Hepatoprotective Effect of Red Grape Seed Extracts Against Ethanol-Induced Cytotoxicity

Hazem M.M. Hassan

Department of Biochemistry, Faculty of Agriculture, Cairo University, Giza, Egypt

Abstract: The hepatoprotective activity of red grape seed extracts (GSEs) was investigated against ethanol-induced cytotoxicity. In this study, liver slice culture model was used to demonstrate hepatoprotective activity of two GSEs (water and ethanol) in vitro. The hepatoprotective activity was evaluated by measuring the levels of lipid peroxidation, protein carbonyls, lactate dehydrogenase (LDH) leakage and antioxidant enzymes (catalase and peroxidase) in liver slice culture. The results revealed that the pre-treatment or treatment along of liver slices with water and ethanol grape seed extracts, significantly inhibited the ethanol-induced oxidative stress in the liver by suppressing lipid peroxidation and protein carbonylation. Parallel to these changes, the GSEs prevented the ethanol induced increases in the LDH leakage from liver cells and maintained the levels of antioxidant enzymes. The ethanol grape seed extract (EGSE) was more effective than water grape seed extract (WGSE) against hepatotoxicity of alcohol. Hepatoprotective activity of the grape seed extracts could be attributed to the antioxidant effect of the constituents and enhanced antioxidant defenses.

Key words: Hepatoprotective • Red grape seed • Ethanol • Cytotoxicity • Liver slice culture

INTRODUCTION

Liver diseases are posing as a major health problem around the world. Hepatitis viral infection, toxic industrial chemicals, alcohol, aflatoxins, water pollutants are the major risk factors of liver diseases [1, 2]. Chronic liver damage is a widespread pathology characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma. The ability of ethanol to increase the production of reactive oxygen species (ROS) and enhance peroxidation of lipids, protein and DNA has been demonstrated in a variety of systems, cells and species including humans [3]. The mechanism of ethanol-induced oxidative stress and cell injury appear to involve redox state changes (decrease in the NAD+/NADH redox ratio) produced as a result of ethanol oxidation by alcohol and acetaldehyde dehydrogenases, production of the reactive metabolite acetaldehyde, one electron oxidation of ethanol to the 1-hydroxy ethyl radical [4-6]. Many of these pathways are not exclusive of one another and it is likely that several systems contribute to the ability of ethanol to induce a state of oxidative stress. As the oxidative stress plays a central role in liver pathologies and progression, the use of antioxidants has been proposed as therapeutic agents, as well as drug coadjuvants, to counteract liver damage [7]. A number of studies have shown that antioxidants including the plant extracts protect against ethanol hepatotoxicity by inhibiting lipid peroxidation and enhancing antioxidant enzyme activity [8, 9]. The liver slice culture system is used to quantitate the cytotoxic effects of ethanol and their reversal by plant extracts in terms of the release of lactate dehydrogenase (LDH) by the cells into the medium and the cellular levels of the antioxidant enzymes (AOEs), superoxide dismutase, catalase and peroxidase. Liver slice culture retains tissue specific micro-architecture with maintained cell diversity, identity and functional heterogeny compared to hepatocytes and therefore resembles more closely to the in vivo situation [10].

