice.

Methods

Animals

CD-1 outbred male mice were used in this experiment. All animals were 3 mo old and housed in groups of 5 mice at the beginning of the experiments in Plexiglas cages (33 × 13 × 14 cm) under standardized conditions with pellet food (enriched standard diet purchased from Mucedola, Settimo Milanese, Italy). Food (Purina Lab Chow # 5015) and water were available *ad libitum*. A 12 L:12 D lighting regime was used.

40 male CD-1 mice were randomly divided into four groups: (i) a group of mice (n = 10) received sucrose dissolved in water at equivalent caloric intake of the ethanol group and was used as control group; (ii) another group of mice (n = 10) received *ad libitum*, as only source of liquid, after an habituation period, ethanol 11% dissolved in water for 90 d; (iii) a third group of mice (n = 10) received polyphenols (20 mg • kg^{-1} • d^{-1}) dissolved in sucrose; and (iv) a further group of mice (n = 10) received polyphenols (20 mg • kg^{-1} • d^{-1}) dissolved in ethanol 11% (again following an habituation period). The four groups are named, respectively: (i) *Ctrl*; (ii) *EtOH*; (iii) *Poly*; and (iv) *EtOH+Poly*. Ethanol used for the preparation of the drinking solution was obtained from Sigma-Aldrich (St. Louis, Missouri, USA) and was of analytical grade. Fluid intake was measured regularly and the amounts consumed were calculated [9]. All groups received pellet food *ad libitum* as above. Food intake was measured regularly and the daily amounts consumed were calculated. Two months after treatment, mice were used for the experiments. 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). Permission number 08-2014 of February 3, 2014.

Polyphenol's blend

The polyphenols used in the present study, as indicated by the manufacturer (www.phenopharm.it), derive from a natural standardized olive pulp (*Olea europaea* L.) extract obtained by an eco-sustainable patented mechanical process (Table 1). It is a by-product of the olive residues (pomace) obtained following olive pressing during the production of extra virgin olive oil. Specifications of the mixture, as indicated by the manufacturer, are shown in a previous study [10]. We used a blend of phenolic compounds because it has been proposed that most of the health benefits associated with virgin olive oil are due to its minor components [5]. Due to the possible synergisms, a combination of biophenols is supposed to possess a stronger action for counteracting different stages of oxidative damage than a single compound [11].

Blood samples

Animals were sacrificed by a guillotine 1 wk after the behavioral experiments. The blood was collected in vials and quickly centrifuged at 10 000 rpm for 15 min for serum preparation (n = 5 per group) or in heparin vials for the measurement of blood ethanol levels (n = 5 per group). Serum aliquots were then stored at −80°C while heparin vials were stored at 4°C.

Table 1
Phenolea Active Complex Composition

Specification of Phenolea_Active Complex as indicated by the manufacturer	
Appearance	Red rubin molasses
Solubility in water, %	>99
Microbiologic	
Salmonellae SPP	absent in 25 g
Escherichia coli	absent in 1 g
Yeast and molds, CFU/g	≤5 × 102
Enterobacteria, CFU/g	≤1 × 102
Total plate count, CFU/g	≤5 × 105
Pesticides	absent
Aflatoxins	absent
Ochratoxin	absent
Polycyclic aromatic hydrocarbons mg/kg	<1
Chemical composition of Phenolea_Active Complex	
Moisture	28%
Carbohydrates	61%
Ashes	6.5%
Proteins	2.5%
Fats	0%
Crude fiber	2%
Phenolic composition of Phenolea_Active Complex	
Total polyphenols	5%
Phenolic families (% on total polyphenols)	
Hydroxytyrosol	30%
Other hydroxytyrosol derivatives	20%
Ligstroside and derivatives	6%
Total secoiridoid acids	14%
Total phenolic acids	10%
Oleocanthal	2%
Other polyphenols (flavonoids, anthocyanins, oleuropein, lignans thyrosol)	18%

