Effect of chronic ethanol exposure on the hepatotoxicity of ecstasy in mice: An ex vivo study

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Abstract

3,4-Methylenedioxymethamphetamine (MDMA) is frequently consumed at “rave” parties by polydrug users that usually take this drug in association with ethanol. In addition, many young people are repeatedly exposed to ethanol, which likely leads to tolerance phenomena. Both compounds are metabolized in the liver, with formation of hepatotoxic metabolites, which gives high relevance to the evaluation of their putative toxicological interaction. Therefore, the aim of this study was to evaluate the toxicity induced by 0.8 and 1.6 mM MDMA to freshly isolated hepatocytes obtained from ethanol-treated mice whose tap drinking water was replaced by a 5% ethanol solution for 1 week and, afterwards, by a 12% ethanol solution for 8 weeks (ethanol group) comparatively to non-treated animals (non-ethanol group). The hepatocytes were incubated under normothermic and hyperthermic conditions in order to simulate in vitro the hyperthermic response induced in vivo by MDMA, a condition that has been recognized as a life-threatening effect associated with MDMA exposure and implicated in its hepatotoxicity. Six mice treated under the same protocol as the ethanol group were used for histological analysis, and compared to non-ethanol-treated animals. The pre-treatment of mice with ethanol caused a significant decrease in the hepatocytes yield in the isolation procedure comparatively to the non-ethanol group, which can be explained by an increase in collagen deposition along the hepatic parenchyma as observed in the histological analysis. The initial cell viability of hepatocytes suspensions was similar between ethanol and non-ethanol groups. However, the ethanol group showed a higher GSH oxidation rate, which was enhanced under hyperthermia. Additionally, a concentration-dependent MDMA-induced loss of cell viability and ATP depletion was observed for both groups, at 41°C. In conclusion, the repeated treatment with ethanol seems to increase the vulnerability of freshly isolated mice hepatocytes towards pro-oxidant conditions, as ascertained by the increase in collagen deposition, lower hepatocyte yield and decreased glutathione levels. However, MDMA toxicity to the isolated hepatocytes was independent of ethanol pre-treatment, while significantly dependent on incubation temperature.

Keywords: Ethanol, 3,4-Methylenedioxymethamphetamine (MDMA); Hepatocytes; Oxidative stress

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA or ecstasy), is a commonly used drug of abuse, whose consumption appears to be spreading in many parts of the world, with a high prevalence among young people (EMCDDA, 2005; SAMHSA, 2005). MDMA consumption results in a plethora of systemic and organ-specific effects, including hepatic toxicity (Maurer et al., 2004) and also hyperthermia (Crean et al., 2006; Izco et al., 2007b) that can be, at least in part, responsible for the hepatic cellular damage (Santos-Marques et al., 2006). Various factors may contribute to MDMA-induced liver damage.
toxicity, including the metabolism of MDMA (de la Torre et al., 2004), the increased efflux of neurotransmitters (de la Torre et al., 2004), the oxidation of biogenic amines (Carvalho et al., 2004b), and hyperthermia (Green et al., 2004). Hyperthermia corresponds to a well-known life-threatening effect of MDMA in vivo (Hall and Henry, 2006) that is highly dependent on ambient temperature (Carvalho et al., 2002a; Von Huben et al., 2007). Our group has already shown that the in vivo toxic effects induced by MDMA in isolated mouse hepatocytes are exacerbated under hyperthermic conditions (Carvalho et al., 1997; Carvalho et al., 2001).

MDMA consumers are often polydrug abusers. Ethanol (EtOH) is, by far, the most popular drug among youth (Smart and Ogborne, 2000; Marques-Vidal and Dias, 2005) and it is often consumed in large amounts along with MDMA use (Tossmann et al., 2001; Barrett et al., 2006). In fact, EtOH is often taken with MDMA, especially at the beginning of the night, to get a stronger/better “high” and, in the last part of the night, EtOH is taken in high dosages to decrease the long-lasting effects of MDMA such as irritability and restlessness that persist beyond its empathogenic and entactogenic effects (Schifano, 2004).

