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DUAL EFFECT OF ETHANOL ON CELL DEATH IN PRIMARY CULTURE OF HUMAN AND RAT HEPATOCYTES
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Abstract — Aims: In-vivo and in-vitro studies have shown that ethanol induces hepatocyte damage. The aim of the present study was to evaluate the effect of a broad range of ethanol concentrations on apoptosis and necrosis in primary culture of human and rat hepatocytes. Methods: Human and rat hepatocytes were isolated from human hepatectomies and male Wistar rats (200–250 g) using the classical collagenase perfusion method. After stabilization of cell culture, ethanol (0–10 mmol/l) was administered and the parameters were measured 24 h after ethanol addition. Apoptosis was studied by DNA fragmentation, iodide propidium–DNA staining, Malondialdehyde (MDA) and GSH/GSSG were used as parameters of oxidative stress. Results: Ethanol enhanced dose-dependently all the parameters associated with apoptosis and necrosis in human and rat hepatocytes. Low or high ethanol concentrations induced an opposite action against cell necrosis in cultured hepatocytes. Low concentrations of ethanol (1–2 mmol/l) reduced LDH release from human and rat hepatocytes. However, the highest ethanol concentration (10 mmol/l) induced a sharp increase in cell necrosis. The effect of ethanol on cell necrosis was related to lipid peroxidation in hepatocytes. Conclusions: Ethanol differentially regulates apoptosis or necrosis in cultured hepatocytes. Although ethanol exerted a dose-dependent induction of apoptosis, low ethanol concentrations were able to reduce basal lipid peroxidation and necrosis in hepatocytes. The highest ethanol concentration (10 mmol/l) induced apoptosis and necrosis in human and rat cultured hepatocytes.

INTRODUCTION
Alcohol-dependent liver disease (ALD) is the most common form of liver dysfunction in the western world. ALD is also the major cause of chronic illness and death associated with alcohol misuse (Frank and Raith, 1985; French, 1996). In addition, excessive alcohol intake is also associated with gastric mucosa damage (Kvietsyt et al., 1990), inhibition of platelet aggregation (Mikhailidis et al., 1983), alteration of red blood cell membranes (Rottenberg, 1986), deregulation of immune response (Devire et al., 1988; Nelson et al., 1989) and inhibition of cell growth (Claren and Smith, 1978). Other experimental studies have also demonstrated a beneficial effect of ethanol against coronary heart disease (Amarasuriya et al., 1992; Miyamae et al., 1998; Xia et al., 1998), drug-related neuronal dysfunction (Boobis et al., 1975; Padilla et al., 1992) and drug-related hepatotoxicity (Tredger et al., 1985).

The aim of the present study was to evaluate the effect of a broad range of ethanol concentrations on cell death in cultured human and rat hepatocytes.

MATERIALS AND METHODS
Materials
All reagents were from Sigma Chemical (St Louis, MO) unless otherwise stated. William’s medium E was from AppliChem (Darmstadt, Germany). Antibiotic–antimycotic solution and fetal bovine serum were from Life Technologies (Paisley, UK). All experimental animals received humane care and the study protocols and human hepatectomies were obtained following the institution’s guidelines and supervised by the Ethic Committee.

