WHY DO NOT POLYPHENOLS OF RED WINE PROTECT AGAINST THE HARMFUL EFFECTS OF ALCOHOL IN ALCOHOLISM?

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The effect of polyphenolic bioactive substances, especially resveratrol (12.03 mg l⁻¹), of an often consumed Hungarian red wine was investigated in a short term rat experiment. Male young Wistar albino rats were treated with high volumes of red wine (matching one bottle of wine/day for a 85 kg man) (N=5) and another alcoholic drink of the same alcohol concentration (N=5), corresponding to the circumstances of alcoholism, and 5 rats were in the control group. A total of 7 routine laboratory parameters were measured from the sera by kits.

The changes of redox homeostasis (H-donor activity, induced chemiluminescence, diene-conjugates, GSHPx) were studied in blood plasma and/or in liver homogenates by spectrophotometric and luminometric methods. Transmethylation property of the liver was measured by overpressured layer chromatography (OPLC) technique. It was proven with in vitro OPLC analytical study that resveratrol reacted with methyl groups, and resveratrol was demonstrated to influence transmethylation processes as well as redox homeostasis. Red wine compounds do not protect from the harmful effects of alcohol, and even by high doses of resveratrol, the liver further deteriorates and the negative effect of alcohol increases. It has been confirmed that high doses of resveratrol do not provide protection against liver damage in those suffering from alcoholism.

Keywords: resveratrol, alcohol, hepatotoxicity, transmethylation, redox homeostasis

Abbreviations:

ADH: alcohol dehydrogenase; ALDH: aldehyde dehydrogenase; ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate transaminase; CHOL: cholesterol; CREAT: creatinine; CYP: cytochrome P450 isoenzymes; DPPH: 2,2-diphenyl-1-picryl-hydrazyl radical; GSH: glutathione; GSHPx: glutathione peroxidase; HCHO: formaldehyde; HDON: H-donor activity; MALDI-MS: matrix-assisted laser desorption/ionization - mass spectrometry; OPLC: overpressured layer chromatography; RLU: relative light unit; ROS: reactive oxidative substances; UA: uric acid; TG: triglyceride

The number of alcoholics and people suffering from diseases related to alcohol consumption increases in the world (HERBERT et al., 2017) despite that several studies have drawn the conclusion that biologically active antioxidant polyphenolic compounds, in particular flavonoids and stilbenes, found in red wine, have favourable physiological effects. Especially

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trans-resveratrol (resveratrol) reduces hazards of various cardiovascular diseases as well as cancer, diabetes, and so on (SZENDE et al., 2000; BRADAMANTE et al., 2004; CHIVA-BLANCH et al., 2013; MORENO-INDIAS et al., 2016; OH & SHAHIDI, 2018).

Resveratrol shows several biological effects, and the compound appears to have multiple molecular targets. For example, it inhibits the activity of various CYP enzymes and their expression through various nuclear factors, induces or inhibits Phase II enzymes, furthermore enhances fibrosis in a continuous gastric infusion rat model of ethanol exposure. Along with alcohol consumption, enhanced autophagy has also been observed (WU et al., 2012; MCGILL et al., 2015).

The *in vivo* antioxidant property of resveratrol is more likely to be attributable to its effect as a gene regulator. Resveratrol increases the expression of various antioxidant enzymes. Some of the gene-regulating effects of resveratrol are mediated by the histone/protein deacetylase sirtuin-1 or by the nuclear factor-E2-related factor-2 (McCUBREY et al., 2017; XIA et al., 2017).

At dosage of 0.02 mg kg⁻¹, resveratrol promoted antioxidant defence by preventing total and reduced GSH depletion caused by ischemia-reperfusion in rat liver, however, at high dosage (20 mg kg⁻¹), it became prooxidant with an aggravation of liver injury, marked by aminotransferase release and histological picture, and associated with a depletion of total/reduced GSH levels and a decrease of antioxidant enzyme activities (HASSAN-KHABBAR et al., 2008).