CFU, colony-forming unit; SPP, species

Blood ethanol levels by gas chromatography/head space procedure

Gas chromatography/Head Space procedure was applied in this research to determine blood alcohol concentration in whole blood samples (n = 5 for each group). In this research, a Clarus 600 Gas Chromatography Perkin Elmer and a TurboMatrix 40 Trap HeadSpace Sampler Perkin Elmer with FID detector was used. Analytical conditions were set up and the method was validated by a previous study [12]. From each blood sample was collected 100 μL of whole blood with a micropipette and transferred this volume into a gas chromatography vial. The gases which are formed inside gas chromatography vial were collected to be analyzed. If the sample was not analyzed in the same day as its collection, it was important to firmly close the vial to prevent the evaporation of ethanol during time and to conserve the vial inside of a refrigerator. Standard solutions were set up for calibration curve at 100 mg, 50 mg, 25 mg, 12.5 mg, 6.2 mg, and 3.1 mg of ethanol and were obtained by consequent dilutions of pure ethanol in distilled water.

Free oxygen radicals defence and free oxygen radicals test

Free oxygen radicals defence (FORD) and free oxygen radicals (FORT) tests were carried out using two specific kits (both purchased by Callegari, Parma, Italy) following the instruction provided by the manufacturer [13,14]. Blood serum was used both for the FORT and FORD determination (n = 5 for each group). FORD test allows the determination of free oxygen radical defense. Briefly, this test uses a preformed stable and colored radical and determines the decrease in absorbance that is proportional to the antioxidant concentration of the sample according to Lambert Beer's law [15]. In the presence of an acidic buffer (pH = 5.2) and a suitable oxidant ($FeCl_3$) the chromogen, which contains 4-amino-*N*,*N*-diethylaniline sulfate forms a stable and colored radical cation photometrically detectable at 505 nm. Antioxidant compounds in the sample reduce the radical cation of the chromogen quenching the color and producing a decoloration of the solution, which is proportional to their concentration. The absorbance values obtained for the samples are compared with a standard curve obtained using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E commonly used as a reference.

By contrast, FORT test allows the determination of free oxygen radicals (ROSs) through a colorimetric assay based on the ability of transition metals, such as iron, to catalyze the breakdown of hydroperoxides (ROOH) into derivative radicals, according to Fenton's reaction [15]. Briefly, when 20 μL of the blood serum sample was dissolved in an acidic buffer, the hydroperoxides reacted with the transition metal ions liberated from the proteins in the acidic medium and were converted to alkoxy- ($RO_{\blacksquare}$) and peroxy- ($ROO_{\blacksquare}$) radicals. The radical species produced by the reaction interact with an additive (phenylendiamine derivative (2 $CrNH_2$)) that forms a colored, fairly long-lived radical cation evaluable by spectrophotometer at 505 nm (linear kinetic-based reaction, 37°C). The intensity of the color correlates directly to the quantity of radical compounds and to the hydroperoxide concentration and, consequently, to the oxidative status of the sample according to the Lambert Beer law (Form CR 2000; Callegari, Parma, Italy).