Preparation of primary hepatocytes and cell culture
Human hepatocytes were isolated from hepatectomies obtained from patients (two men and two women; 52 ± 15 years old) submitted to surgical intervention for the resection of primary or secondary liver tumours. Rat hepatocytes were isolated from livers of male Wistar rats (seven animals, 200–250 g) anaesthetized by intraperitoneal administration of sodium thiopental. Human and rat hepatocytes were isolated by in-situ collagenase perfusion of liver (Seglen, 1976). Livers were perfused first with an oxygenated solution I (10 mmol/l HEPES, 6.7 mmol/l KCl, 145 mmol/l NaCl and 2.4 mmol/l EGTA), pH 7.4 at 37°C, and then with solution II (100 mmol/l HEPES, 6.7 mmol/l KCl, 67 mmol/l NaCl, 10 g/l albumin, 4.8 mmol/l CaCl₂ and 0.05% collagenase A), pH 7.4 at 37°C. In order to reduce ischaemia-reperfusion syndrome during human hepatocyte isolation antioxidants were added (100 μmol/l sorbitol, 100 μmol/l manitol and 100 μmol/l GSH) to solution I and II. Thereafter, tissue was gently minced and cell suspension filtered through a nylon mesh. Hepatocytes were centrifuged and washed three times at 50 g for 5 min in William’s medium E, pH 7.4, supplemented with 1 μmol/l insulin, 0.6 μmol/l hydrocortisone, 15 mmol/l HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, 2 mmol/l glutamine and 26 mmol/l NaHCO₃. Cell viability determined by trypan blue exclusion was consistently higher than 85%. Hepatocytes (150 000 cells/cm²) were plated in a Petri dish coated with collagen type I (Iwaki, Gyouda, Japan) and cultured in supplemented William’s medium E, pH 7.4, containing 5% fetal bovine serum. After 2 h, culture medium was removed and replaced by fresh supplemented medium without fetal bovine serum and the culture was maintained for 24 h without treatment. After stabilization of cell culture, ethanol...
ethanol and cell death in hepatocytes

...KCl, 4.3 mmol/l Na2 HPO4) pH 7.4 at 4°C in phosphate buffer solution (PBS) (137 mmol/l NaCl, 2.7 mmol/l KCl, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 0.6% Nonidet NP-40, 5 µg/ml aprotinin, 10 µg/ml leupeptin, 0.5 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l DTT) for 10 min on ice. After, samples were vortexed and centrifuged at 15 000 g for 1 min at 4°C. The supernatant, as cytoplasmatic fraction, was recovered and frozen at −80°C for the further measurement of caspase-3-associated activity. Caspase-3-associated activity was measured in samples (25 µg protein) by a fluorometric assay using the peptide-based substrate ac-N-acetyl-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC) (Bachem, Bubendorf, Switzerland). The linear increase in fluorescence (Ex 400 nm, Em 505 nm) of enzymatically released AFC was recorded over a period of 2 h at 37°C using a GENios Reader (TECAN, Salzburg, Austria).

Measurement of annexin V binding

The binding of annexin V to phosphatidylserine sites in human and rat hepatocytes was followed using a commercial kit (Sigma Chemical). This assay is based on the binding of annexin V-FITC to hepatocytes and further quantitative determination by flow cytometry. The inclusion in the kit of propidium iodide to label the cellular DNA in necrotic cells allowed us to evaluate the percentage of apoptotic hepatocytes that binds annexin V.

Measurement of lactate dehydrogenase release

Lactate dehydrogenase (LDH) in the culture medium and hepatocytes was measured by modification of a colorimetric routine laboratory method (Taffs and Sitkovsky, 1991). Briefly, a volume of culture medium or cell lysate was incubated with 0.2 mmol/l β-NADH and 0.4 mmol/l pyruvic acid diluted in PBS pH 7.4. It was also confirmed that addition of ethanol to the incubation mixture did not alter the oxidation of β-NADH. LDH concentration in the sample was proportional to the linear decrease in the absorbance at 343 nm. LDH concentration was calculated using a commercial standard. The percentage of LDH released from hepatocytes was calculated as LDH present in culture medium in relation to total LDH obtained in culture medium and hepatocytes.

Evaluation of lipid peroxidation

The presence of malondialdehyde (MDA) in culture medium was used as an index of lipid peroxidation in hepatocytes following a procedure described previously (Wasowicz et al., 1993). Briefly, the samples (100 µl) were treated with trichloroacetic acid (10%) and centrifuged at 20 800 g at 4°C for 5 min. EDTA (1.34 mmol/l) and GSH (0.65 mmol/l) was added to the supernatant to prevent further lipid peroxidation caused during the assay. The samples were treated with 1 ml HCl (25%) and 1 ml of thiobarbituric acid (1% diluted in 50 mmol/l NaOH), and the mixture was heated at 100°C for 1 h. MDA was evaluated measuring the absorbance of the samples at 532 nm in a DU® 64 Spectrophotometer (Beckman Coulter, California, USA). Standard curve was prepared daily using 1,1,3,3-tetraethoxypropane (Sigma Chemical) diluted in ethanol as source of MDA.