An 8-week resveratrol administration (daily 3000 mg) has not benefited overweight/obese patients with non-alcoholic fatty liver disease in an Australian clinical study. ALT and AST increased significantly, but resveratrol was well-tolerated (CHACHAY et al., 2014).

Ethanol is converted into acetaldehyde through three major metabolic pathways. In the cytosol ALD, in the microsomes inducible CYP2E1, and in the peroxisomes catalase are involved in the transformation (LIEBER, 1997). During alcohol metabolism, primary and secondary ROS can be observed. Acetaldehyde accelerates GSH depletion and ROS mediated toxicity (WORRAL et al., 1993). CYP2E1 is a major ROS generator, because ethanol reacts with H₂O₂, formed at detoxification, meanwhile forming a 1-hydroxyethyl free radical (DUPONT et al., 1998).

GSH, which can inhibit ROS reactions, is rapidly eliminated in alcoholism. This is due to the lack of sufficient methionine for the synthesis of cysteine, the main factor of GSH synthesis. Since the concentration of S-adenosylmethionine decreases in the cirrhotic liver considerably, the GSH regeneration slows down. In the absence of S-adenosylmethionine, many other vital metabolic pathways are also hindered. It is also proven that transfer of a methyl group happens via the formation of HCHO during the endogenous transmethylation processes. The abnormalities of the HCHO cycle may be potential risk factors (TYIHÁK et al., 1998).

Resveratrol is a concentration-dependent HCHO capture molecule. In oxidizing environment, ROS can be formed in the reaction of resveratrol with HCHO. Quantum chemical calculations support the possibility of interaction between resveratrol and HCHO in biological systems, in which methoxy and formyl derivatives can be formed as presented in the study of MOLNÁR and co-workers (2008). TYIHÁK and co-workers (2011) analysed the reaction products of HCHO and resveratrol as well as HCHO and heart tissue by OPLC and MALDI-MS.

In this experiment, we were curious to know whether the polyphenol (1004 mg l⁻¹), flavonoid (quercetin 13.7 mg l⁻¹, catechin 54.2 mg l⁻¹, anthocyanins 73 mg l⁻¹), and resveratrol

rich (12.03 mg l⁻¹) red wine (alcohol concentration: 10.5%) consumption can modify the redox-balance involving GSHPx antioxidant enzyme activity and transmethylation processes.

1. Materials and methods

1.1. Materials

Egri cuvee red wine was purchased from Wine-cellars König (Hungary).

HCHO, DPPH, methanol, resveratrol, luminol, H₂O₂, microperoxidase, and bovine serum albumin were obtained from Sigma (USA). Routine laboratory kits were Reanal kits. All other reagents were of analytical grade, purchased from Reanal (Budapest).

1.2. Animal experiment

Male Wistar rats of 150–200 g were involved in the experiment. The animals were divided into three groups. Each group contained 5 rats. All animals were kept on normal rat chow. The controls were given tap water to drink. Alcohol-water solution of 10.5 v/v% or wine with the same alcohol concentration was added to the rats in alcohol treated groups. The rats were treated daily 8 ml kg⁻¹ of body weight alcoholic solutions or wine for 10 days (which matches one bottle of wine/day for a 85 kg man). The animals were exsanguinated through the abdominal vein in deep pentobarbital narcosis (35 mg/bw kg i.p) on day 10, and blood was collected. Liver, sera, and plasma were prepared for the measurements. Measurements with kits were carried out and chemiluminescence as well as diene conjugates were detected promptly and consecutively, and all samples were stored at –20 °C for other measurements. Permission Number: 770/004/04.

1.3. Methods

Blood was collected into tubes containing citrate for the plasma and native tubes for the sera (Greiner), and was centrifuged immediately at 4 °C at 3000 r.p.m. for 10 min using standard method.

The liver was taken out, washed, and broken into small pieces, then washed with isotonic KCl solution. The bloodless liver pieces were homogenised with Potter-Elvehjem homogeniser at 0–4 °C. The homogenates were standardised by protein content by the method of LOWRY and co-workers (1951) with bovine serum albumin.