Grape is one of the most commonly consumed fruits in the world. It has various biological functions, due to its rich polyphenol ingredients, most of which are contained in its seeds (60-70%) and skin (30%). However, large quantities of grape seed wastes are produced annually by the food processing industry-wine, juice etc [11]. Grape ingredients also have healthful properties. Phenolic bioflavanoids from grape seeds and skins are powerful...
antioxidants. In particular, grape seeds contain two-thirds of the phenols of the grape, 5-8% by weight. Catechin, epicatechin and epicatechin gallate and gallic acid are the monomeric compounds identified in grape seeds. These, along with dimers, trimers and oligomers of catechin and epicatechin are referred to as procyanidins [12]. When used with either vitamins C or E, the extract possesses double the antioxidant capacity [13]. Polyphenols in grape seeds have also been reported to have a variety of biological activities, including antioxidant, antithrombotic, antitumor, antibacterial, antiviral, anti-inflammatory, antiallergic, protection against X-ray and ultraviolet rays, chemoprevention, vasodilatory actions and cardioprotective effects [14-21]. Grape seed extract (GSE) has a protective effect on oxidant-induced production and deposition of extracellular matrix components, which results in hepatic fibrosis [22]. It also improves hepatic ischemia-reperfusion injury and reduces the size of the infarct in cardiac ischemia in the rat [23, 24]. Several studies have indicated that extracts obtained from grape seed inhibit enzyme systems that are responsible for the production of free radicals and that they are antimutagenic and anticarcinogenic [25]. Jamshidzadeh et al. [26] studied the ex vivo hepatoprotective effect of grape seed extract against acetaminophen and ethanol toxicity. The results showed that grape seed extract does have protective effect against acetaminophen and ethanol. Cetin et al. [27] evaluated the possible protective effect of grape seed extract on liver toxicity induced by radiation therapy (RTx, X-rays and γ-rays) in the rat liver. The results showed the levels of antioxidant parameters on RTx-induced liver toxicity were restored to control values with grape seed extract therapy. Grape seed extract may be promising as a therapeutic option in RTx-induced oxidative stress in the rat liver. Shin et al. [28] investigated the protective effect of grape seed procyanidins on hepatic injury induced by dimethylnitrosamine (DMN) in rats. The results demonstrated that procyanidins exhibited in vivo hepatoprotective and anti-fibrogenic effects against DMN-induced liver injury. It suggested that grape seed procyanidins may be useful in preventing the development of hepatic fibrosis. Pan et al. [29] studied the effect of grape seed extract (GSE), an antioxidant dietary supplement, on arsenic-induced liver injury. The results from this study revealed that GSE co-treatment significantly attenuated arsenic-induced low antioxidant defense, oxidative damage, proinflammatory cytokines and fibrogenic genes. Mirzaei et al. [30] evaluated the protective effect of remedy of grape seed with Jaft extract with equal ratio on liver toxicity caused by carbon tetrachloride in rats. The results showed that grape seed with Jaft extracts with equal ratio may have hepatoprotective effect, because of antioxidative component.

The present study was aimed to investigate the hepatoprotective effect of grape seed extracts against ethanol hepatotoxicity in vitro model.

MATERIALS AND METHODS

Materials
Plant Material: Grape (Vitis vinifera L., variety Roumy Ahmer), as large clusters with red berries, was purchased from a local market at Giza, Egypt.

Animals: Male Swiss albino mice, weighing about 28-30 g were purchased from Research Institute of Ophthalmology, Giza, Egypt.

Chemicals: Folin–Ciocalteu reagent, guaiacol and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co., USA. Lactate dehydrogenase (LDH) kit was obtained from Centronic GmbH, Germany. All other chemicals were of analytical reagent grade.

Methods
Preparation of Grape Seed Extracts (GSEs): Two different extracts were prepared from red grape seeds using the procedure described by Badavi et al. [31] with some modifications as follows: Grape seeds were separated from the grapes manually, air dried (in shade, 25-30°C) for one week and milled to fine powder. To prepare water grape seed extract (WGSE), 0.2 g of grape seed powder was macerated in 20 ml of distilled water (DW) for 24 h at 5°C and was stirred three times. The mixture was filtered with cheese cloth and the resulting filtrate was used as WGSE. The same method was used for the extraction of grape seed with ethanol 80%. After extraction with ethanol, the solvent was removed from the obtained extract by evaporation. The residue was redissolved in the same volume of DW. The obtained solution was used as ethanol grape seed extract (EGSE).