To study the putative interaction between these two compounds, it is crucial to consider that many young people are exposed to EtOH early in life, sometimes even repeatedly, which means that many may have developed some tolerance to EtOH when taking MDMA for the first time and concomitantly with EtOH (Hamida et al., 2006). Thus, the study of EtOH–MDMA interaction should not be restricted to the acute consumption of these substances. It is of extreme importance to develop an experimental model considering this common chronic abuse pattern, since the chronic exposure to EtOH prior to the first contact with MDMA may induce deleterious effects that can influence MDMA toxicity.

Both compounds, EtOH and MDMA, are metabolized in the liver leading to the formation of hepatotoxic metabolites. Most EtOH biotransformation occurs in the liver, mainly via oxidation catalyzed by alcohol dehydrogenase (ADH) and by a cytochrome P450 isoform (CYP2E1) into acetaldehyde (Gemma et al., 2006). The metabolism of MDMA involves N-demethylation to 3,4-methylenedioxyamphetamine (MDA) and both MDMA and MDA are O-demethylated to catechols [N-methyl-a-methyldopamine (N-Me-a-MeDA) and a-methyldopamine (a-MeDA), respectively] that can undergo oxidation into the corresponding o-quinones (Carvalho et al., 2004b). These highly reactive molecules can alkylate crucial cellular proteins and/or DNA and further: (i) redox cycle originating semiquinone radicals, leading to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS); (ii) undergo irreversible 1,4-intramolecular cyclization with subsequent formation of aminochromes and (iii) conjugate with glutathione (GSH) to form a glutathionyl adduct, which remains redox active and can further react with GSH and protein thiols (Carvalho et al., 2004b).

Previous studies on MDMA and EtOH interactions have focused on pharmacokinetic (Bilsky et al., 1990; Rezvani et al., 1992; Hernández-López et al., 2002; Ramaekers and Kuypers, 2006), behavioural (Cassel et al., 2004; Cassel et al., 2005; Hamida et al., 2006; Ben Hamida et al., 2007; Izco et al., 2007a) and physiologic and psychologic (Pacifici et al., 2001) consequences of these drugs combination. No hepatic toxicological interactions between these compounds have been reported yet.

Thus, the aim of the present study was to evaluate, under normothermic (37 °C) and hyperthermic (41 °C) conditions, the toxic effects of MDMA in an ex vivo model, trying to simulate the most common pattern of EtOH and MDMA use and the hyperthermic response that occurs in vivo as a consequence of MDMA consumption. For this purpose, the toxicity induced by MDMA to freshly isolated hepatocytes obtained from EtOH-treated mice was evaluated. The study included the quantification of the following parameters: hepatocytes yield in the isolation procedure and initial cell viability, reduced (GSH) and oxidised glutathione (GSSG) content, adenosine triphosphate (ATP) levels and lactate dehydrogenase (LDH) leakage. Furthermore, one separate group of ethanol-treated mice (that followed the same administration protocol as the ethanol group) was used for histological analysis, and compared to the control group.

2. Materials and methods

2.1. Chemicals

All reagents used in this study were of analytical grade. Collagenase (type I), bovine serum albumin (fraction V), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), β-nicotinamide adenine dinucleotide reduced form (β-NADH), β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), pyruvic acid, 2-vinylpyridine, reduced glutathione (GSH), oxidised glutathione (GSSG), glutathione reductase (EC 1.6.4.2), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), trypan blue solution, trizma® hydrochloride, d-luciferin sodium salt and luciferase (EC 1.13.12.7) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Perclohydrochloric acid, trichloroacetic acid, 2-thiobarbituric acid, absolute ethanol (EtOH) and all other chemicals were purchased from Merck (Darmstadt, Germany). 3,4-Methylenedioxyamphetamine (HCl salt) was extracted and purified from high purity MDMA tablets that were provided by the Portuguese Criminal Police Department. The obtained salt was pure and fully characterized by NMR and mass spectrometry methodologies.