Serum hydroxytyrosol measurement

Hydroxytyrosol, also known as DOPET, a derivate of dopamine, is naturally present in the mouse serum [5]. Indeed, the serum for hydroxytyrosol and its derivates measurement was immediately prepared by centrifugation at 1500 g for 30 min at 20°C. Before analysis, the aliquots of serum from each animal were thawed and pooled (1 aliquot for each treatment). The sample were acidified with formic acid and diluted to 0.8 mL with milliQ water (water purified using Millipore MilliQ lab water system, Billerica, MA, USA); salicylic acid (20 ng) was added as internal standard. The samples were centrifuged at 13000 rpm for 30 min at 4°C, and supernatants were purified by SPE using Oasis HLB 30 mg cartridges (Waters, Milford, MA). The eluted fractions were evaporated by a gentle flow of nitrogen, and the residues were dissolved in 0.2 mL of methanol, filtered through 0.20 μm PVDF filters and injected. Quantitative online HPLC-ESI-MS/MS analyses were performed using HPLC system interfaced to an Applied Biosystems (Foster City, CA, USA) API3200 Q-Trap instrument working with triple quadrupole analyser in Multiple Reaction Monitoring (MRM) mode. LC analyses were conducted using a system equipped with a 200 binary pump (PerkinElmer, Waltham, MA, USA). Samples were injected (10 μL) into an Atlantis column T3 (Waters) (150 × 2.1 mm, 3 μm) and eluted at flow rate of 0.2 mL/min. Mobile phase A was H_2O containing 0.1% formic acid while mobile phase B was acetonitrile containing 0.1% formic acid. Elution was carried out using a linear gradient commencing at 0% B and changing to 50% B in 10 min, isocratic 50% B in 5 min, then to 80% B in 1 min, isocratic 80% B for 4 min, and finally to 0% B in 1 min. Column was then equilibrated for 10 min at 0% B for 10 min. The column was kept at 30°C, using a Peltier Column Oven Series 200 (PerkinElmer). The flow from the chromatograph was injected directly into the ESI source. The API 3200 ES source was tuned by infusing solutions of hydroxytyrosol, tyrosol and salicylic acid (1 ng/μL in methanol) into the source at a flow rate of 1 μL/min. The MS operated with an electrospray voltage at −4500 V and with source temperature of 450°C. Nitrogen was used as ion spray (GS1), dryng (GS2), and curtain gas at 20, 40 and 40 arbitrary units, respectively. The declustering potential (DP), collision energy (CE) and entrance potential (EP) were −32, −20, and −7 V for hydroxytyrosol; −32, −20, and −7 V for tyrosol; and −42, −15, and −8 V for salicylic acid, respectively. Hydroxytyrosol, tyrosol, hydroxytyrosol sulfates, and salicylic acid were detected with MRM transition of 153/123, 137/106, 233/153, and 137/93 $[M–H]^-$, respectively. Data acquisition and processing were performed using Analyst software 1.5.1. The quantification of phenolic compounds was performed by using a calibration curve; due to lack of standard, the quantification of hydroxytyrosol sulfates was tentatively quantified using the calibration curve of hydroxytyrosol. The chromatographic analysis of the serum revealed two different hydroxytyrosol sulfates (named HTS1 and HTS2), this was probably due to the presence of sulfate group in different positions.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) considering as main factors ethanol and polyphenols administration. Post-hoc comparisons were performed using the Tukey's HSD test. Data are illustrated as mean ± SEM.

Results

Polyphenols effects on food and liquid intake and body weight

Under the present experimental conditions, despite having ingested more liquid and food than the other two groups ($F [3,12] = 47.98$; $F [3,12] = 11.74$, respectively; $P < 0.005$), the polyphenols' group did not show significant changes in body weight as shown at the end of the treatment ($F [3,36] = 1.35$; $P = 0.27$) (Table 2). The ethanol blood levels in the ethanol groups ranged from 3.2 to 20.8 mg/100 mL.

Table 2
Mouse body weight and mean daily food and liquid consumption

	Ctrl		EtOH		Poly		EtOH+Poly	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Mouse body weight								
Day of treatment								
0	34.44	0.9	34.51	0.6	35.83	0.8	35.74	0.7
30	40.48	1.1	40.17	0.7	42.37	1.0	41.88	0.6
60	44.66	1.0	43.64	0.9	46.94	1.2	45.04	0.7
90	45.99	1.3	44.57	0.8	47.53	1.2	45.73	0.8
Mean daily food and liquid consumption per cage								
Mean food consumption	94.41	5.1	98.97	5.4	112.2	6.0	101.38	5.5
Mean liquid consumption	66.66	3.0	61.57	2.7	91.85	4.2	62.45	2.7

SEM, standard error of the mean

Oxidative stress evaluation

In order to evaluate whether polyphenols might exert antioxidant effects in alcoholic mice we used two well established assays, the FORT (free oxygen radical test) and the FORD (free oxygen radical defense) tests [15] (Fig. 1). We found that FORT levels in blood serum were significantly higher in *EtOH* mice as compared to controls. Quite interestingly, the presence of polyphenols seems to have partially counteracted this effect ($F [3,16] = 299.95$, $P < 0.01$ in the ANOVA: see Fig. 1A for post-hoc comparisons). However, the levels of FORD in blood serum were significantly lower in both group of ethanol-treated mice (*EtOH* and *EtOH+Poly*) as compared to *Ctrl* and *Poly* animals ($F [3,16] = 64.8$, $P < 0.01$ in the ANOVA: see Fig. 1B for post-hoc comparisons). Quite interestingly, polyphenols supplementation increased the natural free oxygen radical defense (FORD) as shown by the significant differences between the polyphenols group versus the control group (see post-hoc comparisons of Fig. 1B).