Assay Against Ethanol Cytotoxicity of Liver Cells in vitro
Liver Slice Culture: Liver slice culture was maintained following the protocol developed by Naik et al. [10] as follows: To prepare liver slice culture, one Swiss albino
male mouse was used. Mouse was dissected open after decapitation and liver lobes were removed and transferred to pre-warmed (37°C) Krebs-Ringer-HEPES (KRH) buffer solution (HEPES 2.5 mM pH 7.4, NaCl 118 mM, KCl 2.85 mM, CaCl₂ 2.5 mM, KH₂PO₄ 1.5 mM, MgSO₄ 1.18 mM and glucose 4.0 mM). Liver was then cut into thin slices using sharp scalpel blades. After weighting the slices, the slice weighing between 7 and 10 mg was used in this experiment. Each experimental system contained 15-17 slices weighing together 120–150 mg. Slices of each system were washed with 10.0 ml KRH buffer solution every 10 min over a period of 1 h. Each system was then pre-incubated for 60 min in small plugged beaker containing 2.0 ml of KRH buffer solution on a shaker water bath at 37°C. In the experiments of grape seed extracts, each medium contained 2500 ppm grape seed extract during the second half of the pre-incubation period. At the end of pre-incubation, the medium was replaced by fresh 2.0 ml of KRH buffer solution and incubated for 2 h at 37°C with ethanol solution (1.37 M) or both ethanol and grape seed extract. Four different experimental conditions were used for treatment with grape seed extracts as follows: WGSE + Eth-1: Water grape seed extract was present for 0.5 h only during pre-incubation; WGSE + Eth-2: Water grape seed extract was present for 0.5 h during pre-incubation and also for next 2 h with ethanol; EGSE + Eth-1: Ethanol grape seed extract was present for 0.5 h only during pre-incubation; EGSE + Eth-2: Ethanol grape seed extract was present for 0.5 h during pre-incubation and also for next 2 h with ethanol. At the end of incubation, each group of slices was homogenized in appropriate volume of chilled potassium phosphate buffer solution (100 mM, pH 7.8) in an ice bath to give a tissue concentration of 100 mg/ml. The culture medium was collected and used for estimation of lactate dehydrogenase (LDH), which was employed as a cytotoxicity marker. The homogenates were centrifuged at 10,000 rpm for 10 min at 4°C and the supernatants assayed for LDH, catalase, peroxidase activities and protein content. Lipid peroxidation and protein carbonyl content were also assayed in obtained homogenates.

Estimation of Proteins: Proteins were estimated according to the method of Lowry-Folin as described by Dawson et al. [32].

Assay of Lipid Peroxidation: Thiobarbituric acid-reactive species (TBARS) formation was used to evaluate lipid peroxidation in the method of Rudnicki et al. [33] as follows: In centrifuge tube, 600 µl of trichloroacetic acid solution (10%, w/v) were added to 300 µl of the liver slices homogenate and centrifuged at 10,000 rpm for 10 min. 400 µl of supernatant were taken in clean test tube then mixed well with 400 µl of thiobarbituric acid solution (0.67%, w/v). After 30 min in a boiling water bath, the reaction mixture was cooled to room temperature then the absorbance (A) was measured at 532 nm using Jenway 6300 spectrophotometer. The malondialdehyde (MDA) content (µmol/ml) was calculated using molar extinction coefficient (156,000 M/cm). The data are expressed as MDA equivalents (µmol/mg protein).

Assay of Protein Carbonyl Content: Protein carbonyl content was assayed by the method of Rudnicki et al. [33] as follows: In a clean test tube, 600 µl of the liver slices homogenate were centrifuged at 10,000 rpm for 15 min and 200 µl of supernatant were mixed with 200 µl of 2,4-dinitrophenylhydrazine solution (DNPH; 10.0 mM in 2 M HCl solution). The mixture was incubated at room temperature for 1 h, followed by the addition of 100 µl of trichloroacetic acid solution (20%, w/v) and centrifugation at 5000 rpm for 3 min. The protein pellets were washed three times with 500 µl of acetone and 1.0 ml of sodium dodecyl sulphate (2% SDS in 20 mM Tris–HCl and 0.1 M NaCl, pH 7.4) was added to solublize the pellet. The absorbance of the solution was measured at 360 nm using Jenway 6300 spectrophotometer. The protein carbonyl content (µmol/ml) was calculated using molar extinction coefficient (22,000 M/cm). The data are expressed as µmol of carbonyls/mg protein.

