Synergistic toxicity of ethanol and MDMA towards primary cultured rat hepatocytes

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1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA; ecstasy) consumption patterns have been widely described in literature (EMCDDA, 2005; Lora-Tamayo et al., 2004; SAMHSA, 2005; Schifano et al., 2006; Topp et al., 1999; Tossmann et al., 2001; Winstock et al., 2001) and are generally characterized as a polydrug consumption scenario (Gouzoulis-Mayfrank and Daumann, 2006; Hopper et al., 2006; Schifano et al., 1998, 2003), often related with fatalities (Barrett et al., 2006; Liechti et al., 2005; Oyefeso et al., 2006; Sanjurjo et al., 2004; Schifano, 2004; Schifano et al., 2003, 2006). One of the most commonly consumed drugs, along with ecstasy, is ethanol (EtOH) (Breen et al., 2006; Smart and Ogborne, 2000; Tossmann et al., 2001), probably due to the fact that EtOH is legal, cheap and widely available. In fact, EtOH is often taken prior or along with MDMA, to increase the acute pleasant effects of MDMA and to decrease the irritability and restlessness that persist after the end of the empathogenic and entactogenic effects associated with ecstasy consumption (Schifano, 2004). Of note, when EtOH is consumed to decrease the long-lasting unpleasant effects of MDMA, it is taken in large amounts (Schifano, 2004) probably because ecstasy users were more likely to engage in several risky behaviours such as binge drinking (Boyd et al., 2003).

Liver toxicity resulting from the concomitant consumption of these two drugs is of higher concern since both compounds are metabolized in the liver leading to the formation of hepatotoxic compounds (Gemma et al., 2006; Green et al., 2003). Accordingly, our group has recently demonstrated that hepatocytes isolated from mice exposed to 12% EtOH for 8 weeks, as drinking fluid, showed increased vulnerability to MDMA-induced hepatotoxicity (Pontes et al., 2008b). EtOH pre-exposure has also been shown...
to modify MDMA pharmacokinetics, which results in increased MDMA plasma concentrations in humans (Hernández-López et al., 2002). Another important feature of MDMA-induced toxicity is hyperthermia, an effect known to be potentiated after continuous exposure to ethanol (Pontes et al., 2008a).

Considering the potential synergic effects of EtOH and MDMA to the liver, the aim of the present study was to evaluate the putative hepatotoxic interaction between EtOH and MDMA in primary cultured rat hepatocytes and to elucidate the mechanism(s) underling the observed effects. For this purpose, the toxicity induced by MDMA to primary cultured rat hepatocytes in absence or in presence of EtOH was evaluated, under normothermic (36.5 °C) and hyperthermic (40.5 °C) conditions, to simulate the hyperthermic response induced in vivo by MDMA consumption. The study included the evaluation of the type of cell death [acidic orange and ethidium bromide (AO/EB) staining, lactate dehydrogenase (LDH) leakage, caspase-3 activation, Bax translocation to the mitochondria and AIF and cytochrome c release from the mitochondria], oxidative stress parameters [reactive oxygen and nitrogen species (ROS and RNS) formation, reduced (GSH) and oxidised glutathione (GSSG) contents, γ-glutamylcysteine synthetase (γ-GCS) and GST activities] as well as cell energy status [adenosine triphosphate (ATP) levels].

2. Materials and methods

2.1. Chemicals

All chemicals 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-aminoethyether)-N,N,N,N-tetracetic acid (EGTA), β-nicotinamide adenine dinucleotide reduced form (β-NADH), β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), pyruvic acid, 2-vinypyrindine, reduced glutathione (GSH), oxidised glutathione (GSSG), trypan blue solution, trizma® hydrochloride, NADPH), pyruvic acid, 2-vinylpyridine, reduced glutathione (GSH), oxidised glutathione (GSSG), trizma® hydrochloride, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), ethyleneglycol-bis-(β-aminoethyether)-N,N,N,N-tetracetic acid (EGTA), β-nicotinamide adenine dinucleotide reduced form (β-NADH), β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), pyruvic acid, 2-aminopyridine, 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, l-nutricin sodium salt and luciferase (EC 131.12.7), antibioptic-antimycotic solution, insulin solution, dexamethasone, acidic orange, ethidium bromide, dihydrodorhadone 123, mono(cyclohexylammonium)phoshonopolyruvate, l-α-aminono-butyrate, l-glutamate, pyruvate kinase from rabbit muscle and l-tactic dehydrogenase from rabbit muscle, adenosine-5-triphosphate (ATP), N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Perchloric acid, EtOH and all other chemicals were purchased from Merck (Darmstadt, Germany). William’s E medium, l-glutamine, foetal bovine serum (FBS) and phosphate buffer solution (PBS) were purchased from Lonza Ltd. (Basel, Switzerland). Collagen G was purchased from Biochrom AG (Berlin, Germany). 1,4-Methylenedioxymethylpheniamine (HCI 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 methods.