Serum hydroxytyrosol and metabolites

Table 3 shows the measurement of hydroxytyrosol (HT), tyrosol (T), hydroxytyrosol sulfates (HTS), and hydroxytyrosol glucuronide (HTG) in the serum of each experimental group. In the serum of *Ctrl* mice and *EtOH* mice, as previously shown [10] low DOPET levels were observed. Both isomers of hydroxytyrosol sulfates (HTS1 and HTS2) were observed in the serum of *Poly* and *Poly+EtOH* mice.

Discussion

This is the first study to demonstrate that olive polyphenols' supplementation in a mouse model of ethanol addiction may confer protection against ethanol-induced oxidative stress by reducing serum free oxygen radicals but not affecting the free oxygen radicals defense. Under the present experimental conditions, our data demonstrate that (i) polyphenols can partially protect from ethanol-induced oxidative stress although further studies analyzing multiple methods of oxidative stress are required; (ii) polyphenols by themselves do not appear to potentiate the natural endogenous antioxidant resources following chronic alcohol exposure; (iii) however, polyphenols administration by themselves may potentiate the natural endogenous antioxidant resources in normal conditions.

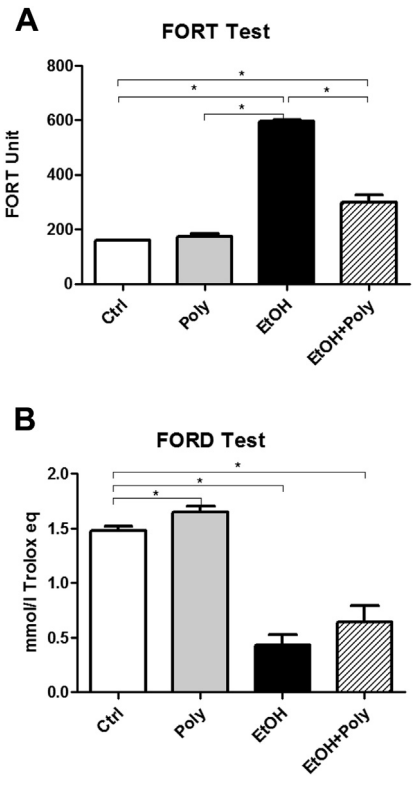

Fig. 1. FORT (A, expressed as FORT unit; see Methods) and FORD levels (B, expressed as mmol/Trolox eq; see Methods) in blood serum of control or alcoholic mice treated or not with olive polyphenols. The vertical lines in the figures indicate pooled standard error means (SEM) derived from appropriate error mean square in the ANOVA. Asterisks indicate significant differences between groups (*$P < 0.05$).

Similar findings were previously observed in aged rats treated with an olive leaf extract [16] showing a decrease in oxidative stress by acting as an antioxidant *per se* but not affecting the antioxidant system. In this study, we administered a blend of polyphenols containing mostly hydroxytyrosol. Hydroxytyrosol [5,17] is a strong inhibitor of metal-induced oxidation of low density lipoprotein. Furthermore, metal-independent oxidation is also significantly counteracted by hydroxytyrosol. The antioxidant activity of hydroxytyrosol, which has been shown to be more effective than butylated hydroxytoluene or vitamin E, was also confirmed by the use of stable free radicals. Likewise, hydroxytyrosol is a scavenger of superoxide anions. In addition, a scavenging effect of hydroxytyrosol was demonstrated with respect to potent oxidants as the hypochlorous acid, produced during inflammatory processes (reviewed by Visioli and Bernardini [5]). Antioxidant activities have also been shown against DNA damage, hydrogen peroxide-induced insult to red blood cells. Quite interestingly, hydroxytyrosol maintains antioxidant activities also in vivo [18]. It protects from second hand smoke-induced oxidation [19], ameliorates lipid profile and decreases atherosclerosis development [20]. Some of these experiments have been performed with blends of olive phenols, in which hydroxytyrosol was the most important ingredient in concentration but not the exclusive one (as in the present study). Hence, a synergy with other olive phenols is highly possible. It was also shown that olive polyphenols in male alcoholics may modulate serum brain-derived neurotrophic factor during withdrawal [14].