Assay of Lactate Dehydrogenase Activity: Lactate dehydrogenase (LDH) was estimated by the method of Weibhaar et al. [34]. Percent release of enzyme from liver slices was calculated as the ratio of LDH activity found in the supernatant to the total LDH (supernatant + homogenate) activity [10].

Assay of Catalase Activity: Catalase (CAT) activity was estimated using the method of Murugan and Pari [35] as follows: To prepare the reaction mixture (1.5 ml), 1.0 ml of phosphate buffer solution (0.01 M, pH 7), 0.1 ml of liver homogenate and 0.4 ml of H₂O₂ solution (2 M) were mixed well. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate solution with glacial acetic acid, 1:3) then the absorbance was measured at 620 nm using Jenway 6300 spectrophotometer. CAT activity was expressed as µM of H₂O₂ consumed/min/mg protein.
Assay of Peroxidase Activity: Peroxidase activity was determined using the method of Bergmeyer [36] as follows: In a clean test tube, 0.1 ml of liver homogenate was added to 3.0 ml of phosphate buffer solution (0.1 M, pH 7), 0.05 ml of guaiacol solution (20.1 mM) and 0.03 ml of H₂O₂ solution (12.3 mM). The mixture was mixed thoroughly and incubated for 1 min at 37°C. The absorbance was measured at 1 min intervals thereafter for 3 min against blank at 436 nm using Jenway 6300 spectrophotometer. The activity unit was defined as the amount of enzyme that produced an increase of an absorbance unit per minute under the assay conditions.

Statistical Analysis: The results were analysed by an analysis of variance (P<0.05) and the means separated by Duncan’s multiple range test. The results were processed by CoStat computer program (1986).

RESULTS AND DISCUSSION

Assessment of Hepatoprotection of GSEs Against Ethanol Cytotoxicity: The hepatoprotective activity of grape seed extracts was investigated by liver slice culture in vitro. The liver slice is a microcosm of the intact liver consisting of highly organised cellular communities in which the different cell types are subject to mutual contact. Liver slice culture is therefore an in vitro technique that offers the advantages of in vivo situation and hence is a more suitable model for the experimental analysis of hepatotoxic events [37, 38]. Employing this model, the ethanol toxicity can be calculated by measuring the release of LDH into the medium by liver slices, lipid peroxidation, protein carbonyls and the activity of antioxidant enzymes.

Lipid Peroxidation and Protein Carboxyls of Liver Tissue: Ethanol is known to generate oxidative stress in cells, which can be measured from the extent of lipid peroxidation and protein carbonyls in liver tissue. Lipid peroxidation and protein carbonyls were measured in liver slices and are expressed as µmol of malondialdehyde (MDA) or protein carbonyl formed/mg protein. The results revealed that the levels of lipid peroxidation and protein carbonyls were significantly reduced when liver cells were pre-treated with water and ethanol grape seed extracts or when water and ethanol grape seed extracts were added along with ethanol in comparison with ethanol treated slices (Table 1). In ethanol treated liver slices, the values of lipid peroxidation and protein carbonyls reached 8.13 and 50.66, respectively. The extent of lipid peroxidation was reduced when liver cells pre-treated, individually with water or ethanol grape seed extracts (5.88 and 5.47 µmol MDA/mg protein, respectively). At the same time, protein carbonyl contents were also reduced when liver cells pre-treated with water or ethanol grape seed extracts (44.75 and 33.81 µmol/mg protein, respectively). Additional reduction in lipid peroxidation (5.21 and 3.22 µmol MDA/mg protein, respectively) and in protein carbonyl (31.57 and 26.12 µmol/mg protein, respectively) was reported when liver cells treated along, individually with water or ethanol grape seed extracts. This reduction in lipid peroxidation and protein carbonyls was found to be near of control untreated slices (3.03 and 23.13, respectively). From the obtained results, it could be concluded that EGSE was more effective than WGSE in reduction of lipid peroxidation and protein carbonyls in liver cells.