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, with some modifications (Pontes et al., 2008b). Sample ATP levels are proportional to the intensity of the light emitted by luciferine, in a reaction catalyzed by luciferase, that was measured using a 96-well Microplate Luminometer (BioTek Instruments, Winooski, VT, USA) [baseline: 485 nm excitation and 528 nm emission]. Results are presented as percentage of control conditions at 36.5 °C.

2.3. Hepatocytes isolation and primary culture

Hepatocytes isolation was performed by collagenase perfusion as previously described (Pontes et al., 2008b), with some modifications. Briefly, after perfusion with a chelation agent to allow the cleavage of the hepatic desmosomes, hepatic collagen was hydrolyzed by ex situ perfusion with a collagenase solution supplemented with its co-factor (calcium) and hepatocytes were dissociated in Krebs–Henseleit buffer.

The obtained hepatocyte suspension was purified by low-speed centrifugations and incubated for 30 min, at 4 °C, with 500 U/mL penicillin G, 0.5 mg/mL streptomycin sulfate and 1.25 μg/mL amphotericin B. The viability of isolated hepatocytes was estimated by the trypan blue exclusion test. Subsequently, a suspension of 0.5 mg/mL viable cells/mL in complete culture medium (William’s E medium supplemented with 2 mM l-glutamine, 0.86 μM insulin, 0.5 mM dexamethasone, 10 mM HEPES, 100 U/mL penicillin G, 0.1 mg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, 5% FBS) was seeded in previously collagen-coated culture flasks in a density of 6 × 10⁴ viable cells/cm². Cells were incubated at 36.5 °C with 5% CO₂, 3–4 h for cell adhesion, washed twice with PBS, and inoculated over-night at 36.5 °C with culture medium without FBS. The day after, cells were added five different concentrations of MDMA (0, 0.2, 0.4, 0.8 and 1.6 mM) in the presence or in the absence of 1.4% (m/v) EtOH, and incubated with 5% CO₂ at 36.5 or 40.5°C. The tested MDMA concentrations were chosen on the basis of previous studies from our group (Carvalho et al., 2003, 2004b) and also in previous reports that found tissue concentrations of MDMA and its metabolites substantially higher (up to 18 times) than blood concentrations (García-Repetto et al., 2003). Considering that in most of MDMA fatalities, blood levels ranged 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, the evaluation of the interactions of MDMA and its metabolites with cellular components is only possible using worst-case approach concentrations. After 24 h incubation, AO/EB staining was performed, and samples were collected to perform Western blotting analysis and to quantify LDH leakage, ROS and RNS formation, ATP levels, GSH and GSSG concentrations and γ-GCS, GST and caspase-3 activities.

2.4. Acidine orange and ethidium bromide staining

The fluorescent DNA-binding dyes ethidium bromide and acidic orange were used to distinguish necrotic from apoptotic cells. After the 24-h incubation period with MDMA, EtOH or MDMA + EtOH, cells were incubated with 2 μg/mL acidic orange and 2 μg/mL ethidium bromide for 5 min before imaging, using a fluorescence microscope with a standard fluorescein excitation filter (Eclipse E400 Nikon, Japan).

2.5. Biochemical analysis

2.5.1. LDH Leakage

LDH leakage to the culture media was evaluated as previously reported (Bergermeyer and Bernt, 1974). The LDH activity was determined by following the rate of oxidation of NADH, measured at 340 nm. Results are presented as percentage of control conditions at 36.5 °C.