2.2. Animals

Animal experiments were licensed by the Portuguese General Directorate of Veterinary Medicine. Housing
and experimental treatment of animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR, 1996).

Adult male CD1 mice (Charles-River Laboratories, Barcelona, Spain), weighing 35–45 g were used. For at least 1 week prior to use, the animals were acclimatized in polyethylene cages, lined with wood shavings, with wire-mesh tops, at an ambient temperature of 20 ± 2 °C, humidity between 40% and 60% and 12 h/12 h light/dark cycle (light on from 8.00 to 20.00 hours), in our animal facilities, having standard chow and tap drinking water ad libitum. After this adaptation period, the animals were divided into two groups: EtOH and non-EtOH groups. In the EtOH group the tap drinking water was replaced by a 5% EtOH solution for 1 week and, afterwards, by a 12% EtOH solution for 8 weeks until the hepatocytes isolation procedure. The replacement of water for 5% ethanol before 12% ethanol corresponds to a required adaptation period, to avoid aversion of mice to a high ethanol concentration in the drinking fluid, since these animals do not have natural preference for 12% ethanol. The non-EtOH group remained drinking tap water ad libitum, during the same period. Surgical procedures for the isolation of hepatocytes were performed under anaesthesia and carried out between 10 a.m. and 11 a.m.

Six mice from the EtOH group and six mice from the non-EtOH group were used for histological analysis. After 8 weeks of treatment, livers were excised under anaesthesia and samples were processed for optic microscopy as indicated in 2.5.

2.3. Hepatocytes isolation and incubation

Hepatocytes isolation was performed by collagenase perfusion as previously described (Carvalho et al., 2004b). Briefly, after anaesthesia, a cannula was introduced in the hepatic portal vein and a Hank solution with EGTA and albumin was perfused to remove the blood from the sinusoids and to chelate calcium, clearing the hepatic desmosomes. In a second stage, the liver was perfused with a Hank solution including collagenase and its co-factor calcium. This enzyme hydrolyses the collagen molecules that ensure the mechanical stability of the liver. The third step of this protocol consisted in a soft mechanical liver dissociation in Krebs–Henseleit solution with albumin and the cellular suspension obtained was then purified by filtration, centrifugation and washings with Krebs–Henseleit buffer supplemented with 12.5 mM HEPES (pH 7.4) to remove non-parenchymatous cells, non-viable hepatocytes and other contaminants from the suspension. The viability of the isolated hepatocytes was estimated by the trypan blue exclusion test and was always higher than 80%.

Aliquots of isolated hepatocytes suspensions containing 10⁶ viable cells per milliliter in Krebs–Henseleit solution supplemented with 12.5 mM HEPES (pH 7.4) were pre-incubated, before the beginning of the experiments, in a shaking water bath (90 oscillations/min) for 30 min at 37 °C and continuously gassed with carbogen (95% O₂/5% CO₂). After this pre-incubation period, cells were divided and the experiments were performed, in parallel, at 37 °C and 41 °C. Sample aliquots were collected at time 0 and then the cells were incubated with MDMA hydrochloride at final concentrations of 0.8 and 1.6 mM. The tested MDMA concentrations were chosen based on previous studies (Carvalho et al., 2001; Carvalho et al., 2004b) and on preliminary experiments, using this experimental model. Additionally, liver concentrations of MDMA and its metabolites have been found to be substantially higher (up to 18 times) than blood concentrations (García-Repetto et al., 2003). Taking into account that most cases of serious toxicity or fatality have involved MDMA blood levels ranging from 2.5 to 50 μM, the concentrations used in the present study are most likely attained in the in vivo situation. On the other hand, it is important to evaluate the interactions of MDMA and its metabolites with cellular components, which is only possible using worst-case approach concentrations. The control cells were incubated under the same conditions with an equivalent volume of Krebs-Henseleit solution. Additional sample aliquots were collected every hour, during a period of incubation of 3 h and were used for the evaluation of cell viability, and quantification of GSH, GSSG, and ATP. The evaluation of cell viability was accomplished in the same day of the experiment, and the remaining samples were kept frozen (−80 °C) until assay.

