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Defatted milled grape seed protects adriamycin-treated hepatocytes against oxidative damage

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Abstract Defatted milled grape seed (DMGS) is a wine by-product obtained from the oil extraction of the grape seed that contains different types of phenolic compounds. The present study was designed to evaluate the possible protective effect of DMGS on toxicity induced by adriamycin (ADR) in isolated rat hepatocytes. The study was carried out by examining the results of lactate dehydrogenase (LDH) release to estimate cytotoxicity; the thiobarbituric acid reactant substances (TBARS) and carbonyl group levels were measured as biomarkers of oxidative stress and ATP and GSH levels as estimation of intracellular effect. The results showed that DMGS extract protects the cellular membrane from oxidative damage and consequently prevents protein and lipid oxidation. The levels of ATP and GSH changes for the ADR toxicity were restored to control value in the presence of DMGS extract. The experimental results suggest that this wine by-product may be used to decrease oxidative stress.

Key words DMGS extract – phenolics – adriamycin – oxidative damage

Abbreviations DMGS: defatted milled grape seed · LDH: lactate dehydrogenase · TBARS: thiobarbituric acid reactant substances · GSH: glutathione · ADR: adriamycin

Introduction

Dietary intake of natural antioxidants can neutralize harmful free radicals and their noxious tissue- and organ-damaging effects and may be an important defense mechanism of our body against oxidative stress [1]. Antioxidants have a wide range of biological effects, including effects on inhibition of tumor initiation, promotion and progression, cell proliferation, and differentiation as well as DNA repair, cell membrane stability and may inhibit low-density lipoprotein (LDL) oxidation and carcinogenesis [2].

Most wine by-products are rich sources of dietary chemical constituents that may have potential for the prevention and treatment of human malignancies [3–7]. These by-products have generated remarkable interest based on positive reports of their antioxidant properties and their ability to serve as free radical scavengers [8]. This is the case with grape seeds or their products such as defatted milled grape seed

(DMGS), a by-product obtained from the grape seed. Several studies have indicated that extracts obtained from grape seed inhibit the oxidation of LDL, inhibit enzyme systems that are responsible for the production of free radicals, are antimutagenic, and anticarcinogenic [6, 9, 10]. For this, the grape seed extracts are widely consumed as a dietary supplement and could be useful in synergizing the efficacy of cancer chemotherapeutic agents in cancer treatment.

Various naturally occurring antioxidants are known to be present in grape seeds, notably phenolic compounds, whose content may range from 5 to 8% by weight [6, 11]. It is well established that grape seeds contain principally flavonoids such as [+]-catechins, [−]-epicatechin and [-]epicatechin-3-*O*-gallate, and dimeric, trimeric and tetrameric procyanidins [12]. These molecules possess a structure that confers on them an antioxidant property, which has been demonstrated to exert a novel spectrum of biological, pharmacological, therapeutic, and chemoprotective effects against oxygen free radicals and oxidative stress [13]. There is a great diversity between commercial seed extracts varying in total phenol concentration, monomers, oligomers and polymers [6, 14]. Such diversity is based upon seed selection and on the processing method employed. Nowadays there is evidence that polyphenols are absorbed by the body and increase total antioxidative capacity of blood plasma or decrease the harmful effects of oxidative stress [15]. This central finding suggests that regular consumption of grape phenols from red wine or wine by-products may result in the long-term effect of reduced incidence of different diseases [16].

The anthracycline antibiotic, adriamycin (ADR), has been in use as a potent antitumoral drug for the treatment of a variety of human cancers including lymphomas, leukemia, and solid tumors [16, 17]. Its action is mediated by the formation of reactive oxygen species (ROS) [16, 18, 19] that stimulate lipid peroxidation, inhibit mitochondrial function and cause cell damage, this representing one of the side effects of the drug [20]. Previous studies have reported the induction of cell toxicity using concentrations of 25–75 μM [21]. Treatment with chemotherapeutic agents in combination with natural dietary supplements may counteract some of the toxic effects of the drugs [10, 16, 22]. For these reasons, this system represents a good model to evaluate the effect of DMGS extract on drug toxicity mediated by ROS [23].