2.5.2. ROS and RNS quantification

Detection and quantification of intracellular reactive species, including RNS and ROS were performed by the dihydrorhodamine 123 (DHR-123) assay as previously reported (Carvalho et al., 2003). Briefly, cells were pre-incubated with 100 μM DHR-123 for 60 min. At the end of the incubation period, cells were washed 2 times with PBS and added with new medium containing the tested compounds. The oxidation of DHR-123 to its fluorescent product, Rhodamine 123, was measured 24 h after incubation with EtOH, MDMA, or MDMA + EtOH, using a fluorescence plate reader (BioTek Instruments, Winooski, VT, USA) [ baseline: 485 nm excitation and 528 nm emission]. Results are presented as percentage of control conditions at 36.5 °C.

2.5.3. GSH/GSSG quantification

The cellular GSH and GSSG levels were determined, after cell suspension in PBS, by the DTNB–GSSG reduction assay as described before (Anderson, 1985), with some modifications (Pontes et al., 2008b). The stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB) was followed for 3 min at 415 nm and compared to a standard curve. The molar GSH levels were calculated by subtracting the GSSG content, determined after derivatization of GSH with 2-aminopyridine, from the total glutathione content (GSH + GSSG). Results are presented as percentage of control conditions at 36.5 °C.

2.5.4. ATP quantification

Cellular ATP levels were evaluated through a luciferase-based bioluminescent technique, as previously reported (Pontes et al., 2008b). Sample ATP levels are proportional to the intensity of the light emitted by luciferine, in a reaction catalyzed by luciferase, that was measured using a 96-well Microplate Luminometer (BioTek Instruments, Vermont, US). Results are presented as percentage of control conditions at 36.5 °C.

2.5.5. γ-GCS activity

γ-Glutamylcysteine synthetase (γ-GCS), also known as glutamate cysteine ligase (GCL), is the first enzyme in the glutathione biosynthesis pathway, and its rate-limiting step. The activity of γ-GCS was evaluated as previously described by Seelig.
et al. (1984) with some modifications (Piner et al., 2007). γ-GCS activity was measured from the rate of ADP formation (assumed to be equal to the rate of oxidation of NADH) as calculated from the change in absorbance at 340 nm in reaction mixtures at 37 °C. Reaction medium included 0.1 M pH 7.75 Tris buffer, 150 mM KCl, 2 mM phosphoenolpyruvate, 10 mM d-glutamate, 20 mM MgCl₂, 10 mM l-α-amino-n-butyrate, 2 mM EDTA, 0.2 mM NADH, 5 mM ATP, 2 U/mL pyruvate kinase, and 2 U/mL lactate dehydrogenase. Results are presented as percentage of control conditions at 36.5 °C.

2.5.6. GST activity

Glutathione-S-transferase (GST) activity was assayed according to the method of Habig et al. (1974). The formation of GST conjugate with 1-chloro-2,4-dinitrobenzene was monitored for 5 min at 340 nm. The GST activity was calculated using an extinction coefficient of 9.6 mM−1 cm−1 and expressed as pmol/min per mg of protein. Results are presented as percentage of control conditions at 36.5 °C.

2.5.7. Caspase-3 activation

Caspase-3 assay was based on a colorimetric assay by following the hydrolysis of the peptide substrate Ac-DEVD-pNA by caspase 3, which results in the release of 2.5.6. GST activity were presented as percentage of controls.

Control. The membrane was then washed and developed with Western blotting macia Biotech) during 1 h. Peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Amersham Phars- during 2 h at room temperature, washed and incubated with secondary horseradish anti-cytochrome c (1:2000; sc-99 mouse monoclonal IgG1; Santa Cruz Biotechnol- or anti-α-tubulin (1:1000; T6074 mouse monoclonal IgG1; Sigma) antibodies during 2 h at room temperature, washed and incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Amersham Pharmacia Biotech) during 1 h. α-Tubulin Western blot is included as a loading protein control. The membrane was then washed and developed with Western blotting chemiluminescence reagents (Amersham Pharmacia Biotech) according to manufacturer’s instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, USA). Optical density was measured and results of all groups were presented as percentage of controls.