2.4. Biochemical analysis

During the course of the experiments, cell viability was determined by the lactate dehydrogenase (LDH) leakage method.

The GSH and GSSG contents in cell suspensions were determined by the DTNB-GSSG reductase recycling assay as described before (Carvalho et al., 2004a), with some modifications. Briefly, cell suspension aliquots were precipitated with equal volume of 10% HClO₄ and centrifuged for 2 min at 16,000g (4 °C). The supernatants were kept frozen at −80 °C until quantification. The thawed acidic supernatant was neutralized with equal volume of 0.76 M KHCO₃ and centrifuged for 2 min at 16,000g (4 °C). For measurement of total glutathione (GSHt), 100 μL/well of the neutralized supernatants, standards or blank were added in triplicate to 96-well microtiter plates, followed by 65 μL/well of freshly prepared reagent containing 0.69 mM NADPH and 4 mM DTNB in 72 mM phosphate buffer. Plates were then incubated at 30 °C in a plate reader (BioTek Instruments, Vermont, US), for 15 min prior to the addition of 40 μL/well of a 10 IU/mL glutathione reductase (GR) solution in phosphate buffer. The stoichiometric reaction of 5-thio-2-nitrobenzoic acid (TNB) was followed for 3 min at 415 nm and compared to a standard curve. For the determination of GSSG, 10 μL of 2-vinylpyridine were added to 200 μL aliquots of acidic
supernatant and mixed continuously for 1 h for derivatization of GSH. GSSG was then measured as described above for total glutathione. The molar GSH levels were calculated by subtracting the GSSG content from the total glutathione content (\( \text{GSH} = \text{GSH}_t - 2 \times \text{GSSG} \)).

ATP levels were evaluated through a bioluminescent technique based on an enzyme, luciferase, that catalyses the formation of light from ATP and luciferin, as described before (Capela et al., 2007). The emitted light intensity is linearly related to the ATP concentration and was measured using a 96-well Microplate Luminometer (BioTek Instruments, Vermont, US). Samples in 5% HClO\(_4\) were neutralized with 0.76 M KHCO\(_3\). After centrifugation for 2 min at 16,000g (4 °C), 100 μL of the neutralized supernatants were added to each well of a 96-well microplate containing 100 μL of luciferin-luciferase assay solution [0.15 mM luciferin, 300,000 light units of luciferase from Photinus pyralis (American firefly), 50 mM glycine, 10 mM MgSO\(_4\), 1 mM Tris, 0.55 mM EDTA, 1%BSA (pH 7.6)]. ATP calibration curves were routinely performed (ATP standard stocks in HClO\(_4\) 5% were kept at −80 °C until the assay).

2.5. Tissue preparation for optic microscopy – histological analysis

For optic microscopy, after excision, livers were sliced in 2–4 mm\(^3\) pieces, approximately, and fixed in 4% formaldehyde. The fixed pieces were dehydrated with graded ethanol and included in paraffin blocks. Benzene was used in the transition between dehydration and impregnation. Semi-thin sections (4 μm) were cut in a microtome, applied on silane-coated slides and deparaffinized. The deparaffinated sections were stained for collagen detection with Weigert’s haematoxylin, acid alcohol (for differentiation) and Van Gieson’s staining solution. This stain evidences collagen fibres with a red coloration.

2.6. Statistical analysis

Results are presented as mean ± S.E.M. (from at least five experiments of different preparations of hepatocytes). For hepatocyte yield analysis, comparisons were made by unpaired t-test with Welch’s correction. All other statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by the Bonferroni’s post-hoc test. Significance was accepted at \( P < 0.05 \).