On this basis we wanted to verify whether DMGS, a by-product obtained from grape seed, has a preventive activity against induced oxidative stress in isolated rat hepatocytes. The protective effect was evaluated by measuring LDH leakage, the inhibition of lipid and protein oxidation and the changes that could take place on the ATP and GSH levels by the DMGS extract.

Materials and methods

Chemicals and reagents

DMGS was obtained from Aceites Borges Pont S.A., Lérida, Spain. The ADR used was obtained from Pharmacia and Upjohn, Milan (Italy). All reagents were of analytical grade and obtained from Merck (Darmstadt, Germany) and Scharlau SL (Barcelona, Spain). Enzymes and coenzymes together with other substrates used in this study were the highest grade available from Boehringer Mannheim and Sigma Chemical Co (St. Louis, MO, USA). Substrate solutions were prepared using Milli-Q (Millipore) double distilled water.

Defatted milled grape seeds

DMGS obtained as a waste from the grape seed extraction industry, were used as raw material. This raw material was subjected to extraction with water in order to obtain an extract interesting from the nutritional point of view due to their feasibility for intestinal absorption. Phenolic compounds were analyzed following the method described by Remy [24]. The equipment used for the HPLC analysis consisted of a DAD and MS detectors (Hewlett Packard 1100 Series). Separation was performed on a reversed-phase (Lichorspher) 100-RP18, (250×4 mm, 5 μm) column at 30°C. A gradient consisting of solvent A (water/formic acid, 95/5) and solvent B (acetonitrile/water/formic acid, 80/5/15) was used. Detection was performed at 280 nm (proanthocyanidins), 320 nm (phenolic acids and aldehydes) and 360 nm (flavonols). Full spectra (detection wavelengths from 220 to 400 nm, step 0.4 nm) were recorded for all peaks. AP-ESI was used for MS detection. Nitrogen was used as nebulizer and drying

Table 1 Concentrations of the different components of the aqueous extract of polyphenols from DMGS

Compound	mg/kg of DMGS
Syringic acid	1.47
Monogalloyl glucose	8.62
Gallic acid	31.72
Flavonols	0.66
Catechin	42.95
Epicatechin	30.36
Epicatechin gallate	2.07

gas with a flow rate of 10 ml/min. Identification and quantification of polyphenols was carried out as previously described by Perez-Magariño et al. [25]. The concentration of each identified component expressed as mg of component per g of DMGS is listed in Table 1.

Antioxidant activity

The antioxidant activity was determined using the ABTS assay according to Miller et al. [26]. ABTS radical cation ($ABTS^{+}$) was produced by adding potassium persulphate to the solution and keeping it in the dark at room temperature for more than 12 h. The decreased absorbance to 734 nm represents the antioxidant activity of DMGS extract. The antioxidant activity was expressed as TEAC (Trolox equivalent antioxidant capacity), which is expressed in mg/g DMGS extract using the calibration curve plotted with different amounts of Trolox.

Animals, hepatocytes and treatment

Wistar male rats were housed in individual cages after weaning. They were maintained under controlled conditions of light cycle (12 h/12 h light–dark) and temperature (22°C). They were fed a standard IPM-20 diet (Panlab, Barcelona, Spain) and water was given ad libitum. In order to carry out the study, we used isolated hepatic cells from male Wistar rats, aged between 3 and 4 months, and weighing between 250 and 300 g. Hepatocytes were isolated by a simplified version of the perfusion method of Berry and Friend [27]. Cell membrane integrity was tested with trypan blue and approx. 95% of the isolated hepatocytes were found to exclude the dye. A volume of 2 ml of hepatocytes suspension containing approximately 2×10^{6} cells in Krebs–Henseleit saline equilibrated with O_2/CO_2; 95/5; v/v were incubated in a shaking water bath at 37°C for 1 h, in 25 ml conical flasks sealed with rubber stoppers. Incubation mixtures containing cells and different concentrations of ADR (25 μM) in absence/presence of DMGS extract (2.5, 10 and 25 μg/ml) were made up to 4 ml final volume with Krebs–Henseleit buffer solution (pH 7.4). Once the incubation time had elapsed, the cells were processed to carry out the relevant tests of oxidative stress.

