



## Chronic exposure to ethanol exacerbates MDMA-induced hyperthermia and exposes liver to severe MDMA-induced toxicity in CD1 mice

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### ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA; ecstasy) is an amphetamine derivative drug with entactogenic, empathogenic and hallucinogenic properties, commonly consumed at rave parties in a polydrug abuse pattern, especially with cannabis, tobacco and ethanol. Since both MDMA and ethanol may cause deleterious effects to the liver, the evaluation of their putative hepatotoxic interaction is of great interest, especially considering that most of the MDMA users are regular ethanol consumers.

Thus, the aim of the present study was to evaluate, *in vivo*, the acute hepatotoxic effects of MDMA (10 mg/kg i.p.) in CD-1 mice previously exposed to 12% ethanol as drinking fluid (for 8 weeks). Body temperature was continuously measured for 12 h after MDMA administration and, after 24 h, hepatic damage was evaluated.

The administration of MDMA to non pre-treated mice resulted in sustained hyperthermia, which was significantly increased in ethanol pre-exposed mice. A correspondent higher increase of hepatic heat shock transcription factor (HSF-1) activation was also observed in the latter group. Furthermore, MDMA administration resulted in liver damage as confirmed by histological analysis, slight decrease in liver weight and increased plasma transaminases levels. These hepatotoxic effects were also exacerbated when mice were pre-treated with ethanol. The activities of some antioxidant enzymes (such as SOD, GPx and Catalase) were modified by ethanol, MDMA and their joint action. The hepatotoxicity resulting from the simultaneous exposure to MDMA and ethanol was associated with a higher activation of NF-κB, indicating a pro-inflammatory effect in this organ.

In conclusion, the obtained results strongly suggest that the consumption of ethanol increases the hyperthermic and hepatotoxic effects associated with MDMA abuse.

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### 1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA; ecstasy) is an amphetamine derivative drug with entactogenic, empathogenic and hallucinogenic properties, commonly consumed at rave parties in a polydrug abuse pattern, especially with cannabis, amphetamine, cocaine, tobacco and ethanol (Green et al., 2003; Schifano, 2004; Breen et al., 2006; Barrett et al., 2006). The common concomitant use of ethanol and MDMA has a great interaction potential since both compounds are metabolized in the liver to hepatotoxic metabolites (Gemma et al., 2006; Green et al., 2003; Carmo et al., 2006) with higher toxicity than their parent compounds

**Abbreviations:** MDMA, 3,4-methylenedioxymethamphetamine, ecstasy; MDA, methylenedioxyamphetamine; ROW, relative organ weight; %DLWC, percent dry lipid weight content; HSF-1, heat shock factor 1; NF-κB, nuclear factor kappa-B; GOT, glutamic oxalic transaminase; GPT, glutamic pyruvic transaminase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; GSH, glutathione (reduced form); GSSG, glutathione (oxidised form); TBARS, thiobarbituric acid reactive substances; IHC, immunohistochemistry.

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(Carvalho et al., 2004; Meier and Seitz, 2008). This metabolic activation observed with MDMA seems to be a common feature of amphetamine derivatives, since it was already described for compounds such as 4-MTA (Carmo et al., 2004b, 2007), 2-CB (Carmo et al., 2004a, 2005) which showed a variable toxicity profile accordingly to the individual metabolic capacity. In addition, ethanol can alter the expression and/or activity of some drug-metabolizing enzymes (Jang and Harris, 2007), including those involved in MDMA metabolism. Furthermore, MDMA users are regular ethanol consumers before their first contact with MDMA (Ben Hamida et al., 2006), which may represent a putative risk factor for the toxic interaction between these two drugs. In fact, our group has recently demonstrated that the repeated exposure of CD1 mice to ethanol seems to increase the vulnerability of freshly isolated hepatocytes towards pro-oxidant conditions (Pontes et al., 2008). Additionally, results reported by Pacifici and Hernández-Lopes in human subjects (Hernández-López et al., 2002; Pacifici et al., 2001) showed that also the physiologic and psychopathologic effects of MDMA could be increased in presence of ethanol. Thus, the objective of the present study was to evaluate, *in vivo*, the acute hepatotoxic effects of MDMA (10 mg/kg *i.p.*) in CD-1 mice previously exposed to 12% ethanol as drinking fluid (for 8 weeks). This experimental approach was designed to simulate the most common pattern of ethanol and MDMA abuse, in which the individuals are exposed to ethanol prior to the first contact with MDMA. To our knowledge, experimental work addressing the *in vivo* effects of chronic ethanol exposure on MDMA-induced hepatotoxicity has not been previously reported.

## 2. Materials and methods

### 2.1. Chemicals

All the reagents used in this study were of analytical grade. Bovine serum albumin (fraction V),  $\beta$ -nicotinamide adenine dinucleotide phosphate reduced form ( $\beta$ -NADPH), pyruvic acid, 2-vinylpyridine, glutathione reduced form (GSH), glutathione oxidised form (GSSG), glutathione reductase (EC 1.6.4.2), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), guanidine hydrochloride, 2,4-dinitrophenylhydrazine, 1-chloro-2,4-dinitrobenzene (CDNB), xanthine, nitroterazolium blue chloride, xanthine oxidase from bovine milk and hydrogen peroxide solution were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). The injectable solution of sodic heparine was obtained from B. Braun (Lisbon, Portugal). Perchloric acid, trichloroacetic acid, 2-thiobarbituric acid, Folin Ciocalteu reagent and all other chemicals were purchased from Merck (Darmstadt, Germany). 3,4-Methylenedioxymethamphetamine (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 (purity > 99%).