2.7. Statistical analysis

Results are presented as means ± SEM (from at least five experiments of different preparations of hepatocytes). 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. Acridine orange and ethidium bromide staining

The ethidium homodimer cannot penetrate intact cellular mem- branes and, therefore, stains the nucleus of cells in red only when the membranes are disrupted, whereas acridine orange is membrane-permeable and stains living cells in green. Therefore, under the ethidium bromide/acridine orange staining (Fig. 1), liv- ing cells appear as cells with a regular-sized green fluorescent nucleus, whereas early apoptotic cells have a green fluorescent condensed, shrunken, or fragmented nucleus, and late apoptotic cells have a red fluorescent condensed, shrunken, or fragmented nucleus. Necrotic cells exhibit a red fluorescent regular-sized or increased nucleus. The pictures in Fig. 1 suggest that MDMA, by itself, did not produce evident changes in cell viability, while EtOH exposure was able to induce some degree of necrotic cell death, especially notorious at hyperthermic conditions. This EtOH-induced necrotic cell death was aggravated by the co-exposure with MDMA under hyperthermic conditions. It was also observed a decrease in cell density due to high rate of cell death, leading to cell detachment.

3.2. LDH leakage

Cells exposed under normothermic conditions to several MDMA concentrations suffered a slight loss of viability at the highest concentration tested [down to 90.5 ± 3.8% of control (P < 0.01 at 1.6 mM MDMA)] (Fig. 2). Loss of cell viability was significantly aggravated under hyperthermia [down to 77.1 ± 4.2% of control (P < 0.001) at 1.6 mM MDMA]. EtOH, by itself, did not have a significant effect on LDH leakage but potentiated MDMA-induced loss of cell viability, which was observed for MDMA concentrations as low as 0.4 and 0.1 mM, at normothermia and hyperthermia, respectively. The extension of this synergic effect was, therefore, dependent on MDMA concentration and on incubation temperature [at 36.5 °C, EtOH + 1.6 mM MDMA decreased cell viability down to 71.3 ± 5.8% of control (P < 0.05), while at 40.5 °C this co-exposure resulted in a cell viability down to 11.1 ± 1.8% of control (P < 0.001)].

3.3. ROS and RNS formation

When cells were incubated under normothermic conditions, none of the treatments resulted in detectable modification in the formation of reactive species (Fig. 3). However, after a 24-h incubation period at 40.5 °C, EtOH increased ROS and RNS formation [to 128.7 ± 8.2% of control (P < 0.01)] and this increase was signifi- cantly potentiated by simultaneous exposure to EtOH and MDMA [to 192.3 ± 17.2% of control (P < 0.001)].

3.4. GSH/GSSG quantification

The changes in GSH/GSSG levels are presented in Fig. 4. Under normothermic conditions, MDMA exposure caused a sig- nificant decrease of intracellular total glutathione (tGSH) and reduced glutathione (redGSH) at the highest concentration [the redGSH levels decreased from 94.2 ± 0.5% in control cells to 82.3 ± 10.1% in cells exposed to 1.6 mM MDMA (P < 0.05) and tGSH decreased to 92.8 ± 11.2%]. This decrease was also verified when cells were co-exposed to EtOH [redGSH decreased from 97.9 ± 8.4% in cells exposed only to EtOH to 51.2 ± 7.4% in cells exposed to EtOH + 1.6 mM MDMA (P < 0.05) and tGSH levels decreased from 103.0 ± 8.9% in cells exposed only to EtOH to 62.7 ± 9.0% in cells exposed to EtOH + 1.6 mM MDMA (P < 0.05)]. This decrease was only aggravated by hyperthermia in cells simultaneously exposed to MDMA and EtOH [redGSH decreased from 84.7 ± 10.2% when cells were exposed only to EtOH at 40.5 °C to 1.78 ± 0.92% when cells were exposed to EtOH + 1.6 mM MDMA (P < 0.001)]. This decrease in redGSH was accompanied by a tGSH decrease [from 93.4 ± 11.0% to 5.49 ± 0.95% (P < 0.01)]. As expected, the intracellular levels of GSSG did not present changes among the considered experimental conditions since, once formed, it is excreted to the extracellular medium (Leier et al., 1996).