AST, ALT, ALP, TG, CHOL, UA, and CREAT were carried out with Hitachi Modular and Advia 12 equipments using Reanal kits. HDON activity was determined by the method of HATANO and co-workers (1988). Chemiluminescence assay was adopted from BLÁZOVICS and SÁRDI (2018). Diene conjugates were determined after 20 hours at λ_{232} nm according to AOAC method (1984). GSHPx antioxidant enzyme activity was measured by RANSEL RS505 Randox kit.

Dimedone is a HCHO capture molecule, therefore, quantification of formaldehyde is via formaldemethone. HCHO, as its dimedone adduct formaldemethone, has been detected with a fast and accurate OPLC by the methods of SÁRDI and TYIHÁK (1998). Molar ratio: resveratrol 1, HCHO 5, dimedone solution 10 in methanol for 24 hours. The reaction mixture was used for chromatographic separations. The chromatographic separations were carried out on Silica gel 60 F₂₅₄ precoated chromatoplates (Merck Co., Germany) using a chloroform-methylene

chloride mixture (35:65, V/V) for formaldemethone determination. Calibration curves were made by means of authentic substances at $\lambda=265$ nm for formaldemethone.

The liver samples were treated with dimedone solution (0.05% dimedone in methanol) for 24 hours. This suspension was centrifuged at 1500 *g* for 10 min at 4 °C. The clear supernatants were used for chromatographic separations. The chromatographic separations were the same as described before.

1.4. Statistical analysis

For comparison of the results, Student's *t*-test or Kruskal–Wallis test and ANOVA were done with Statistica 13 software. The significance level was determined at $P<0.05$. Mean $\pm$ SD and c.v. % were calculated from the data.

2. Results and discussion

In rats that consumed red wine, significantly higher ALP and UA values were measured. There were no changes in lipids, TG, and CHOL concentrations or in the CREAT of sera (Table 1). Red wine significantly increased non-enzymatic antioxidant levels in the plasma, while GSHPx levels were significantly reduced in both alcohol treated groups compared to the controls. Alcohol reduced and red wine increased free radical levels (RLU) in plasma compared to the controls.

Table 1. Changes of routine laboratory parameters during alcohol and red wine treatments

Groups (mean $\pm$ SD)	AST (U l ⁻¹)	ALT (U l ⁻¹)	ALP (U l ⁻¹)	TG (mmol l ⁻¹)	CHOL (mmol l ⁻¹)	UA (μ mol l ⁻¹)	CREAT (μ mol l ⁻¹)
Control (N=5)	63.16 $\pm$ 5.30	32.83 ^a $\pm$ 3.76	545 ^a $\pm$ 42	0.84 $\pm$ 0.40	1.65 $\pm$ 0.18	3.87 ^a $\pm$ 2.16	30.16 $\pm$ 1.47
Alcohol treated (N=5)	55.20 $\pm$ 5.44	26.80 ^b $\pm$ 3.27	490 ^b $\pm$ 49	0.63 $\pm$ 0.20	1.46 $\pm$ 0.15	8.20 ^b $\pm$ 2.58	29.20 $\pm$ 1.78
Red wine treated (N=5)	56.20 $\pm$ 7.98	30.60 $\pm$ 3.50	560 ^c $\pm$ 70	0.70 $\pm$ 0.17	1.50 $\pm$ 0.33	12.00 ^b $\pm$ 7.11	27.60 $\pm$ 1.81

Significance $P<0.05$: a vs b, b vs c

The transmethylation abilities of livers were different among the groups. In both treated groups, liver HCHO concentrations (in formaldemethone form) were significantly lowered compared to the control group. The strong ROS reactions (RLU) and the moderated activity of enzymatic defence led to formation of diene conjugates of lipids (Table 2), even though concentration of antioxidant molecules (HDON) in treated groups was high.

Figure 1 shows the changes in formaldemethone concentrations without and with resveratrol in an in vitro experiment. When the resveratrol-formaldehyde reaction product with dimedone in OPLC system was detected, the molar ratio was: resveratrol 1, HCHO 5, dimedone 10.