### 2.2. Animals

Adult male CD1 mice (Charles-River Laboratories, Barcelona, Spain), weighing 30–40 g, were used in all experiments. For at least 1 week prior to the treatment, animals were acclimatized in polyethylene cages with wire-mesh tops, lined with wood shavings, at an ambient temperature of  $20 \pm 2^\circ\text{C}$ , humidity between 40 and 60% and 12 h/12 h light/dark cycle (light on from 8.00 to 20.00 h), having standard chow and tap drinking water *ad libitum*. After this period, animals were divided into two main groups: ethanol ( $n = 12$ ) and non-ethanol ( $n = 12$ ). In the ethanol main group the tap drinking water was replaced by a 5% ethanol solution for 1 week and, afterwards, by a 12% ethanol solution for 8 weeks. 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-ethanol main group had drinking tap water *ad libitum* for the same period. There were no differences in body weight between the main groups after the 8 weeks of the experiment (data not shown). During the 24-h period after MDMA administration, animals were deprived of food but the drinking solution was allowed *ad libitum*. Surgical procedures for blood collection and liver excision were performed under anaesthesia and carried out between 9 and 11 a.m. Animal experiments were licensed by 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 (Institute for Laboratory Animal Research, 1996). The experiments complied with the current Portuguese laws.

### 2.3. MDMA challenge

Mice were housed in four groups of six animals (control, ethanol, MDMA and ethanol + MDMA groups) and were injected intraperitoneally with saline (0.9% NaCl) or 10 mg/kg MDMA (dissolved in saline) in a volume of 0.1 mL/30 g body weight between 9:00 and 10:00 a.m. The experiment was performed under controlled ambient temperature fixed at  $20^\circ\text{C}$  ( $\pm 2^\circ\text{C}$ ). Subcutaneous temperature of animals was measured repeatedly after MDMA administration (see measurement of body temperature). After 24 h, animals were anaesthetized and blood was removed from the inferior vena cava and collected into heparinized tubes. The liver was excised and weighed, after body perfusion with 0.9% NaCl solution, and used for biochemical analysis. Samples were kept frozen ( $-80^\circ\text{C}$ ) until assay. Liver sections were also collected for histological examination. The relative organ weight (ROW), an indicator of tissue harm, was also calculated for each animal as a percentage of the total body weight at the sacrifice day.

### 2.4. Measurement of body temperature

Subcutaneous temperatures of mice were measured using BioMedic programmable temperature transponders (microchips BMDS IPTT-100, Plexx BV, 6660 AE ELST, Netherlands). The microchips were implanted subcutaneously between the shoulder blades, using the BioMedic implant device, 2 days before the experiment. Readings of subcutaneous temperature were taken immediately prior to MDMA or saline injection and for 12 h thereafter using a hand held Biomedic Pocket Scanner (DAS-5007). Temperature was recorded every 15 min during the first hour, every 30 min during the second and third hours and every hour thereafter.

### 2.5. Plasma biochemical analysis

#### 2.5.1. Plasma transaminases

Plasma activities of glutamic oxalic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were evaluated as biomarkers hepatic damage. These determinations were performed in a Reflotron Analyser (Boehringer Mannheim, Indianapolis, IN) using 32  $\mu\text{L}$  of plasma for each analysis (GOT, Cat no. 10745120, Roche Diagnostics; GPT, Cat no. 10745138, Roche Diagnostics).

#### 2.5.2. Oxidative stress parameters

**2.5.2.1. Sample preparation.** For the quantification of protein carbonyl groups, lipid peroxidation extent and GSH and GSSG concentrations, tissue samples were homogenized (1:4 m/v) in ice-cold 5% perchloric acid with an Ultra-Turrax® homogenizer. After the homogenization, aliquots were taken to determine protein carbonyl groups content and protein levels. The remaining homogenates were centrifuged at  $3200 \times g$  for 10 min at  $4^\circ\text{C}$  and the supernatants were used for the quantification of lipid peroxidation and GSH/GSSG.

For measurement of antioxidant enzymes activities, liver samples were homogenized (1:4 m/v) in ice cold 0.1% Triton X-100 in 50 mM phosphate buffer (pH 7.4). The homogenates were centrifuged at  $3200 \times g$  for 10 min at  $4^\circ\text{C}$ , and the supernatants collected for the enzymatic and protein assays.

**2.5.2.2. Protein carbonyl groups.** Protein carbonyl groups (ketones and aldehydes) were quantified according to Levine et al. (1994). Results were expressed as nmol of DNPH incorporated per mg of protein, calculated by using an extinction coefficient of  $2.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ .

**2.5.2.3. Non-specific lipid peroxidation.** The extent of lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) assay at 535 nm (Buege and Aust, 1978; Carvalho et al., 1997). TBARS were expressed as malondialdehyde equivalents per milligram of protein, calculated by using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

**2.5.2.4. GSH and GSSG.** Tissue GSH and GSSG contents were determined by the DTNB-GSSG reductase recycling assay as described before (Pontes et al., 2008).

**2.5.2.5. Catalase.** Catalase activity was measured according to the method of Aebi (1984), by monitoring the decomposition of  $\text{H}_2\text{O}_2$  at 240 nm, calculated by using an extinction coefficient of  $39.4 \text{ M}^{-1} \text{ cm}^{-1}$ . The enzyme activity was expressed as  $\mu\text{mol}$  of  $\text{H}_2\text{O}_2$  consumed per min and per mg of protein.

**2.5.2.6. Glutathione-S-transferase (GST).** Glutathione-S-transferase (GST) activity was assayed according to the method of Habig et al. (1974). The formation of GSH conjugate with 1-chloro-2,4-dinitrobenzene was monitored for 5 min at 340 nm. The GST activity was calculated by using an extinction coefficient of  $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$  and expressed as  $\mu\text{mol}$  per min per mg of protein.