Measurement of LDH leakage

Cell viability assessment was made by quantification of the lactate dehydrogenase released into the extracellular medium [28]. These were centrifuged at slow speed to spin down the cells and appropriate dilutions of the cell-free supernatants were used for determination of activity. Oxidation of NADH was followed spectrophotometrically at 340 nm at 25°C. Hepatocytes treated with 10 mg/ml of digitonine were used as control.

Determination of thiobarbituric acid reactant substances (TBARS)

Damage caused to lipids and the extent of lipid peroxidation was determined by measuring TBARS according to the spectrophotometer method of Stacey and Priestly [29] with 1,1,3-tetramethoxypropane used as standard. For the assay, a 0.4 ml of hepatocytes was mixed with 1 ml of 20% (p/v) trichloracetic acid (TCA) and 2 ml of 0.67% (p/v) thiobarbituric acid (TBA) and heated at 100°C for 15 min. The mixture was then cooled and its absorbance at 535 and 520 nm was measured.

Protein carbonyl measurement

Protein oxidation was measured by an estimation of carbonyl groups formed during the incubation using the method of Levine et al. [30] and modified by Tian et al. [31]. For assays 1 ml of cellular suspension were centrifuged for 10 min at 3000 g and treated with 0.4 ml of 10 μM 2,4-dinitrophenylhydrazine (DNPH). The samples were incubated for 1 h at room temperature. The samples were precipitated with 20% TCA and washed twice with ethanol/ethyl acetate (1:1). The pellets were then dissolved in 1 ml of 6 M guanidine HCl and incubated for 1 h at 37°C. Protein concentration was calculated from spectrophotometric absorption at 373 nm and calculated using a molar absorption coefficient of 22.000 M^{-1} cm^{-1}.

Adenosine triphosphate (ATP) determination

At the end of the incubation period, metabolic reactions were stopped by the addition of 0.1 ml of 20% (v/v) perchloric acid and placed on a tray containing ice. Acidified incubated samples were centrifuged to precipitate proteins and the supernatants were neutralized with potassium hydroxide for the enzymatic determination of ATP. The ATP levels were determined on isolated rat hepatocytes by measuring spectrophotometrically at 340 nm NADPH oxidation in the presence of glucose by Lamprecht and Trautschold method [32].

Measurement of GSH levels

The levels of reduced glutathione was determined in samples neutralized with sodium bicarbonate. The assay of reduced levels of GSH was assayed by the method of the glutathione-S-transferase according to Brigelius et al. [33].

Protein measurement

Total protein concentration was evaluated using bovine albumin as a standard by Lowry method [34] and modified by Markwell et al. [35].

Statistical analysis

Results are expressed as mean values ± SD. Comparison of the means of four measurements using a significance level of $P<0.05$ was performed by one-way analysis of variance (ANOVA) using the Statgraphics Computer System.

Results

The study of the oxidative stress biomarkers is needed to investigate whether the DMGS extract is capable of protecting the oxidative damage in isolated hepatocytes induced by ADR. The phenolic composition of the DMGS extract is in Table 1. It contains phenolic acids, flavonols, flavanol monomers and dimers. The phenolic compounds more abundant in the extract are the flavonoids catechin and epicatechin with values of 42.95 and 30.36 mg/kg of DMGS followed by gallic acid. The minority phenolic compounds were the flavonols with levels of 0.66 mg/kg of DMGS. The antioxidant activity of the extract was also measured and the TEAC value obtained was 942 mg/g DMGS extract.

The study was carried out in isolated rat hepatocytes incubated with ADR (25 μM) in absence and presence of different concentrations of DMGS extract (2.5, 10, 25 μg/ml).