Table 2. Changes of antioxidant profile of plasma and liver homogenates during alcohol and red wine treatments

Groups (mean±SD)	Plasma HDON (mmol l ⁻¹)	Plasma GSHPx (U l ⁻¹)	Plasma (RLU)	Diene- con- jugates (Abs. ₂₃₃ /ml)	Liver HDON (mmol l ⁻¹)	Liver (RLU)	HCHO (µg g ⁻¹)
Control (N=5)	1.067 ^a ±0.21	3955 ^a ±500	1.274×10 ^{6a} c.v.<0.5%	0.191 ^a ±0.057	46.84 ±8.91	1.983×10 ^{6a} c.v.<0.5%	7.29 ^a ±1.29
Alcohol treated (N=5)	1.004 ±0.23	4263 ^b ±476	7.26×10 ^{5b} c.v.<0.5%	0.239 ±0.053	48.55 ±7.80	2.388×10 ^{6b} c.v.<0.5%	5.72 ^b ±1.04
Red wine treated (N=5)	1.215 ^{b,c} ±0.11	3585 ^c ±458	1.811×10 ^{6b,c} c.v.<0.5%	0.308 ^b ±0.053	52.21 ±5.07	3.082×10 ^{6b,c} c.v.<0.5%	5.22 ^b ±0.41

Significance P<0.05: a vs b, b vs c

Fig. 1. Detection of resveratrol-formaldehyde reaction product with dimedone system (Molar ration: resveratrol 1: HCHO 5: dimedon 10)

The HCHO concentration in formaldemethone without resveratrol was 28.2 µg g⁻¹ ± c.v. 5% and with resveratrol was 21.6 µg g⁻¹ ± c.v. 5%.

Since it is known that the concentration of S-adenosylmethionine, the main methylation agent in the body, is reduced in alcoholism, the question is, whether the bioactive components (flavonoids and resveratrol) of red wine can contribute to a moderation in alcoholic liver diseases? According to the literature, the answer is linked to resveratrol (TYIHÁK et al., 1998; 2011). Egri cuvée contains a high concentration of resveratrol, almost 1.5–3 times higher than the commercially available red wines (VRHOVSEK et al., 1995; SOUTO et al., 2001).

It was verified in an in vitro dimedone reaction system that resveratrol can react with bound HCHO, also confirmed by MOLNÁR and co-workers (2008).

The equivalent alcohol content of red wine and alcoholic drink did not damage the liver functions significantly in this short term, 10-day-long experiment, which is clearly apparent from the routine laboratory results, but the redox parameters indicate that red wine surprisingly favours lipid peroxidation processes.

Among plasma redox parameters, H-donor activity indicated that red wine polyphenols could be absorbed in rats. Non-specific antioxidant protection diminished the activity of GSHPx significantly. The plasma inducible ROS level increased due to the consumption of red wine. Interestingly, the alcohol diminished its level, which could be due to the fact that the antioxidant compounds of the rat chow could be absorbed better by the alcohol, while the excess antioxidant compounds of wine could already modify the redox equilibrium negatively.

The effectiveness of red wine-derived antioxidants could be detected by the moderately higher H-donor activity in the liver. At the same time, lipid peroxidation processes were intensified. Higher diene conjugation concentration and significant inducible chemiluminescence intensity indicated ROS formation in the livers of both treated groups. Meanwhile, a significant decrease was observed in methylation capacity in both treated groups.

To prove that resveratrol was the cause of moderated transmethylation ability during red wine treatment, an in vitro experiment was conducted.

The primary cause was found to be the decreased concentration of methyl groups accompanying the metabolism of alcohol, and the reaction of resveratrol with HCHO was the next. The HCHO molecule is in connection with the redox homeostasis, playing an important role with ROS in the biological system. According to our in vitro and in vivo data, it is assumed that resveratrol HCHO reaction might affect the methylation level, causing changes in enzyme activity of glutathione peroxidase via lower glutathione concentration through the formaldehyde cycle (TYIHÁK et al., 1998).

3. Conclusions

Damage caused by the alcohol content of the wines cannot be avoided by other bioactive compounds of the wines in case of alcoholism. Our results confirm that resveratrol is a Janus faced bioactive molecule.

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