3. Results

3.1. Hepatocytes yield and initial cell viability

During the perfusion step of the isolation procedure, the livers of EtOH pre-treated animals offered high resistance to perfusion, reducing the efficiency of this process, and resulting in significantly fewer viable cells at the end of the isolation procedure [42.7 ± 2.8 millions for the non-EtOH group versus 31.1 ± 2.4 millions for the EtOH group (\( P < 0.01 \)]. Accordingly, in the histological analysis it was observed that liver sections of EtOH pre-treated animals presented a higher deposition of collagen fibres than the control animals along the hepatic parenchyma (Fig. 1). However, there were no statistical differences between cell viabilities of the hepatocytes suspensions obtained from mice of non-EtOH (87.8 ± 1.3%) or EtOH pre-treated (86.3 ± 1.1%) groups.

3.2. Toxicity parameters

The toxicity of MDMA to freshly isolated hepatocytes obtained from non-EtOH mice and mice repeatedly exposed to 12% EtOH was evaluated, under normothermic and hyperthermic conditions, by measuring (i) the redox status of the hepatocytes suspensions by the quantification of total glutathione (GSHt), GSH and GSSG levels, (ii) the energy status by determination of cellular ATP concentrations and (iii) the cell death-rate through the quantification of LDH leakage to the suspension buffer.

3.2.1. Redox status of the hepatocytes suspensions

3.2.1.1. Effect of EtOH pre-treatment. Both GSH and GSSG levels presented variations, which were dependent on mice EtOH pre-treatment, MDMA concentration, temperature and time of incubation (Fig. 2). The initial levels of total and reduced glutathione were significantly decreased in the hepatocytes from the EtOH-treated group (Fig. 2b and d). The non-EtOH group presented initial GSHt levels of 162.3 ± 11.4 nmol/10\(^6\) cells and 137.6 ± 11.2 nmol/10\(^6\) cells of GSH while the EtOH group presented 101.1 ± 5.9 nmol/10\(^6\) cells of GSHt (\( P < 0.01 \)) and 71.0 ± 6.2 nmol/10\(^6\) cells of GSH (\( P < 0.01 \)). These results correspond to a depletion of the GSHt levels and GSH levels of approximately 40% and 50%, respectively, in the EtOH-group. In accordance, the GSSG levels in the EtOH group were significantly increased, around 25% (15.1 ± 1.0 nmol of GSSG/10\(^6\) cells for the EtOH group versus 12.4 ± 0.6 nmol of GSSG/10\(^6\) cells for the non-EtOH group). However, it is interesting to notice that the variation trends of GSH and GSSG levels during the 3 h period of hepatocytes incubation were similar between the hepatocytes obtained from EtOH and non-EtOH groups. In both groups, GSH decreased and GSSG increased, in a time-, concentration- and temperature-dependent manner, although to a different extension.

3.2.1.2. Effect of hyperthermia. GSH oxidation was clearly enhanced under hyperthermic conditions and significantly increased when mice were pre-treated with EtOH. In fact, after 3 h of incubation, the hepatocytes obtained from the non-EtOH group showed a GSH depletion more severe at 41 °C than at 37 °C (135.5 ± 48.0 nmol/10\(^6\) cells at 37 °C versus 87.6 ± 30.3 nmol/10\(^6\) cells at 41 °C, for control cells). Considering the EtOH-group, under normothermic conditions the GSH concentrations in the hepatocytes are
approximately half of the concentrations of the non-EtOH group \((55.2 \pm 21.2 \text{ nmol GSH/10}^6 \text{ cells for EtOH group versus } 135.5 \pm 48.0 \text{ nmol GSH/10}^6 \text{ cells for non-EtOH group, considering control cells at } 37 ^\circ \text{C and } t = 3 \text{ h})\). Under hyperthermia, these concentrations can reach values 6 times lower than those in the hepatocytes obtained from mice not exposed to EtOH \((19.9 \pm 7.4 \text{ nmol GSH/10}^6 \text{ cells for EtOH group versus } 87.6 \pm 30.3 \text{ nmol GSH/10}^6 \text{ cells for non-EtOH group, considering control cells at } 41 ^\circ \text{C and } t = 3 \text{ h})\) (Fig. 2).