The results of the effect of DMGS alone on the hepatocytes are present in the Table 2. The parameters studied, LDH, GSH and carbonyl group did not show any significant change when the hepatocytes were incubated only in the presence of DMGS extract. However, the levels of TBARS decreased significantly with respect to the control hepatocytes (Table 2). Thus, DGMS were found to be non-toxic to hepatocytes over a range of 0–25 μg/ml.

As illustrated in Fig. 1 we studied cell viability through measurement of the LDH leakage. The hepatocytes treatment with 25 μM ADR was highly toxic with a significant increase ($P<0.005$) in LDH leakage as compared to control hepatocytes. When the isolated rat hepatocytes were co-incubated with ADR and DMGS extract for 60 min, the levels of LDH decreased significantly ($P<0.05$) at all concentrations of DMGS extract studied.

Adriamycin is known to generate oxidative stress in hepatocytes, which can be measured from the

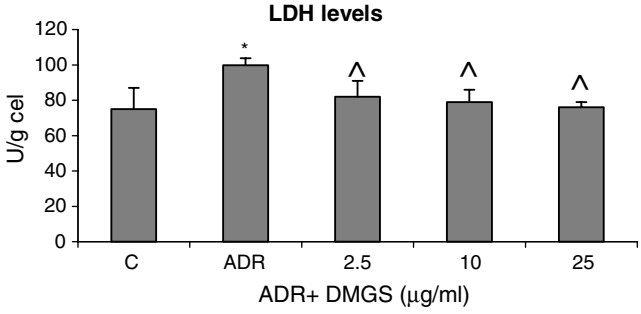

Fig. 1 Effect of DMGS extract on LDH leakage induced by ADR in isolated rat hepatocytes. Hepatocytes were incubated with ADR 25 μM in the absence or presence of 2.5, 10 and 25 μg/ml DMGS extract. Data are reported as the mean ± SD of six experiments. Pairwise comparisons were conducted employing Student's *t*-test. Differences were considered significant at *$P<0.05$ comparing with the control. ^$P<0.05$ comparing DMGS + ADR extract with ADR alone

Table 2 Levels of LDH leakage, TBARS, carbonyl protein groups, ATP and GSH levels in hepatocytes incubated with DGMS alone

	TBARS	LDH	GSH	Carbonyl group
Control	280 ± 48	84.7 ± 17.3	1.06 ± 0.39	1.40 ± 0.38
DMGS extract				
2.5 μg/ml	293 ± 57	80.2 ± 11.9	1.06 ± 0.43	0.96 ± 0.14
10 μg/ml	180 ± 36*	75.8 ± 10.1	0.92 ± 0.24	1.36 ± 0.32
25 μg/ml	91 ± 17*	81.5 ± 7.9	0.99 ± 0.47	1.25 ± 0.02

Hepatocytes were incubated in the absence (control) and in presence of DMGS extract for 1 h to 37°C. Results are expressed as mean ± SD of 8 separate experiments

Differences were considered significant at *$P<0.05$ compared with the control

Table 3 Effect of DMGS extract on the generation of lipid peroxides in isolated hepatocytes measured as TBARS

	TBARS nmol/g cell
Control	287±48
ADR	445±90*
ADR+2.5 (μg/ml) DMGS	268±55[a]
ADR+10 (μg/ml) DMGS	170±17[b]
ADR+25 (μg/ml) DMGS	92±5[c]

Hepatocytes were incubated with ADR 25 μM in the absence or presence of 2.5,10 and 25 μg/ml DMGS extract. Data are reported as the mean ± SD of six experiments. Pairwise comparisons were conducted employing Student's *t*-test Differences were considered significant at *$P<0.05$ compared with the control. Values with different alphabetical letters are significantly different from each other and from + ADR ($P<0.05$)

Table 4 Effect of DMGS on GSH and ATP levels of isolated rat hepatocytes treated with ADR