**2.5.2.7. Selenium-dependent glutathione peroxidase (GPx).** Selenium-dependent glutathione peroxidase (GPx) activity was assayed by NADPH oxidation at 340 nm

(Flohe and Gunzler, 1984). The enzyme activity was calculated by using an extinction coefficient of  $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$  and expressed as nmol of NADPH oxidized per min per mg of protein.

**2.5.2.8. Superoxide dismutase (SOD).** Copper/zinc superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD) were assayed using the method of Flohé and Ötting (1984), with some modifications previously reported before (Dinis-Oliveira et al., 2006).

#### 2.5.3. Protein quantification

Protein was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard.

#### 2.5.4. Dry lipid weight content

The total liver lipid content was determined according to Folch et al. (1957) and expressed on a dry weight percentage (%DLWC).

### 2.6. Nuclear extracts and fEMSA

Transcriptional activations of NF- $\kappa$ B and HSF-1 were determined by fluorescent electrophoretic mobility shift assay (fEMSA) using nuclear protein extracts according to a previously reported method (Dinis-Oliveira et al., 2007). The nuclear protein extracts were obtained from tissue homogenates (1:2.5 m/v) in cell lysis buffer (AC Buffer) containing 10 mM HEPES, 1.5 mM  $\text{MgCl}_2$ , 10 mM KCl, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.25 mM phenyl methylsulfonyl fluoride (PMSF) and 20  $\mu\text{L/mL}$  Igepal (pH 7.9).

To obtain the nuclear extracts, the homogenates were exposed to a freeze/thaw cycle and centrifuged for 10 min at  $850 \times g$ ,  $4^\circ\text{C}$ . The pellets were washed again with AC Buffer, incubated for 15 min on ice, and centrifuged at  $16,000 \times g$ ,  $4^\circ\text{C}$ , for 30 s. The obtained nuclear pellets were resuspended in complete lysis Buffer (BC Buffer, containing 20 mM HEPES, 20% (m/v) Glycerol, 420 mM NaCl, 1.5 mM  $\text{MgCl}_2$ , 0.5 mM EDTA, 1 mM DTT, 0.25 mM PMSF, 20  $\mu\text{L/mL}$  Igepal and 5  $\mu\text{g/mL}$  of a protease inhibitors cocktail containing 1 mg/mL aprotinin, 1 mg/mL leupeptin and 1 mg/mL pepstatin), incubated for 30 min on ice on a rocking platform at 150 rpm and centrifuged for 10 min at  $16,000 \times g$  at  $4^\circ\text{C}$ . Aliquots of the supernatant were stored at  $-80^\circ\text{C}$  until assay. Protein quantification was determined by the Lowry assay and each extract aliquot was diluted to 2 mg/mL of protein.

Thereafter, to perform the fEMSA electrophoresis, 20  $\mu\text{g}$  of protein from nuclear extracts were incubated with Cy5-labeled NF- $\kappa$ B or HSF-1 consensus probe in assay buffer (200 mM HEPES, 500 mM KCl, 10 mM EDTA and 10 mM DTT) supplemented with glycerol, poly(dI-dC), DTT and Igepal, at  $4^\circ\text{C}$  over-night. Specificity of the DNA-protein complex was confirmed by the addition of a 50-fold excess of an unlabeled specific competitor (SC) (equal to the specific probe but without the Cy5 label). Nine microliters of each mixture were loaded on a 5% non-denaturing polyacrylamide gel at  $10^\circ\text{C}$ , 800 V, 50 mA and 30 W, in TBE buffer  $1 \times$  (90 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.3) using an ALFexpress II DNA Analyser (Amersham Pharmacia Biotech, Sweden). The temperature was regulated by an external ALF-express II Cooler system (Amersham Pharmacia Biotech, Sweden). The following Cy5-labeled specific double stranded probes were used for NF- $\kappa$ B and HSF-1 gel assays: NF- $\kappa$ B, 5'-GCC TGG GAA AGT CCC CTC AAC T-3'; HSF-1, 5'-GAT CCT CGA ATG TTC GCG AAA AG-3'.

### 2.7. Tissue preparation for light and transmission electron microscopy

For light microscopy, after excision, liver was sliced in 2–4 mm<sup>3</sup> pieces, approximately, and fixed in 4% formaldehyde. The fixed pieces were, then, dehydrated with graded ethanol and included in paraffin blocks. The compound used in the transition between dehydration and impregnation was benzene. Semi-thin sections (4  $\mu\text{m}$ ) were cut in a microtome, applied on silane-coated slides and deparaffinated. The deparaffinated sections were treated for different staining protocols (Haematoxylin-Eosin, Van Gieson) and for immunohistochemistry assays for the detection of NF- $\kappa$ B.

The haematoxylin-eosin staining was performed by immersion in Mayer's haematoxylin solution for 3–4 min followed by immersion in 1% eosin solution for 7 min, dehydration with graded alcohols through xylene, and mounting with DPX.

To perform the NF- $\kappa$ B immunohistochemical detection, NF- $\kappa$ B p50 (NLS) rabbit polyclonal antibody (SC-114, 1:20, Santa Cruz Biotechnology Inc., California, USA) was applied on deparaffinated liver sections and these samples were incubated at  $37^\circ\text{C}$  for 2 h in a humidified chamber. The same samples were then incubated with a secondary anti-immunoglobulin goat anti-rabbit IgG, F(ab')<sub>2</sub> conjugated with alkaline phosphatase (SC-3838, 1:50, Santa Cruz Biotechnology Inc., California, USA), under the same conditions, for 1 h. SIGMAFAST® Fast Red TR/Naphthol AS-MX Tablets were used as substrate according to manufacturer's instructions. The sections were counterstained with Mayer's haematoxylin. The primary antibody was replaced by PBS for negative control sections. Wistar rat 14th day placenta slides were used as positive control. All stained sections were mounted on glass slides using Aquatec®. For the light microscopy study, an optic photomicroscope Nikon, Model Eclipse E400 was used.