3.5. ATP quantification

At 36.5 °C, when cells were incubated with four different MDMA concentrations, ATP levels remained unchanged compara- tively to control cells (Fig. 5). However, co-incubation with EtOH induced a significant decrease in ATP levels whose extension was dependent on MDMA concentration [ATP levels decreased from 87.4 ± 4.1% when cells were exposed only to EtOH, to 62.3 ± 4.3% when cells were exposed to EtOH + 1.6 mM MDMA (P < 0.01)]. Under hyperthermia (40.5 °C), MDMA itself was able to significantly decrease ATP levels at the highest concentration.
Fig. 1. Fluorescence microscope photographs from ethidium bromide/acridine orange staining of primary rat hepatocytes cultures exposed for 24 h to 1.4% (m/v) EtOH, 1.6 mM MDMA or both compounds simultaneously. The upper row corresponds to cells incubated under normothermia (36.5°C) and the lower row corresponds to cells incubated under hyperthermia (40.5°C). The columns correspond, from left to right, to control cells, and cells incubated with EtOH, MDMA and EtOH + MDMA, respectively. For each condition, the left image corresponds to a 200 times magnification, and the right image corresponds to a 400 times magnification.

Fig. 2. Effect of MDMA and hyperthermia on viability of primary cultured rat hepatocytes in presence or in absence of EtOH (W/EtOH and W/O EtOH, respectively). Results are represented as mean ± SEM of the percentage of control cells (exposed neither to MDMA nor to EtOH at 36.5°C) from at least five experiments. Control cells presented 88.1 ± 2.3% of cell viability. (*) Compared to control cells at 36.5°C; (#) compared to 0 mM MDMA of the respective curve; (×) compared to normothermia; (+) compared to W/O EtOH.

Fig. 3. Effect of MDMA and hyperthermia on reactive species production (ROS and RNS) in primary cultured rat hepatocytes in presence or in absence of EtOH (W/EtOH and W/O EtOH, respectively). Results are represented as mean ± SEM of the percentage of control cells (exposed neither to MDMA nor to EtOH at 36.5°C) from at least five experiments. (*) Compared to control cells at 36.5°C; (#) compared to 0 mM MDMA of the respective curve; (×) compared to normothermia; (+) compared to W/O EtOH.
Fig. 4. Effect of MDMA and hyperthermia on tGSH, GSH and GSSG levels in primary cultured rat hepatocytes in presence or in absence of EtOH (W/EtOH and W/O EtOH, respectively). Results are represented as mean ± SEM of the percentage of control cells (exposed neither to MDMA nor to EtOH at 36.5 °C) from at least five experiments. Control cells presented 63.5 ± 5.4 nmol of tGSH/mg protein, 59.9 ± 5.3 nmol of redGSH/mg protein and 1.80 ± 0.16 nmol of GSSG/mg protein. (*) Compared to control cells at 36.5 °C; (#) compared to 0 mM MDMA of the respective curve; (×) compared to normothermia; (+) compared to W/O EtOH.

Fig. 5. Effect of MDMA and hyperthermia on ATP levels in primary cultured rat hepatocytes in presence or in absence of EtOH (W/EtOH and W/O EtOH, respectively). Results are represented as mean ± SEM of the percentage of control cells (exposed neither to MDMA nor to EtOH) from at least five experiments. Control cells presented 16.5 ± 2.0 nmol of ATP/mg protein. (*) Compared to control cells at 36.5 °C; (#) compared to 0 mM MDMA of the respective curve; (×) compared to normothermia; (+) compared to W/O EtOH.

[From 108.7 ± 9.6% in control cells to 76.0 ± 6.3% when cells were exposed to 1.6 mM MDMA (P < 0.05)] being this decrease drastically potentiated by EtOH co-exposure [ATP levels decreased to 0.25 ± 0.16% when cells were co-exposed to EtOH and 1.6 mM MDMA (P < 0.001)].

3.6. γ-GCS activity

As it can be observed in Fig. 6, MDMA and EtOH, by themselves, did not change γ-GCS activities, neither at 36.5 °C nor 40.5 °C, at any of the tested concentrations. The co-incubation resulted in a slight
tendency for a decrease of γ-GCS activity (down to 70.7 ± 6.3%), reaching statistical significance under hyperthermic conditions, in which γ-GCS activities practically disappeared at the two highest MDMA concentrations tested (P < 0.001).