	GSH (μmol/g of cells)	ATP (nmol/g cell)
Control	1.00±0.35	2.07±0.37
ADR 25 μM	0.77±0.08*	1.25±0.18*
" + DMGS 2.5 μg/ml	1.22±0.36**	1.61±0.12**
" + DMGS 10 μg/ml	0.99±0.19**	1.51±0.12**
" + DMGS 25 μg/ml	1.13±0.44**	1.55±0.10**

Isolated hepatocytes were incubated with 25 μM of ADR at 37°C for 60 min in the absence or presence of different concentrations of DMGS extract. Results are expressed as mean ± SD of 6 separate experiments
Statistical significance was evaluated by Student's *t*-test. *$P<0.05$, compared with the control and **$P<0.05$ compared with the ADR group

extent of lipid peroxidation in the tissue. Lipid peroxidation was measured in terms of TBARS (Table 3). In ADR-treated hepatocytes the amount of lipid peroxidation increased significantly compared with its control. The levels of lipid peroxidation were significantly lower and reached nearly control levels when hepatocytes were co-treated with ADR and DMGS. At the concentration of 25 μg/ml the inhibition value with respect to the hepatocytes incubated with ADR alone was 83%. The DMGS protective effect was dose-dependent ($P<0.05$).

Protein oxidation was quantified by measuring protein carbonyl levels (Fig. 2). Like other oxidative stress biomarkers, the levels of the hepatocytes carbonyl groups increased significantly in presence of ADR. The addition of DMGS extract at the same time as the drug mitigated the cytotoxicity response, and a significant decrease ($P<0.005$) of the carbonyl groups at the concentrations of 10 and 25 μg/ml was observed.

The intracellular effects of the DMGS extract were studied by measuring the levels of antioxidant GSH and the metabolite ATP (Table 4). The treatment of hepatocytes with ADR significantly ($P<0.05$) reduces the GSH and ATP levels by about 25 and 39% respectively, compared with their control. The hepatocytes co-incubated with ADR and DMGS significantly preserved the hepatocellular GSH and ATP levels.

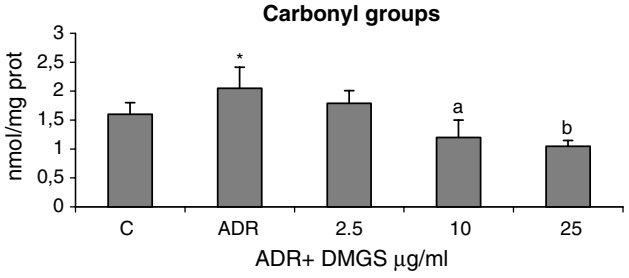

Fig. 2 Effect of DMGS extract on the protein carbonyl content in rat hepatocytes treated with ADR. Hepatocytes were incubated with ADR 25 μM in the absence or presence of 2.5, 10 and 25 μg/ml DMGS extract. Data are reported as the mean ± SD of six animals. Pairwise comparisons were conducted employing Student's *t*-test. Differences were considered significant at *$P<0.05$ compared with the control. Values with different alphabetical letters are significantly different ($P<0.05$) from each other and from ADR alone

Discussion

There is a growing interest in the utilization of wine by-products for their dietary and pharmacological properties. Grape seed extract containing flavonoids is being used as a nutritional supplement [6, 8, 36, 37]. As well as their antioxidant benefits, seed extracts have been shown to exert chemo-preventive and anticancer effects [10, 38].

DMGS is a wine by-product obtained from grape seed and it contains a variety of biologically active species used for protection against oxidative stress induced by free radicals and active oxygen species [39]. According to its chemical composition, DMGS might have a digestive behavior similar to that of other grape by-products [40]. In relation to their polyphenol compounds, as shown by our results, the DMGS contain mainly flavonoids, all involved in ameliorating oxidative stress in vitro and in vivo [41–43]. To evaluate their potential as antioxidants we have studied the effect of the extract on oxidative damage and on antioxidant defense of hepatocytes exposed to oxidative stress.