For electron microscopy, 1 mm<sup>3</sup> tissue pieces were fixed in 2% glutaraldehyde (in 0.2 M sodium cacodylate) for 2 h at  $4^\circ\text{C}$ . After three 15 min washes with 0.2 M sodium cacodylate buffer at  $4^\circ\text{C}$ , the pieces were post-fixed with 2% osmium tetroxide (in

0.2 M sodium cacodylate), for 2 h at  $4^\circ\text{C}$ . After a new wash with sodium cacodylate (5 min), the samples were submitted to a dehydration process using increasing concentrations of ethanol (from 50% to 100%) for 90 min. In the impregnation stage, the pieces were placed in mixtures containing absolute ethanol and LR White, with increasing concentrations of resin (50% and 75% (v/v) of absolute ethanol) for 30 min each. In the next stage, samples were kept for two periods of 30 min each, in 100% LR White, and afterwards were mounted on covered gelatine capsules. The inclusion was processed in a drying oven at  $60^\circ\text{C}$  for 22–26 h. Ultra-thin (thickness equal to 500–600 Å) sections were cut and contrasted with a saturated aqueous solution of uranyl acetate, for 30 min, and with a solution of lead citrate, for 15 min, with washes at the beginning and at the end of each one of these procedures. For the electron microscopy study a transmission electron microscope Zeiss EM 10 A, at 60 Kvolts was used.

### 2.8. Statistical analysis

Results are presented as mean  $\pm$  S.E.M. (from six animals) and were tested for normality with the Kolmogorov-Smirnov test. For temperature analysis, comparisons were made by analysis of variance (ANOVA) with repeated measures, followed by the Bonferroni's post-hoc test. The other statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Significance was accepted at  $P < 0.05$ .

## 3. Results

### 3.1. Body temperature

After administration of 10 mg/kg MDMA, animals from the MDMA group showed a typical amphetaminic reaction characterized by hyperthermia and hyperactivity. However, in the ethanol + MDMA animals, hyperactivity was not present and a marked prostration was observed.

The ethanol group presented a similar body temperature profile to that of the control group. However, the ethanol + MDMA group showed a sustained increase in body temperature that was significantly higher than that observed for MDMA alone (Fig. 1A). Statistical differences were found during the first 7 h after MDMA administration.

### 3.2. Heat shock factor-1 (HSF-1) activation

The ethanol + MDMA group presented a significantly higher increase of heat shock transcription factor (HSF-1) activation (Fig. 1B, lane 4), when compared to that induced by MDMA alone (Fig. 1B, lane 3).

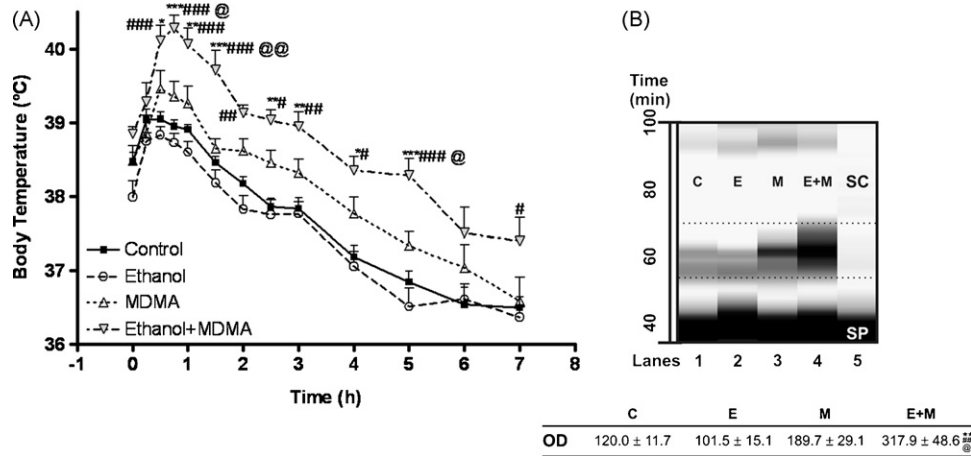
### 3.3. Nuclear factor kappa-B (NF- $\kappa$ B) activation

As shown in Fig. 2, the ethanol + MDMA group presented a significant sharp activation of NF- $\kappa$ B in mouse liver (lane 4) comparatively to control (lane 1), ethanol (lane 2) and MDMA (lane 3) groups. This increase in NF- $\kappa$ B activation was also visible in the immunohistochemistry for NF- $\kappa$ B in liver sections since in the ethanol + MDMA group a high number of red stained nucleus, corresponding to activated NF- $\kappa$ B, was observed when compared to the other groups (Fig. 2).