Quantification of GSH/GSSG ratio

GSH and GSSG were quantified in hepatocytes following the procedure described by Asensi et al. (1994). The whole hepatocyte population, including the floating cells obtained...
from collected culture medium, was treated with precipitating solution (12% perchloric acid, 40 mmol/l N-ethylmaleimide and 2 mmol/l bathophenanthroline disulfonic acid) at 4°C for 5 min. The samples were centrifuged at 20 800 g at 4°C for 5 min. Afterwards, 50 μl of glutamyl glutamate (1 mmol/l) (Sigma Chemical) as internal standard and 10 μl of m-cresol purple (1 mmol/l) (Sigma Chemical Co.) as pH indicator were added to the samples (500 μl). The pH of the solution was adjusted to 8.0–8.5 with KOH (2 mol/l) containing MOPS (0.3 mol/l) to prevent excessive alkalization. After centrifugation the samples at 20 800 g at 4°C for 5 min, a volume (25 μl) was derivatized with 50 μl 1-fluoro-2,4-dinitrobenzene (1%) (Sigma Chemical) in a small glass tube. After 45-min incubation in the dark at room temperature, samples were dessicated under vacuum and stored at −20°C until injection. Afterwards, the samples were dissolved in 50 μl of methanol (80%) and injected (25 μl) into the HPLC system (Beckman Instruments) equipped with a Sperisorb NH2 column (20 × 04 cm, 5 μm particles) (Teknokroma, Barcelona, Spain). The flow rate was set at 1 ml/min. Two mobile phases were used: solvent A (80% methanol) and solvent B (0.5 mol/l sodium acetate in 64% methanol). After injection of the sample, the mobile phase was held at 80% solvent A and 20% solvent B for 5 min followed by a 10-min linear gradient up to 1% solvent A and 99% solvent B. Later, the mobile phase was held at 99% solvent B until GSSG had eluted. The concentration of GSH and GSSG was quantified using the areas below the corresponding HPLC peaks of the sample. Standard curve was drawn using commercial GSH and GSSG (Sigma Chemical).

Statistical analysis

Results are expressed as means with their corresponding standard errors of four to seven separate experiments. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the least significant differences (LSD) test as multiple comparison analysis.

RESULTS

Induction of apoptosis by ethanol exposure in cultured rat hepatocytes

The effect of ethanol (0–10 mmol/l) in the degree of apoptosis in rat hepatocytes was evaluated. Ethanol gradually increased DNA fragmentation (Fig. 1) and caspase-3-associated activity (Fig. 2) in cultured hepatocytes. The measurement of histone-associated DNA fragments in cell extract also showed that ethanol gradually enhanced the presence of mono- and oligonucleosomes in cultured rat hepatocytes. In this sense, ethanol (1 mmol/l) significantly raised DNA fragments (2.34-fold ± 0.164) in comparison with control hepatocytes (P ≤ 0.05). The measurement of DNA content in hepatocytes by flow cytometry showed that the percentage of hypoploidy cells was significantly enhanced at the highest ethanol concentration (10 mmol/l) (Table 1) (P ≤ 0.05). The same method showed that hepatocytes in apoptosis came from hepatocytes in the G2 + M cell cycle stage (Table 1). An early parameter of apoptosis such as annexin V binding to hepatocyte was also evaluated (Table 1). In this assay, intermediate ethanol concentrations (2 mmol/l) significantly enhanced the percentage of apoptotic hepatocytes that binds annexin V (P ≤ 0.05). Interestingly, hepatocytes treated with the highest ethanol concentration (10 mmol/l) reduced their ability to bind annexin V (P ≤ 0.05).
Induction of necrosis by ethanol exposure in cultured rat hepatocytes

The measurement of LDH released from hepatocytes is an index of cell necrosis (Fig. 3). Interestingly, LDH release was significantly reduced at intermediate ethanol concentration (0.2–5 mmol/l) (P ≤ 0.05). At 10 mmol/l ethanol a sharp increase in LDH release was observed in cultured rat hepatocytes (Fig. 3).