3.2.1.3. Effect of MDMA. Hepatocytes incubation with MDMA also increases their oxidative stress status. The cells obtained from the non-EtOH group showed a GSH oxidation dependent on MDMA concentration, incubation time and temperature conditions (Fig. 2). Under normothermic conditions there was a significant depletion of GSH observed after 3 h of hepatocytes incubation with 1.6 mM MDMA [from \(137.6 \pm 11.2 \text{ nmol GSH/10}^6 \text{ cells at } t = 0 \text{ h to } 66.2 \pm 20.1 \text{ nmol GSH/10}^6 \text{ cells at } t = 2 \text{ h } (P < 0.05) \text{ and to } 20.1 \pm 19.0 \text{ nmol GSH/10}^6 \text{ cells at } t = 3 \text{ h } (P < 0.001)]\) and also observed after 3 h for hepatocytes incubation with 0.8 mM MDMA [GSH levels varied from \(137.6 \pm 11.2 \text{ nmol GSH/10}^6 \text{ cells at } t = 0 \text{ h to } 61.6 \pm 8.4 \text{ nmol GSH/10}^6 \text{ cells } (P < 0.05)\)]. In hepatocytes obtained from EtOH pre-treated mice, the GSH depletion induced by MDMA was more pronounced, especially under hyperthermic conditions \((44.4 \pm 17.1 \text{ nmol GSH/10}^6 \text{ cells at } 37 ^\circ \text{C versus } 18.3 \pm 5.8 \text{ nmol GSH/10}^6 \text{ cells at } 41 ^\circ \text{C, for } 1.6 \text{ mM MDMA at } t = 3 \text{ h})\).

Differences in GSH depletion between MDMA concentrations were not evident. However, hepatocytes obtained from the EtOH-treated group showed, for both MDMA concentrations, a higher depletion of GSH cellular levels compared to the non-EtOH group. The initial levels of GSSG were significantly higher for the EtOH-treated group \([15.1 \pm 1.0 \text{ nmol GSSG/10}^6 \text{ cells for EtOH group versus } 12.4 \pm 0.6 \text{ nmol GSSG/10}^6 \text{ cells for non-EtOH group } (P < 0.01)]\). A time-dependent GSSG increase was

![Fig. 1. Histological analysis of liver sections obtained from control (a and b) and EtOH pre-treated animals (c and d) with Van Gieson staining for collagen. The deposition of collagen fibres is evidenced by an increase of red coloured filaments.](image)
observed in both groups (51.0 ± 7.8 nmol GSSG/10⁶ cells for non-EtOH group and 1.6 mM MDMA at 37°C and \( t = 3 \) h) and were significantly potentiated by hyperthermia on the non-EtOH group (93.1 ± 8.0 nmol GSSG/10⁶ cells for the non-EtOH group and 1.6 mM MDMA at 41°C and \( t = 3 \) h).

### 3.2.2. Cellular energy status

Fig. 3 illustrates the energy status of the isolated hepatocytes under the different experimental conditions evaluated by the quantification of ATP levels. No significant differences were observed in the initial levels of ATP between non-EtOH and EtOH groups. In both groups, under normothermic conditions, ATP levels remained unchanged during all the experiment for control cells and both MDMA concentrations tested. Under hyperthermic conditions ATP levels showed a significant concentration- and time-dependent decrease. However, no differences were observed between EtOH and non-EtOH groups. In fact, for both groups, the ATP concentration, at \( t = 0 \) h, was about 45 μM decreasing, at \( t = 3 \) h, to levels nearly 20 μM for control cells, 10 μM for cells incubated with 0.8 mM MDMA and 2 μM for cells incubated with 1.6 mM MDMA.