### 3.4. Structural and ultra-structural analysis

The histological analysis of hepatic sections by light and electron microscopy (Fig. 3) revealed, in all groups, a preserved lobular structure and ultrastructure. The control group presented no relevant changes in the various cellular organelles while the ethanol, MDMA and ethanol + MDMA groups showed diffuse cytoplasmic vacuolization, lysosomal activation, mitochondrial swelling and a dilatation of the cytoplasmic reticulum and the perinuclear cisterna, alterations particularly evident in the ethanol + MDMA group. A severe dilatation of the hepatic centrilobular sinusoids was evident in ethanol and ethanol + MDMA groups. All the groups presented abundant cytoplasmic lipid droplets although in the control and

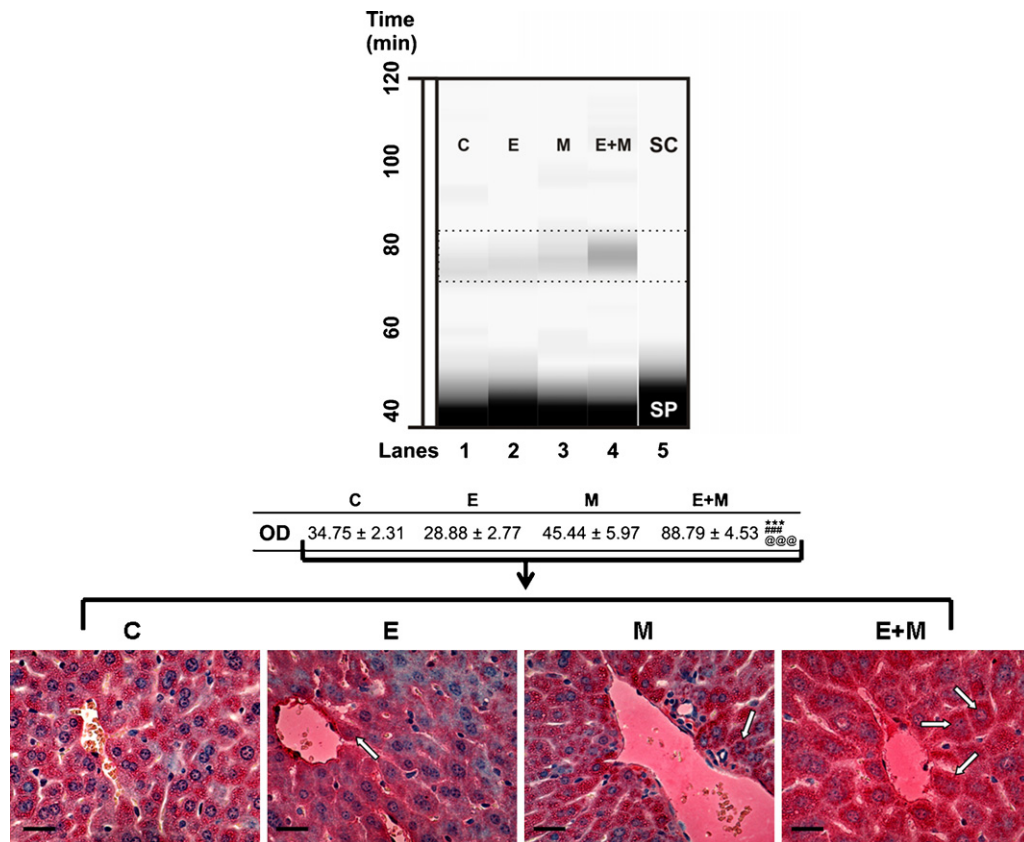




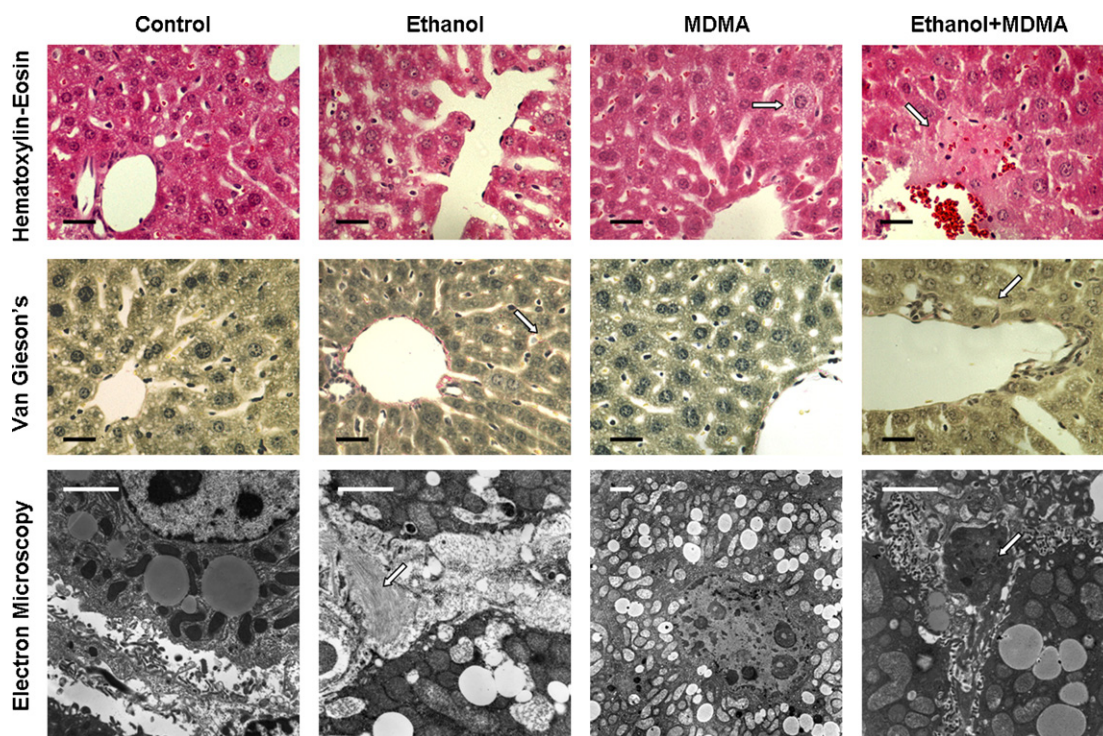
**Fig. 1.** Effect of ethanol pre-treatment on the hyperthermic effect induced by MDMA (A) and in the consequent HSF-1 transcriptional activation (B) in the liver, detected by semi-quantitative fluorescent electrophoretic mobility shift assay (fEMSA). The illustrated gel is representative of the specific HSF-1 bands (highlighted by the dotted line) obtained for the control (C), ethanol (E), MDMA (M) and ethanol + MDMA (E + M) groups by using fluorescence labeled specific probes (SP). Lane 5 represents a competitive experiment with a 50-fold molar excess of a specific competitor (SC, unlabeled specific probe). The optical density (OD) of the bands  $\pm$  S.E.M. of six animals is indicated below. Body temperature results are presented as mean  $\pm$  S.E.M. of six animals. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared to control group; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$ , compared to ethanol group; @ $P < 0.05$ , @@ $P < 0.01$ , compared to MDMA group.