3.7. GST activity

The effects of ethanol, MDMA, as well as their interaction under normothermia and hyperthermia on GST activity are presented in Fig. 7. In the absence of ethanol, GST activity remained unchanged, independently on MDMA concentration and on incubation temperature. When cells were exposed to ethanol, a significant decrease in GST activity was observed (down to 70.7 ± 6.2%) when compared to control (P < 0.05). Under hyperthermic conditions, co-exposure to ethanol and MDMA was able to aggravate the decrease induced by ethanol, in an extent dependent on MDMA concentration [GST activity decreased to 62.7 ± 13.0% when cells were exposed to ethanol + 0.4 mM MDMA (P < 0.05), to 44.4 ± 15.6% when cells were exposed to ethanol + 0.8 mM MDMA (P < 0.001) and to 3.88 ± 1.95% when cells were exposed to ethanol + 1.6 mM MDMA (P < 0.001)].

3.8. Western blot analysis

At 36.5 °C, the release of mitochondrial components (AIF and cytochrome c) seems to be inexistent. However, at 40.5 °C, a significant release of AIF and cytochrome c from the mitochondrial extract was observed, under MDMA EtOH co-exposure (Fig. 8).

There was no translocation of Bax molecule from the cytoplasm to the mitochondria (data not shown).

3.9. Caspase-3 activity

There were no statistical differences in caspase-3 activities among the different incubation conditions (data not shown).

4. Discussion

The data obtained in this study clearly demonstrated that the simultaneous incubation of primary cultured rat hepatocytes with MDMA and EtOH for 24 h, leads to high cell death ratios, mainly through necrosis, accompanied by a significant disturbance of cellular redox status and decreased energy levels.
Both EtOH and MDMA are metabolized in the liver, where EtOH can alter the expression or activity of some drug-metabolizing enzymes (Jang and Harris, 2007) and where both drugs share some metabolic pathways involving the cytochrome P450 isoenzymes and originating metabolites (Gemma et al., 2006; Green et al., 2003) with higher toxicity than their parent compounds, as previously reported for other amphetamines (Carmo et al., 2007). Notwithstanding the knowledge about the decrease of the activities of cytochrome P450 system isoenzymes during the culture of rat hepatocytes, the present experimental model is adequately adapted to the study of the interactions between ethanol and MDMA since the remaining CYP enzyme activities are still sufficient to study drug metabolism in vitro during the 24-h incubation period used in the present study (Gebhardt et al., 2003; Hewitt et al., 2007). While this metabolic interaction may represent an explanation for most of the synergic toxic effects found, other factors are also involved and the hyperthermic effect contributes significantly to the aggravation of the observed toxicity. The MDMA-induced decrease on GSH levels is in agreement with results previously reported by our group on freshly isolated mouse hepatocytes (Pontes et al., 2008b) and can be explained by oxidation of GSH to GSSG by reactive oxygen and nitrogen species, and formation of GSH adducts with MDMA metabolites by GST (Hiramatsu et al., 1990), but also by impaired GSH synthesis, which is also a known effect associated with EtOH exposure (Lauterburg et al., 1984). In the present study, we demonstrated, for the first time, that the impairment of GSH synthesis mediated by ethanol was dramatically aggravated by MDMA co-exposure under hyperthermic conditions, with an almost absolute inhibition of γ-GCS activity for the two highest MDMA concentrations. The decrease on GST activity can be related to the oxidation or alkylation of sulfhydryl groups present in critical aminoacid residues within the enzyme active site, an effect already reported for MDMA metabolites (Carvalho et al., 2004a) and for quinones and catecholamines (Monks and Lau, 1992; Bindoli et al., 1992; van Ommen et al., 1991), formed after exposure to MDMA. A significant decrease in GST activity was also previously reported for ethanol (Gyamfi and Wan, 2006). GST catalyzes the nucleophilic attack of GSH on reactive electrophilic substrates, thereby protecting various cell components from their potentially damaging effects (Mannervik and Danielson, 1988). Thus, its higher inactivation by simultaneous exposure to ethanol and MDMA can increase cell susceptibility to the deleterious effects of reactive electrophiles. These impairments in glutathione homeostasis induced by ethanol and MDMA mixtures can contribute to their synergistic toxicity.