### 3.2.3. Cell death-rate

Fig. 4 represents the death-rate of hepatocytes obtained from non-EtOH and EtOH-treated mice, incubated under the different conditions considered in this study, as evaluated by the LDH leakage. When hepatocytes were incubated at 37°C (Fig. 4a and b), LDH leakage was similar to the control for EtOH and non-EtOH groups and for both MDMA concentrations tested. The hepatocytes suspensions showed a time-dependent loss of cell viability expressed by an increase of LDH leakage, which reached significance at the 3 hour time-point (23.9 ± 0.9% of LDH leakage at \( t = 0 \) h, compared to 43.2 ± 1.4% at \( t = 3 \) h for control cells). Under hyperthermic conditions, the time-dependent loss of cell viability was aggravated and dependent on MDMA concentration. For example, LDH leakage results were 55.1 ± 3.3%, 82.3 ± 3.9% and 91.2 ± 0.4% for cells incubated with 0, 0.8 and 1.6 mM MDMA respectively, for EtOH group at \( t = 3 \) h (\( P < 0.001 \) comparing to \( t = 0 \) h and to normothermic conditions).
conditions). However, there were no significant differences between the non-EtOH and EtOH groups.

4. Discussion

Our experimental observations clearly showed that the repeated *in vivo* treatment of mice with 12% EtOH for 8 weeks compromised the hepatocyte yield after isolation. This handicap for the isolation of hepatocytes from ethanol-treated animals was not reported before, although this lower hepatocyte yield can be explained by the occurrence of collagen deposition during chronic EtOH consumption (Seitz and Stickel, 2006) as confirmed by the histological data presented in Fig. 1, which can affect the hepatic perfusion of the collagenase solution and, therefore, the hepatocytes isolation. In spite of the well known LDH releasing effects of ethanol (Henzel et al., 2004), the present study, the pre-treatment of mice with EtOH did not result in statistical differences between the initial viability of hepatocytes, obtained from EtOH and non-EtOH groups, which is likely due to a selective isolation of EtOH tolerant hepatocytes since, during the washing steps of the isolation procedure, damaged cells were removed.

The toxic effects of EtOH pre-treatment were also evidenced by the GSH content at the beginning of the experiment (137.6 ± 11.2 nmol GSH/10⁶ cells for non-EtOH group versus 71.0 ± 6.2 nmol GSH/10⁶ cells for EtOH group) and during the hepatocytes incubation period as expressed by a severe depletion of glutathione levels, which were further depleted upon MDMA exposure. In the non-EtOH group, the cells incubated with MDMA under hyperthermic conditions presented a higher depletion of GSH levels and a higher increase of GSSG levels than the correspondent control cells (neither exposed to ethanol nor to MDMA). GSH depletion after MDMA exposure was previously reported in isolated rat (Carvalho et al., 2004b) and mouse (Carvalho et al., 2001) hepatocytes, as it happened in the present study. However, the GSH depletion induced by MDMA and/or hyperthermia was not accompanied by a proportional increase in GSSG levels, which means that this GSH decrease was due, not only to glutathione oxidation, but mainly to the formation of glutathione conjugates with MDMA reactive metabolites.
(Carvalho et al., 2002b; Carvalho et al., 2004a). Since GSH is a crucial endogenous antioxidant in cell protection (Han et al., 2006), its depletion may increase the cell susceptibility to the deleterious effects of reactive compounds, ROS and RNS, formed within the cells during the metabolism of MDMA and EtOH. However, the GSH depletion elicited by EtOH pre-treatment (Fig. 2) did not aggravate the MDMA-induced cell death as demonstrated by the similar LDH leakage in both groups (Fig. 4). A possible explanation is the decreased formation of toxic GSH conjugates with the catechol metabolites of MDMA, since less GSH is available for conjugation. As already confirmed by our group, these conjugates are more toxic than their parent compounds for primary cultures of rat and human renal proximal tubular cells (Carvalho et al., 2002b) and for rat cortical neuronal serum-free cultures (Capela et al., 2006).