MDMA groups the distribution of these droplets was preferentially centrilobular, while in the ethanol and ethanol + MDMA groups this distribution had a diffuse pattern along the lobule. In control and MDMA groups the interstitial space and the hepatocytes microvilli

of the bile canaliculi and of the sinusoids presented a normal structure while the ethanol and the ethanol + MDMA groups presented a pronounced deposition of collagen fibres between the hepatocytes and the endothelial cells of the sinusoids, resulting in an apparent



**Fig. 2.** Effect of ethanol pre-treatment on NF-κB transcriptional activation in the liver detected by semi-quantitative fluorescent electrophoretic mobility shift assay (fEMSA). The illustrated gel is representative of the specific NF-κB bands (highlighted by the dotted line) obtained for the control (C), ethanol (E), MDMA (M) and ethanol + MDMA (E + M) groups by using fluorescence labeled specific probes (SP). Lane 5 represents a competitive experiment with a 50-fold molar excess of a specific competitor (SC, unlabeled specific probe). The optical density (OD) of the bands  $\pm$  S.E.M. of six animals is indicated below. \*\*\* $P < 0.001$ , compared to control group; ### $P < 0.001$ , compared to ethanol group; @@@ $P < 0.001$ , compared to MDMA group. Representative light micrographs of hepatic immunohistochemistry for NF-κB detection are also shown (the magnification bar represents 20  $\mu$ m). A cytoplasmic red staining is observed in all images, being more pronounced in the E, M and E + M pictures where some red stained nuclei are also depicted (arrow).



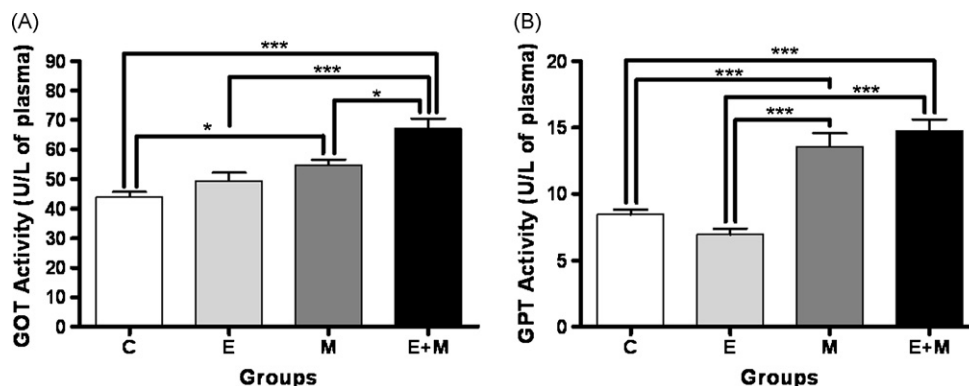
**Fig. 3.** Transmission electron micrographs and light microscopy images (haematoxylin–eosin stain and Van Gieson's stain) of liver sections from control, ethanol, MDMA and ethanol + MDMA groups. In the light microscopy images, a preserved lobular structure is observed. The ethanol illustrations present dilatation of the centrilobular sinusoids. Necrotic cells are punctually observed in MDMA images (arrow). More confluent necrotic areas are evident in the ethanol + MDMA slide (arrow). In the slides stained with Van Gieson, collagen content is poorly pronounced in control and MDMA images and more marked in ethanol exposed groups (arrow). The transmission electron micrographs reveal a mainly lipidic cytoplasmic vacuolization and mitochondrial swelling in all pictures, more pronounced in ethanol, MDMA and ethanol + MDMA images. Large collagen contents between endothelial and parenchymal cells are depicted in the ethanol picture (arrow). In the MDMA micrograph numerous nucleoli are also observed. An activated Kupffer cell is also shown in the ethanol + MDMA picture (arrow). The magnification bar on the light microscopy images represents 20  $\mu\text{m}$ . The magnification bar on the electron microscopy images represents 2  $\mu\text{m}$ .

reduction of the size and density of the hepatocytes microvilli in the sinusoids and in the bile canaliculi. This high collagen deposition was also evidenced by the Van Gieson stain, which revealed a higher deposition of collagen fibres (coloured in red) in both ethanol and ethanol + MDMA groups. In the ethanol group some nuclei presented chromatin condensation on their peripheral regions. In the MDMA group, nuclei with numerous and voluminous nucleoli were observed and in both ethanol and MDMA groups it was also noticed the existence of osmiophobic confluent areas, suggesting intracellular oedema. In the MDMA group, some isolated necrotic cells were observed, while in the ethanol + MDMA group some apparently necrotic regions were found, as well as cell fragments in the

interstitial space. Kupffer cells with indicative signs of activation, suggested by high amounts of lysosomes, were also observed in the ethanol + MDMA group.