Evaluation of lipid peroxidation after ethanol exposure in cultured rat hepatocytes

The presence of MDA in the culture medium was used as an index of lipid peroxidation in hepatocytes (Fig. 4). MDA concentration was significantly reduced at an intermediate concentration of ethanol (0.2–5.00 mmol/l) (P ≤ 0.05). The highest ethanol concentration (10 mmol/l) induced a rise in MDA concentration over control hepatocytes (Fig. 4) (P ≤ 0.05).

Effect of ethanol on GSH/GSSG ratio in cultured rat hepatocytes

The antioxidant status was evaluated by the ratio of GSH and GSSG in hepatocytes. Ethanol gradually decreased GSH/GSSG in hepatocytes, reaching statistical significance at the highest ethanol concentration (10 mmol/l) (93 ± 3.8) in comparison with the value obtained in control hepatocytes (144 ± 15.6) (P ≤ 0.05).

Effect of ethanol on cell death in cultured human hepatocytes

We present data corresponding to the effect of ethanol (0–10 mmol/l) in the degree of apoptosis and necrosis in cultured human hepatocytes. Ethanol (10 mmol/l) induced a significant increase in the presence of histone-associated DNA fragments (3.01-fold ± 0.300) and the percentage of hypodiploid cells (Table 2) in comparison with the values obtained in control hepatocytes (P ≤ 0.05). The presence of an early marker of apoptosis, such as annexin V binding, was also
annexin V (enhanced the percentage of apoptotic hepatocytes that binds intermediate ethanol concentrations (2 mmol/l) significantly assessed in cultured human hepatocytes (Table 2). In this assay, experiments. 

release from human hepatocytes. Data are the mean ± SD of four experiments. *P ≤ 0.05 versus hepatocytes treated with lower ethanol concentration, #P ≤ 0.05 versus hepatocytes treated with lower ethanol concentration.

Data are the mean ± SD of four experiments. *P ≤ 0.05 versus hepatocytes treated with lower ethanol concentration. The effect of ethanol on necrosis in human hepatocytes was similar to that observed in rat hepatocytes. The administration of intermediate (1–2 mmol/l) or high (10 mmol/l) ethanol concentration exerted cytoprotection or exacerbation respectively against cell necrosis in cultured human hepatocytes (Fig. 5) (P ≤ 0.05).

Table 2. Effect of ethanol on the percentage of hepatocytes in hypodiploid and G2+M stage, as well as the binding of annexin V to apoptotic cells in primary culture of human hepatocytes.

<table>
<thead>
<tr>
<th></th>
<th>Hypodiploid cells (%)</th>
<th>G2+M cells (%)</th>
<th>Annexin V binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28 ± 4.1</td>
<td>44 ± 2.4</td>
<td>2.5 ± 0.71</td>
</tr>
<tr>
<td>Ethanol (2 mM)</td>
<td>32 ± 2.5</td>
<td>46 ± 2.7</td>
<td>11.7 ± 0.95*</td>
</tr>
<tr>
<td>Ethanol (10 mM)</td>
<td>42 ± 4.2*</td>
<td>36 ± 4.4*</td>
<td>0.4 ± 0.11*</td>
</tr>
</tbody>
</table>

Fig. 5. Lactate dehydrogenase (LDH) release in human hepatocytes treated with ethanol (0–10 mmol/l). Data correspond to the percentage (%) of total LDH released by hepatocytes to the culture medium. The administration of low doses of ethanol (1–2 mmol/l) reduced LDH release, but 10 mmol/l ethanol induced a sharp increase in LDH release from human hepatocytes. Data are the mean ± SD of four experiments. *P ≤ 0.02 versus hepatocytes treated with lower ethanol concentration; #P ≤ 0.05 versus hepatocytes treated with lower ethanol concentration.