The GSH depletion that results from the EtOH pre-treatment is in accordance with the results previously obtained by other groups, which evidenced that long-term incubation of isolated rat hepatocytes (Cobreros et al., 1997) and primary cultures of human and rat hepatocytes (Castilla et al., 2004; Yang et al., 2005) with high concentrations of EtOH (≥100 mM) increased oxidative stress markers such as lipid peroxidation and GSH depletion. Other studies demonstrated that either acute or chronic EtOH administration to experimental animals decreased tissue levels of antioxidants, including GSH, in the liver (Nordmann et al., 1992), suggesting that the impairment of cellular antioxidant defences along with the formation of ROS plays a role in causing oxidative damage associated with EtOH hepatotoxicity (Yang et al., 2005). On the other hand, recent reports indicate that low EtOH concentrations (<5 mM) were able to reduce the basal level of intracellular oxidative stress markers in primary cultures of human and rat hepatocytes (Castilla et al., 2004) while, Deaciuc and colleagues described that GSH and GSSG contents in hepatocytes were not affected when rats were fed during 7 weeks with an alcohol-containing liquid diet (Deaciuc et al., 1999).
Another toxic event observed in cells exposed to MDMA and EtOH was a concentration- and time-dependent depletion of cellular ATP levels that was significant under hyperthermic conditions. This depletion confirmed the results already described for MDMA alone (Carvalho et al., 2004b) and denotes an inhibition of mitochondrial function under these experimental conditions (Burrows et al., 2000; Wu and Cederbaum, 2003; Quinton and Yamamoto, 2006). This effect may result from altered thiol homeostasis, and toxic metabolites formation from MDMA and/or EtOH that can affect mitochondrial energy processes by damaging mitochondrial membranes and/or proteins (Seitz and Stickel, 2006). Additionally, the mitochondrial dysfunction underlying the observed ATP depletion could be directly instigated by hyperthermic conditions, as already described for tumoral cell lines and cardiomyocytes (Yuen et al., 2000; Qian et al., 2004; Zhao et al., 2006).

Concluding, the results from the present study evidenced, for the first time, that mice pre-treatment with 12% EtOH during 8 weeks increases the vulnerability of freshly isolated mouse hepatocytes towards pro-oxidant conditions, as ascertained by the lower hepatocytes yield and decreased GSH levels and increased GSSG levels. This depletion of GSH content in the isolated hepatocytes alters their susceptibility to one of the early consequences of MDMA metabolism (disruption of thiol homeostasis), which may result in loss of protein function and the initiation of a cascade of events leading to cellular damage. However, MDMA toxicity to the isolated hepatocytes, evaluated by LDH leakage, was independent of EtOH pre-treatment, while significantly dependent on incubation temperature. Thus, the in vitro mimicking of the hyperthermia induced in vivo by MDMA consumption was of extreme importance since it significantly enhanced the detrimental effects induced by MDMA at 37 °C for both non-EtOH and EtOH groups.

Notwithstanding the obtained results, caution should be exercised in comparing in vitro and in vivo situations, because there are some in vivo factors that could not be reproduced in this experimental model, such as uptake and metabolism of different cell types, blood flow variations, hormones and liver zonal differences in oxygen tension and enzymatic distribution.

The relevance of this study is unequivocal because the simultaneous consumption of MDMA and EtOH has been associated with MDMA-related fatalities (Schifano et al., 2003) and road accidents (Drummer et al., 2003; Cheng et al., 2005; Kuypers et al., 2006). To our knowledge this is the first report on the hepatic toxicological interaction of MDMA and EtOH, whose hepatic metabolism to toxic derivatives is already extensively described. Previous studies on the association of these drugs have mainly focused on physiologic or psychologic consequences to humans (Pacifici et al., 2001; Hernández-López et al., 2002; Ramakers and Kuypers, 2006).

Thus, the possible relationship of such findings requires further in vivo testing to clarify the putative effects exerted by the combined use of MDMA and EtOH.

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