### 3.5. Plasma transaminases

Concerning to GOT levels (Fig. 4A), they were slightly increased in the ethanol group compared to the control group. The administration of MDMA produced a significant increase of plasma levels of this transaminase in both MDMA and ethanol + MDMA groups, the increase in this last group being significantly higher (increase of approximately 35%) than in the MDMA group (increase of



**Fig. 4.** Effect of 3,4-methylenedioxymethamphetamine (MDMA) on plasma levels of glutamic oxalic transaminase (GOT (A)), glutamic pyruvic transaminase (GPT (B)) of control and ethanol pre-treated animals. C: control, E: ethanol, M: MDMA and E + M: ethanol + MDMA groups. Results are presented as mean  $\pm$  S.E.M. from six animals. \* $P < 0.05$ , \*\*\* $P < 0.001$ .

**Table 1**

Hepatic percent dry lipid weight content (%DLWC) and liver relative organ weight (ROW) of mice from the control, ethanol, MDMA and ethanol + MDMA groups

Group	Liver	
	ROW	%DLWC
Control	4.359 ± 0.198	22.38 ± 1.89
Ethanol	5.582 ± 0.293**	19.91 ± 1.57
MDMA	3.686 ± 0.165###	30.93 ± 2.546*.,##
Ethanol + MDMA	4.222 ± 0.261##	21.84 ± 1.53®

Results are presented as mean ± S.E.M. from six animals. All groups were compared, however, only the statistically differences were indicated. \**P* < 0.05, \*\**P* < 0.01, compared to control group; ###*P* < 0.01, ###*P* < 0.001, compared to ethanol group; ®*P* < 0.05, compared to MDMA group.

approximately 30%) when compared to ethanol and control groups, respectively.

The levels of GPT (Fig. 4B) were significantly increased in MDMA and ethanol + MDMA groups, especially in the ethanol + MDMA which presented a twofold increase in GPT levels when compared to the ethanol group. The increase of GPT present in the MDMA group corresponds to a 50% increase when compared to control group.

### 3.6. Relative organ weight (ROW) and hepatic percent dry lipid weight content (%DLWC)

As it can be observed in Table 1, the ethanol group presented a significant increase in liver ROW, and the ethanol + MDMA group showed a marked decrease of this parameter when compared to the ethanol group. The MDMA group presented a slight decrease in liver ROW without statistical significance. The MDMA group also presented a significant increase in the liver %DLWC when compared to both control and ethanol groups.

### 3.7. Oxidative stress biomarkers (TBARS, carbonyl groups, GSH/GSSG)

No significant changes in hepatic lipid peroxidation were observed between control and MDMA groups, but a significant increase in TBARS levels was observed in liver of animals from the ethanol group (compared to control group) that was not visible in the ethanol + MDMA group (Table 2). The same occurs with the hepatic GSH/GSSG ratio that suffers a significant reduction in the ethanol group (comparing to control group), not observed in the ethanol + MDMA group.

Additionally, there were found no important changes in hepatic protein carbonyl groups.

### 3.8. Hepatic anti-oxidant enzymes

The MDMA group presented a significant increase in GST activity while the ethanol + MDMA group had a significantly reduced GST activity compared to control group (Table 3). Hepatic GPx and catalase activities did not present significant differences between the control, ethanol and MDMA groups, but evidenced a significant

**Table 2**

Oxidative stress parameters in the control, ethanol, MDMA and ethanol + MDMA groups

Groups	GSHred/GSSG ratio	TBARS (nmol MDA/mg protein)	Carbonyl groups (nmol/mg protein)
Control	9.35 (0.62)	0.080 (0.004)	5.36 (0.33)
Ethanol	6.73 (0.47)**	0.129 (0.015)**	5.76 (0.28)
MDMA	7.75 (0.88)	0.097 (0.004)	4.63 (0.18)
Ethanol + MDMA	7.43 (0.26)	0.073 (0.004)®	5.04 (0.37)

Results are presented as mean ± (S.E.M.) from six animals. All groups were compared, however, only the statistically differences were indicated. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, compared to control group; ®*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001, compared to ethanol group; ®*P* < 0.05, ®®*P* < 0.01, compared to MDMA group. MDA: malondialdehyde.

decrease in the ethanol + MDMA group. Ethanol group presented reduced activities of both MnSOD and Cu/ZnSOD, when compared to control group, and the ethanol + MDMA group showed a significant enhancement in MnSOD and Cu/ZnSOD activities.

## 4. Discussion

The results obtained in the present study clearly demonstrate that the chronic exposure to 12% ethanol prior to MDMA administration potentiates the hyperthermic and hepatotoxic effects induced by MDMA, which was evidenced by a significant increase in several biochemical and histopathological biomarkers of toxicity.

The administration of MDMA was followed by a sharp increase in body temperature. This is an expected effect (Carvalho et al., 2002), which can be attributed to several central and peripheral mechanisms (Walubo and Seger, 1999; Dafters and Lynch, 1998). However, this is the first report of a potentiation of MDMA-related hyperthermia by ethanol pre-treatment resulting not only in significantly higher body temperature scores but also in more sustained hyperthermia than the one induced by MDMA alone. A corresponding higher increase of hepatic heat shock transcription factor (HSF-1) activation was observed (Fig. 1). This reflects a natural reaction of the liver to high temperatures and functions as a defence mechanism towards the hyperthermic aggression by increasing the synthesis of heat shock proteins (HSPs). HSPs are highly conserved proteins with an important role in protein folding (acting like molecular chaperones), signal transduction, cell growth and differentiation, and in the regulation of the actin cytoskeleton, contributing for cellular homeostasis (Pirkkala et al., 2001), counteracting heat shock and developing adaptation to oxidative stress to avoid cell death (Santos-Marques et al., 2006). This defence system may be disrupted if the hyperthermic effect surpasses the normal physiological variations, or in the presence of other risk factors. Indeed, animals exposed simultaneously to ethanol and MDMA and affected by sustained hyperthermia, may be more prone to deleterious phenomena.