Table 3
Serum Hydroxytyrosol and its metabolites

Groups	T (ng/mL)	HT (DOPET) (ng/mL)	HTS1 (ng/mL)	HTS2 (ng/mL)
Ctrl	n.d.	<1.0	n.d.	n.d.
EtOH	n.d.	<1.0	n.d.	n.d.
Poly	n.d.	<1.0	11 ± 1	9 ± 2
EtOH+Poly	n.d.	<1.0	23.4 ± 0.5	22.0 ± 0.4

HT (DOPET), hydroxytyrosol; HTS1, hydroxytyrosol sulfate 1; HTS2, hydroxytyrosol sulfate 2; n.d., not detected; T, tyrosol

Our findings show that olive polyphenol supplementation determines the appearance in the serum of hydroxytyrosol metabolites (i.e., hydroxytyrosol sulphate 1 and hydroxytyrosol sulphate 2), demonstrating that olive polyphenols are absorbed in the mouse gastrointestinal tract. Basal hydroxytyrosol was detected in the serum of all groups of mice regardless polyphenol's administration. The hydroxytyrosol is not only the main phenolic compound found in olives and olive products but it can also be produced endogenously [13] as a product of dopamine oxidative metabolism known as DOPET (3,4-dihydroxyphenylethanol) [21]. It has been also shown that alcohol intake might lead to an increase in DOPET due to the interaction between ethanol and the dopamine oxidative metabolism [22,23]. Furthermore, the hydroxytyrosol bioavailability depends on an extensive metabolic pathway producing different metabolites as the glucuronide and sulfates conjugates. Another minor metabolic pathway is the conversion of hydroxytyrosol into homovanillyl alcohol catalyzed by COMT (catechol-*O*-methyltransferase) [21].

Our results provide evidence that in the serum of *Ctrl* mice and *EtOH* mice no hydroxytyrosol metabolites were detected while both isomers of hydroxytyrosol sulfates (HTS1 and HTS2) were observed in the serum of *Poly* and *Poly+EtOH* mice with highest levels in the *Poly+ETOH* group, thus confirming the enhancement of hydroxytyrosol metabolism [24] as an effect of ethanol administration. Furthermore, both in physiological condition (*Ctrl*) and in oxidative stress situation (due to alcohol intake) we did not observe hydroxytyrosol metabolites. We do believe that, possibly due to the low levels of endogenous hydroxytyrosol, the ethanol administration did not potentiate the dopamine oxidative metabolism. However, hydroxytyrosol metabolites were detected in the serum of polyphenols-administered mice. Quite interestingly, the concomitant presence of polyphenols and ethanol appears to counteract the *EtOH*-induced oxidative effect as indicated by the reduction of FORT serum level. Moreover, the larger presence of hydroxytyrosol metabolites following *EtOH* ingestion represents the first indication that the antioxidant effect of polyphenols could be significantly increased under *EtOH*-induced oxidative stress. The highest levels of sulphate metabolites of hydroxytyrosol in the *Poly+EtOH* mice seems to be due to a greater efficacy and absorption of hydroxytyrosol as a potential inducer of cell defense responses to oxidative insult. Furthermore, there is evidence that nanomolar concentrations of polyphenols may elicit their actions acting as signaling molecules and through the upregulation of the endogenous antioxidant enzyme system (see Kim et al. [25] for review).

Conclusions

Although further investigations are necessary it may be speculated that, since both polyphenols and alcohol consumption are key factors of the Mediterranean diet and alcohol abuse is also a devastating problem in Western countries, the present study may represent a step forward in the attempt to

disclose some biomolecular processes and behavioral responses underlying the antioxidant properties of polyphenols. These results may be of interest for studies in the fields of human nutrition and human addiction.

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