In the present study, we generated oxidative stress in vitro in isolated rat hepatocytes using ADR. It is reported that ADR is a useful compound for the study of oxidative stress because its toxicity is mediated by free radicals [18]. Furthermore, the antioxidant properties of flavonoids have been suggested to play a role in the protection against ADR-induce toxicity [44]. The effect of ADR on perfused rat liver or on isolated rat hepatocytes was documented in previous

studies [20, 23, 45]. In our study, the exposure of rat hepatocytes to ADR, resulted in an increased oxidative damage in lipids and proteins and decreased ATP levels.

Our results showed that co-incubation with DMGS extract reduces the oxidant action of ADR on isolated rat hepatocytes. In fact, we have calculated ADR toxicity by measuring the release of LDH into the medium by hepatocytes. LDH is a cytosolic enzyme mainly present in periportal hepatocytes and released when the cells are lyzed by hepatotoxin. The amount of enzyme released is proportional to the extent of damage caused to the cell. ADR treated hepatocytes released 25% more enzyme into the medium than untreated hepatocytes. DMGS added to hepatocytes with ADR lowered the enzyme release.

Biomarkers of oxidative lipid peroxidation and protein oxidation can be used to establish the role of DMGS extract on protection against oxidative stress [46–48]. An increase in the levels of TBARS and protein carbonyl groups in the hepatocytes indicates an enhancement in peroxidation leading to a loss of membrane integrity and oxidative modifications of amino acid side chains, etc. [48]. DMGS treatment considerably lowered the formation of TBARS and carbonyl groups in hepatocytes and this effect may be due to the phenolic composition of DMGS and their antioxidant activity.

The energy status of the cells was also evaluated by measuring ATP levels. The ATP levels in the cell are linked with the increase or decrease of cell proliferation, changing immune response, or with the induction of apoptotic cell death [49, 50]. We observed a significant preservation of the hepatocellular ATP level when the hepatocytes were incubated with DMGS in presence of ADR. These results suggest that one of the important effects of DMGS is attenuating oxidative stress at the mitochondrial membrane by scavenging ROS, resulting in the preservation of the intracellular ATP concentration and a possible role in the ATP-dependent signaling pathways. Various studies have shown the involvement of grape seed extracts in cell apoptotic process [10, 51].

Flavonoids may also exert antioxidant abilities through protection or enhancement of endogenous antioxidants. GSH is also known to be closely involved in ADR toxicity [52]. The free radicals activated by ADR are scavenged by GSH, and the conjugation of ADR and GSH advances the detoxification of the anthracycline drug. In agreement with previous reports we found that GSH diminished in ADR-treated hepatocytes [19, 53] while the combination with DMGS restored the level of GSH. The concentrations of cellular thiols, such as GSH, play an important role in the maintenance of cellular redox state. Numerous flavonoids have been shown to alleviate oxidative stress by increasing the endogenous antioxidant status protecting cells against free-radical damage by increasing resistance to oxidative stress [54, 55]. In our study, GSH depletion was almost inhibited when cells were co-incubated with DMGS. These data lead to the conclusion that oxidative stress is one of the mechanisms of ADR cytotoxicity and that DMRS have protective effects against such oxidative damage.

In our study of hepatocytes, we observed that the release of LDH is significantly reduced and that lipid peroxidation, ATP and GSH levels are similar to the control group. This indicates that DMGS, by its antioxidant activity, reduces the oxidative stress induced by ADR and protects the hepatocytes in vitro.

In summary, our results demonstrate a good effect of the DMGS–ADR combination in isolated rat hepatocytes. In this regard, recent reports show that naturally occurring dietary supplements with known anti-cancer activity could be used in combination with chemotherapy to reduce the toxicity of the chemotherapeutic agents [10, 22]. Therefore, based on the data shown in the present study, DMGS could be useful in synergyzing the cancer chemotherapeutic efficacy of ADR treatment. Further studies are needed to ensure also their efficacy *in vivo*.

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