The enhancement of the MDMA-induced hyperthermic response by ethanol may seem unexpected since ethanol is known to induce vasodilatation and decrease body temperature (Huttunen

**Table 3**

Hepatic antioxidant enzymes activities in the control, ethanol, MDMA and ethanol + MDMA groups

Groups	GST (U/mg protein)	GPx (U/mg protein)	Catalase (U/mg protein)	SOD (U/mg protein)	
				Mn SOD	Cu/Zn SOD
Control	1112 ± 64	252.5 ± 21.4	182.9 ± 5.9	0.233 ± 0.024	3.071 ± 0.180
Ethanol	1080 ± 123	191.2 ± 11.5	187.7 ± 9.5	0.150 ± 0.011**	2.252 ± 0.128**
MDMA	1626 ± 121*.,#	231.5 ± 19.3	183.1 ± 7.1	0.239 ± 0.020#	3.463 ± 0.185###
Ethanol + MDMA	885 ± 109®®	173.9 ± 13.0*	149.0 ± 13.6#	0.259 ± 0.010##	3.123 ± 0.214#

Results are presented as mean ± S.E.M. from six animals. All groups were compared, however, only the statistically differences were indicated. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, compared to control group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001, compared to ethanol group; ®*P* < 0.05, ®®*P* < 0.01, compared to MDMA group.



et al., 1998). However, this hypothermic effect is only evident for acute ethanol administration and it is affected by tolerance phenomena that can appear as early as between two consecutive exposures with a delay of 24 h after the first one (Khanna et al., 1996). The result obtained in the present study is in agreement with a previous report of Cassel et al., who showed that ethanol inhibits the MDMA-induced hyperthermia by the first day of treatment, but not on subsequent treatment days, suggesting a tolerance effect on ethanol-induced hypothermia (Cassel et al., 2004).

Ethanol pre-treatment was also able to exacerbate MDMA-induced hepatotoxicity, which could be ascertained by the significant increase of plasma transaminases activities, biomarkers of hepatic lesion, when animals were exposed to ethanol and MDMA. The increase in GOT and GPT activities were already described for both compounds in humans (Ellis et al., 1996; Yue et al., 2006) and rats (Beitia et al., 2000; Montet et al., 2002). This hepatotoxic effect was also confirmed by the decrease in liver weight when MDMA was administered to ethanol pre-exposed mice and by histological analysis of liver sections by light and electron microscopy, which gives further evidences that the concomitant exposure to MDMA and ethanol results in a marked aggravation of the hepatotoxic effects exerted by each one of the compounds at isolated exposures.

The marked increase in the activation of NF- $\kappa$ B, one of the transcription factors involved in the activation of immediate early response genes in response to injurious and inflammatory stimuli (Chen and Shi, 2002), indicates a pro-inflammatory effect as ascertained by the observation of activated Kupffer cells and corresponds to another cellular defence mechanism by increasing the activity of antioxidant enzymes such as SOD (Lenart et al., 2007). However, the activation of both NF- $\kappa$ B and HSF-1 was not sufficient to protect cells from the toxicity of MDMA and ethanol despite being efficient in avoiding sharp modifications in oxidative stress parameters such as GSH/GSSG ratio, protein carbonyl groups content and TBARS. Unchanged glutathione levels were also detected in rats after a single dose of MDMA by Beitia et al. (2000). The increase in SOD activity due to MDMA administration verified in this study was previously reported in the kidney by Ninković et al. (2008). The decrease of SOD activity following chronic ethanol consumption is also in accordance with previous reports by Chen et al. (2002). The increase in SOD activity is a specific adaptive mechanism to a stress condition due to an increased superoxide generation associated with MDMA exposure. Increased expression of SOD leads to removal of reactive superoxide radical anions, minimizing the generation of cytotoxic peroxynitrite and terminating lipid peroxidation-induced chain reactions.

Catalase and GPx are other antioxidant enzymes whose activities protect cells from the toxicity of H<sub>2</sub>O<sub>2</sub>. The observed decrease of their activities in the ethanol+MDMA group evidenced that the hepatic oxidative injury was significantly more intense when MDMA was administered to ethanol pre-exposed mice when compared to the other studied groups. The effect of MDMA itself on the activities of antioxidant enzymes confirmed the results previously obtained by our group showing no significant changes in the activities of hepatic Mn SOD, Cu/Zn SOD, GPx and Catalase by 10 mg/kg MDMA (Carvalho et al., 2002). The only exception was GST, which presented an increase in its activity never reported before. The pre-treatment with 12% ethanol seems to avoid the observed increase on GST activity provoked by the administration of MDMA, which can be explained by the GST-lowering effect already described for ethanol (Pari and Suresh, 2008).

Our results also confirm the results of previous reports that addressed an increase in hepatic lipid content to MDMA consumption (Beitia et al., 2000) probably due to an increase of  $\beta$ -adrenergic receptors-dependent lipolysis in the skeletal muscle tissue (Unger,

1995), and the subsequent increase of circulating levels of free fatty acids (Sprague et al., 2007) that are thereafter taken up by the liver. However, in the present study, ethanol was not able to modify this effect despite being widely described that the chronic exposure to ethanol leads to an increase in hepatic lipid content (de la Monte et al., 2008; Pritchard and Nagy, 2005).

In conclusion, the obtained results strongly suggest that the consumption of ethanol increases the toxic effects induced by MDMA exposure, with special relevance to its hyperthermic and hepatotoxic effects. If fact, signs of hepatic necrosis and inflammatory response were evident and consistent with drug related hepatitis.

These results will certainly contribute for the understanding of the health risks undertaken by polydrug abusers who combine ecstasy and ethanol and who were already associated with MDMA-related fatalities (Schifano et al., 2003).

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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