LDH Leakage of Liver Tissue: LDH is a cytosolic enzyme mainly present in periportal hepatocytes and released when the cells are lysed by hepatotoxin. The amount of enzyme released is proportional to the extent of damage caused to the cell. In the liver slice culture system, leakage of LDH was used as a marker to study the hepatotoxicity of ethanol. The results presented in Table 2 revealed that in case of slices treated with ethanol alone there was more LDH in the medium (72.98%) compared to untreated liver slices (24.27%). In other trial, the liver slices were pre-treated with WGSE and EGSE for 0.5 h. This treatment resulted in reduction of LDH leakage to 52.09% and 44.17%, respectively. When grape seed extracts
Table 2: Effect of water grape seed extract (WGSE) and ethanol grape seed extract (EGSE) on the activity of catalase and peroxidase in liver slice culture against ethanol-induced cytotoxicity

<table>
<thead>
<tr>
<th>Treatment* (control (KRH)</th>
<th>LDH leakage%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (KRH)</td>
<td>24.27±1.31</td>
</tr>
<tr>
<td>Ethanol</td>
<td>72.98±2.40</td>
</tr>
<tr>
<td>WGSE1+ethanol</td>
<td>52.09±1.05</td>
</tr>
<tr>
<td>WGSE2+ethanol</td>
<td>41.31±3.27</td>
</tr>
<tr>
<td>EGSE1+ethanol</td>
<td>44.17±0.42</td>
</tr>
<tr>
<td>EGSE2+ethanol</td>
<td>31.13±0.57</td>
</tr>
<tr>
<td>L.S.D</td>
<td>5.594</td>
</tr>
</tbody>
</table>

Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at P<0.05. KRH: Krebs-Ringer-HEPES buffer solution.

Table 3: Effect of water grape seed extract (WGSE) and ethanol grape seed extract (EGSE) on the activity of catalase and peroxidase in liver slice culture against ethanol-induced cytotoxicity

<table>
<thead>
<tr>
<th>Treatment* (control (KRH)</th>
<th>Catalase activity (U/mg protein)</th>
<th>Peroxidase activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (KRH)</td>
<td>6.71±0.018</td>
<td>0.078±0.003</td>
</tr>
<tr>
<td>Ethanol</td>
<td>4.53±0.040</td>
<td>0.017±0.001</td>
</tr>
<tr>
<td>WGSE1+ethanol</td>
<td>5.61±0.054</td>
<td>0.043±0.001</td>
</tr>
<tr>
<td>WGSE2+ethanol</td>
<td>6.51±0.017</td>
<td>0.059±0.003</td>
</tr>
<tr>
<td>EGSE1+ethanol</td>
<td>5.87±0.023</td>
<td>0.052±0.002</td>
</tr>
<tr>
<td>EGSE2+ethanol</td>
<td>6.64±0.032</td>
<td>0.068±0.003</td>
</tr>
<tr>
<td>L.S.D</td>
<td>0.103</td>
<td>0.0066</td>
</tr>
</tbody>
</table>

Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at P<0.05. KRH: Krebs-Ringer-HEPES buffer solution.

Antioxidant Enzymes of Liver Tissue: Catalase and peroxidase are antioxidant enzymes (AOEs) that protect cells from oxidative stress of highly reactive free radicals. These enzymes are induced on the generation of free radicals in cells. Activities of these enzymes were checked in liver slice culture treated with ethanol alone or ethanol and grape seed extracts together or untreated liver slices (control). The activities were checked at the end of 2 h of treatment and expressed as ratios of U/mg protein.

The results presented in Table (3) revealed that in the presence of ethanol the activities of catalase and peroxidase were found to be decreased (4.53 and 0.017 U/mg protein, respectively) compared to control (6.71 and 0.078 U/mg protein, respectively). The activity of catalase was increased when liver cells pre-treated, individually with WGSE or EGSE (5.61 and 5.87 U/mg protein, respectively). At the same time, peroxidase activity was also increased when liver cells pre-treated with WGSE or EGSE (0.043 and 0.052 U/mg protein, respectively). The effect of WGSE and EGSE individually was better when it was present continuously with ethanol compared to when it was added only for 0.5 h during pre-incubation period. When EGSE presented along with ethanol incubation, further increased in the activities of catalase and peroxidase was recorded (6.64 and 0.068 U/mg protein, respectively) in comparison with WGSE presented along with ethanol incubation (6.51 and 0.059 U/mg protein, respectively). Thus, it is clear that pre-treatment with GSEs for 0.5 h protects liver tissue against ethanol cytotoxicity, but prolonged treatment with GSEs offers better protection.