It was previously reported that either EtOH or MDMA is able to increase ROS and RNS formation, in a variety of systems, cells, and species (Das and Vasudevan, 2007; Quinton and Yamamoto, 2006). The increased formation of reactive species after incubation with ethanol may be derived from the induction of cytochrome P450 2E1 activity (Morimoto et al., 1993) or by mitochondrial dysfunction after reduction of GSH content and ATP generation (Cunningham et al., 1990; Colell et al., 1998). MDMA can lead to the formation of...
ROS and RNS during the redox cycles associated with its metabolic conversion (Colado et al., 1997) or during the conjugation of MDMA metabolites with GSH molecules, a pathway also accompanied by the formation of reactive species (Hiramatsu et al., 1990; Monks et al., 2004). However, a synergism in the ROS and RNS formation, when cells were co-exposed to both compounds, was also never reported before.

Concerning ATP levels, MDMA was previously reported to decrease ATP in rat hepatocytes (Carvalho et al., 2004b; Beitia et al., 1999) and EtOH leads to decreased ATP levels and ATP synthesis in rats (Helzberg et al., 1987; Piquet et al., 2004). The ATP depletion caused by ethanol is probably due to mitochondrial damage induced by an overproduction of anion superoxide (O$_2^•$−) at mitochondrial complexes I and III, and consequently of H$_2$O$_2$ and other reactive oxygen species (ROS). This effect results from the excessive NADH formation during acetaldehyde metabolism in liver mitochondria that is used by mitochondria complex I (NADH dehydrogenase) as the first electron donor of the electron transport chain (Sastré et al., 2007). The potentiation of this depletion by the co-incubation with EtOH and MDMA is in accordance with our previous ex-vivo study (Pontes et al., 2008b). This depletion is an indicator of probable mitochondrial damage confirmed by the decrease of cytochrome c and AIF in the mitochondrial extract.

The decrease of GSH and ATP levels was accompanied by a loss in cell viability. Evaluation of apoptotic/necrotic features provided clear evidences that the cell death resulting from the co-incubation with EtOH and MDMA occurs through a necrotic pathway, confirmed by a significant increase of LDH leakage, nuclei staining by the ethidium bromide and acridine orange technique and AIF release that was reported to be on the basis of a necrotic programmed cell death pathway, explored by the groups of Boujrad et al. (2007) and Lorenzo and Susin (2007). Hepatic necrosis was also previously reported after in vitro exposure of mouse hepatocytes (Pontes et al., 2008b), and in vivo exposure of mice (Johnson et al., 2002) and humans to MDMA (Milroy et al., 1996) or after the exposure of human and rat hepatocytes to high EtOH concentrations (Castilla et al., 2004). In this particular case of association between MDMA and EtOH in hepatocytes the classic apoptotic via, already described for MDMA in neural cells (Capela et al., 2007, Alvaro et al., 2008) and hepatic stellate cells (Montiel-Duarte et al., 2004), is discarded since there is no translocation of BAX to mitochondria and no activation of effectors apoptotic pathways such as caspase 3, notwithstanding the cytochrome c release from the mitochondria (Fadeel et al., 2008). Therefore, the type of cell death subsequent to MDMA incubation is probably affected by EtOH co-exposure. Note-worthy, hepatocytes exposed to 1 mM MDMA concentrations were previously reported to suffer an apoptotic type of cell death and not a necrotic cell death as reported in the present study (Montiel-Duarte et al., 2002). This apparent discrepancy can be explained by the ATP requirements of apoptosis, especially in the steps mediated by protein kinases that use ATP as a phosphoryl donor for phosphorylation of some proteins involved in apoptosis (Skulachev, 2006). Since in the present study, a dramatic decrease in ATP levels was observed, these apoptotic pathways are probably impaired.

All the evaluated parameters were significantly aggravated in the hepatocytes cultured under hyperthermia, which is known to be an in vivo life-threatening effect associated with ecstasy consumption (Green et al., 2004) and exacerbates the toxicity of many types of drugs and environmental toxicants (Gordon, 2007), including MDMA (Carvalho et al., 2001). The increase of EtOH and MDMA toxicity by hyperthermia is particularly notorious in the present study by the dramatic decrease in cell viability, GSH content, γ-GCS activity and ATP.

In conclusion, EtOH associated with MDMA definitely enhances the MDMA-related toxicity towards hepatocytes through a decrease in antioxidant defences, an increase in reactive species formation, and enhanced cell death through a necrotic pathway in a temperature-dependent extent.

These findings represent an important step in the elucidation of the mechanisms underlying the EtOH-induced increase on the toxicity to hepatocytes caused by MDMA consumption.

Conflict of interest

None.

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