DISCUSSION

The present study showed that ethanol administered at concentrations below 10 mmol/l exerts a differential regulation on apoptosis and necrosis in cultured human and rat hepatocytes. In this sense, ethanol enhances apoptosis but reduces necrosis, probably through a protection against basal lipid peroxidation.

Alcohol-dependent liver disease (ALD) is the most frequent cause of cell death associated with alcohol misuse (Frank and Raicht, 1985). Several reports have shown that ethanol enhances in-vivo (García-Ruiz et al., 1994; Reinke et al., 1997; Rouach et al., 1997) and in-vitro (Lamb et al., 1994; Kurose et al., 1997; Bailey et al., 1998) hepatocyte cell death through stimulation of oxidative stress. Although free radicals from stimulated inflammatory cells may play an important role in alcohol-dependent liver toxicity (Koop et al., 1997), it seems that enhanced intracellular oxidative stress in hepatocytes during ethanol exposure limits cell viability. In ethanol-induced cytotoxicity, free radicals may be derived from the induction of cytochrome P450E1 activity (Morimoto et al., 1993) or by mitochondrial dysfunction after reduction of GSH content and ATP generation (Cunningham et al., 1990; Collell et al., 1998). Necrosis and apoptosis have been demonstrated in rat hepatocytes treated with ethanol (50 mmol/l or higher) (Lamb et al., 1994; Higuchi et al., 1996). In our conditions, ethanol was able to induce apoptosis and necrosis in human and rat hepatocytes. Nevertheless, ethanol showed differential regulatory properties against apoptosis and necrosis. In this sense, ethanol increased cell apoptosis in a concentration-dependent fashion. In contrast, the administration of intermediate ethanol concentrations, 1–2 mmol/l and 0.2–5 mmol/l in human and rat hepatocytes respectively, reduced cell necrosis. The highest ethanol concentration (10 mmol/l) induced a sharp increase in hepatocyte necrosis. Although we have not measured intracellular free radical production, our data suggest that oxidative stress in terms of lipid peroxidation and antioxidant status induced by ethanol was more related to necrosis than to apoptosis in rat hepatocytes.

The administration of an acute dose of ethanol has been shown to exert cytoprotection in different human and experimental liver dysfunction (Sato et al., 1981; Banda and Quart, 1982). In this sense, although chronic ethanol consumption increases the risk of acetaminophen-associated liver injury, low doses of ethanol consumption prior to the ingestion of acetaminophen leads to a reduction of its related reactive metabolite in humans (Banda and Quart, 1982). The incubation of microsomes with ethanol has not been shown to significantly affect paracetamol activation (Tredger et al., 1985). It has been proposed that acute ethanol administration decreases the availability of NADPH required as cofactor for monoxygenation (Reinke et al., 1980). In our experiments, the addition of 0.2–5 mmol/l ethanol reduced MDA in culture medium, suggesting that ethanol was reducing the basal level of intracellular oxidative stress in hepatocytes. Cytochrome P450E1, an isof orm mostly involved in intracellular free radical production (Albano et al., 1991), activates acetaminophen to reactive metabolites which cause cell toxicity (Dai and Cederbaum, 1995). In our studies, the...
expression of cytochrome P452E1 was maintained for 48 h without decline after hepatocyte isolation (data not shown). These results suggest that the protection by ethanol against hepatocyte necrosis is probably due to a reduction of intracellular free radical production in a similar way by which ethanol is able to protect against acetalophenetin cytotoxicity (Reinke et al., 1980; Banda and Quart, 1982; Tredger et al., 1985). Other factors, such as nitric oxide, which have been shown to inhibit cytochrome P4502E1 catalytic activity and its reactive oxygen radical formation (Gergel et al., 1997), do not seem to play a role in our system. We have not observed any changes in nitrite-plus-nitrate concentration in culture medium during ethanol treatment (data not shown).

In conclusion, the present study shows that although high ethanol concentration induces apoptosis and necrosis in human and rat hepatocytes, the exposure to low ethanol concentrations reduces cell necrosis, probably through a reduction of intracellular oxidative stress. More studies should be done to fully elucidate the intracellular mechanism by which ethanol exerts this cytoprotective effect.

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REFERENCES