Alcohol-induced oxidative stress in the liver cells plays a major role in the development of alcoholic liver disease. This condition results from several processes related to alcohol metabolism: (a) changes in the NAD/NADH ratio resulting from alcohol breakdown by alcohol dehydrogenase, (b) production of reactive oxygen species (ROS) during alcohol metabolism by the microsomal ethanol-oxidizing system, (c) depletion of GSH and (d) decreased activity of antioxidant enzymes. Increased ROS production and decreased antioxidant potential, among other harmful effects, causes lipid peroxidation which leads to damage to liver cells, membrane lesions and loss of LDH from cells. One of the major consequences of oxidative stress is irreversible protein modification such as generation of carbonyls or loss of thiol residues. These oxidative modifications alter the biological properties of proteins leading to their fragmentation, increased aggregation and enzyme dysfunction [9].

In the present study, increased malondialdehyde (MDA), a product of lipid peroxidation, observed in case of liver slices treated with ethanol indicated excessive formation of free radicals resulting in hepatic damage. The hepatoprotective effect of certain plant extracts against ethanol-induced liver injury possibly involves mechanisms related to free radical scavenging effects [39]. Pre-treatment or treatment along with grape seed extracts (WGSE and EGSE) prevented lipid peroxidation which...
could be attributed to their antioxidant and free radical scavenging activities [20, 40, 41]. However, a significant increase in the protein carbonyl content was observed in case of liver slices treated with ethanol which is consistent with earlier reports [42]. Ethanol-induced protein carbonylation in the liver cells was prevented by pretreatment or treatment along with GSEs which could be attributed to their antioxidant activity. Ethanol-induced hepatic damage is characterized by the raised level of LDH which reflects the severity of liver injury [43]. In this study, ethanol treated liver slices released 3 times more enzyme into the medium than untreated cells over a period of 2 h. The leakage of the LDH into the medium is attributed to the hepatic damage. However, ethanol-induced increase leakage of this enzyme was considerably reduced by pretreatment or treatment along with GSEs, implying that the extracts protected the liver against ethanol-induced damage. It has been shown that antioxidants or plant extracts having antioxidant activity exhibit hepatoprotective activity [44, 45]. The defense of cells against oxidative stress involves both non-enzymatic antioxidants such as glutathione, ascorbic acid and alpha-tocopherol which directly react with free radicals as well as antioxidant enzymes like catalase and peroxidase which scavenge free radicals. Treatment of liver cells with ethanol lowered the antioxidant capacity of the rat liver as reflected in the decreased activity of the antioxidant enzymes which is in agreement with earlier reports [46]. Pretreatment with GSEs restored the antioxidant enzyme profile in the liver slices. Further, treatment along with GSEs significantly boosted the antioxidant enzyme activities in the liver slices. Induction of antioxidant enzymes in the liver by plant-derived polyphenols has been reported [47, 48].

In conclusion, red grape seed extracts effectively protected against ethanol-induced oxidative damage to the liver. The hepatoprotective activity of the extracts could, at least partly, be due to the antioxidant and free radical scavenging properties or enhanced antioxidant capacity of the liver [49-51]. The higher hepatoprotective activity of ethanol grape seed extract in comparison with water grape seed extract was associated with its antioxidant activity [20]. The bioactive antioxidant principles of the grape seed extracts, polyphenol substances such as proanthocyanidins, could be responsible for the observed hepatoprotective effect [14, 15, 52, 53].

Concluding Remarks: Finally, it could be concluded that red grape seed extracts possess a protective effect against ethanol-induced hepatotoxicity.

REFERENCES


