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ORGAN TOXICITY AND MECHANISMS

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Is hyperthermia the triggering factor for hepatotoxicity induced by 3,4-methylenedioxymethamphetamine (ecstasy)? An in vitro study using freshly isolated mouse hepatocytes

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Abstract The consumption of 3,4-methylenedioxymethamphetamine (ecstasy; MDMA) may cause hepatocellular damage in humans, a toxic effect that has been increasing in frequency in the last few years, although the underlying mechanisms are still unknown. The metabolism of MDMA involves the production of reactive metabolites which form adducts with intracellular nucleophilic sites, as is the case with glutathione (GSH). Also, MDMA administration elicits hyperthermia, a potentially deleterious condition that may aggravate its direct toxic effects. Thus, the objective of this study was to evaluate the extent of MDMA-induced depletion of GSH, induction of lipid peroxidation and loss of cell viability in freshly isolated mouse hepatocytes under normothermic conditions (37 °C) and to compare the results with the effects obtained under hyperthermic conditions (41 °C). By itself, hyperthermia was an important cause of cell toxicity. A rise in incubation temperature from 37 °C to 41 °C caused oxidative stress in freshly isolated mouse hepatocytes, reflected as a time-dependent induction of lipid peroxidation and consequent loss of cell viability (up to 40–45%), although the variations in GSH and GSSG levels were similar to those under normothermic conditions. MDMA (100, 200, 400, 800 and 1600 μ*M*) induced a concentration- and time-dependent GSH depletion at 37 °C but had a negligible effect on lipid peroxidation and cell viability at this temperature. It is particularly noteworthy that hyperthermia (41 °C) potentiated MDMA-induced depletion of GSH, production of lipid peroxidation and loss of cell viability (up to 90–100%). It is therefore concluded that hyperthermia potentiates MDMA-induced toxicity in freshly isolated mouse hepatocytes.

Key words MDMA · Hyperthermia · Freshly isolated mouse hepatocytes · Hepatotoxicity

This paper is dedicated by the authors to Professor Margarida Alice Ferreira on the occasion of her retirement in recognition of her contribution to the development of the pharmaceutical sciences.

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Introduction

3,4-Methylenedioxymethamphetamine (MDMA; ecstasy) is a phenylethylamine derivative with structural similarities to both mescaline and amphetamine. MDMA has acquired notoriety as a potential tool for the treatment of psychiatric disorders, but has achieved popularity as a drug of abuse by virtue of its euphoria-inducing and stimulant properties (Karch 1993). Although some misinformation available to the public describes this drug as relatively harmless, reports of its toxicity have been increasing in frequency during the last decade, notably hepatocellular damage in humans (for a review see Jones and Simpson 1999). However, in spite of these reports, little is known about the respective biological mechanisms involved, probably due to its complexity, which involve various factors, namely its metabolism, the increase in neurotransmitters efflux, the oxidation of biogenic amines, and hyperthermia.

It is well known that the administration of MDMA to laboratory animals causes a rise in body temperature, and it must be stressed that hyperthermia is a pro-oxidant aggressive condition, which leads to irreversible hepatocellular injury in vitro (Carvalho et al. 1997; Skibba et al. 1991). On the other hand, MDMA is biotransformed into a reactive intermediate that conjugates with glutathione (GSH) (Hiramatsu et al. 1990), which may cause its depletion, a situation known to expose cells to the aggression of pro-oxidant toxicants. Taking into account the concomitant appearance of GSH depletion and hyperthermia as a consequence of MDMA consumption, it seems plausible that they may have

synergistic deleterious effects. Thus, the aim of this study was to evaluate and compare the extent of MDMA-induced depletion of GSH, lipid peroxidation and loss of cell viability in freshly isolated mouse hepatocytes under normothermic and hyperthermic conditions (37 °C and 41 °C, respectively).

Materials and methods

Chemicals

All reagents were of analytical grade. Collagenase (grade I), bovine serum albumin (fraction V), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Tris(hydroxymethyl)aminomethane (Tris), ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide [NAD(H)], pyruvic acid and 2-thiobarbituric acid, were obtained from Sigma Chemical Company (St. Louis, Mo.). Chloroacetic acid, methanol (gradient grade), perchloric acid and all other chemicals were obtained from Merck (Darmstadt, Germany). Water for HPLC was deionized (µs/cm µ0.055) (Seral Water Purification Systems, Munich, Germany). 3,4-Methylenedioxymethamphetamine was a generous gift from the United Nations Drug Control Program (Vienna, Austria).

Animals

Charles-River mice, weighing 25–30 g, were used as the source of hepatocytes. The mice were acclimatized in polyethylene cages lined with wood shavings with wire mesh at the top at an ambient temperature of 20 ± 2 °C, under a humidity between 40% and 60% and under a 12/12-h light/dark cycle. The animals had free access to standard rat chow and drinking water, and were kept in our animal house for at least 2 weeks prior to use. Surgical procedures, which were performed under diethyl ether anesthesia, were always carried out between 9:00 a.m. and 10:00 a.m.

Isolation and incubation of hepatocytes

Hepatocyte isolation was performed by collagenase perfusion as described by Moldéus et al. (1978), with the necessary adaptations for the animal species used (Mouse). The foremost adaptations were a smaller cannula diameter (0.8 × 25 mm), a lower collagenase concentration (0.038%) and the fact that the perfusion was performed in situ. Cell viability at the beginning of the experiments was between 85% and 97%.

Incubations were performed in a shaking water bath (90 oscillations/min) at 37 °C, using 10^6 cells/ml in Krebs-Henseleit buffer supplemented with 25 m*M* HEPES (pH 7.4), and gassed continuously with an air stream of humidified carbogen. The isolated hepatocytes were always preincubated for 30 min at 37 °C before the beginning of the experiments. Cells were then incubated with MDMA chloride (0, 100, 200, 400, 800 and 1600 µ*M*). These experiments were performed both at 37 °C and at 41 °C. Sample aliquots were taken at time 0 and after each 60 min during 3 h of incubation for evaluation of cell viability, lipid peroxidation, and reduced and oxidized glutathione.

Cell viability was determined after isolation by the trypan blue exclusion test. During the course of the experiments cell viability was determined by the LDH leakage method, which was randomly confirmed by the trypan blue exclusion test. GSH and GSSG measurement was performed by HPLC with a dual-electrode coulometric detection (Remião et al. 2000). Briefly, cell suspension aliquots (500 µl) were precipitated with 500 µl perchloric acid (5% final acid concentration) and centrifuged for 10 min at 13,000 rpm in a refrigerated centrifuge. Aliquots of the samples were then injected into the HPLC system with dual-electrode coulometric detection. The extent of lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) assay at 535 nm as described previously (Carvalho et al. 1997).

Statistical analysis

Results are given as means ± SEM ($n = 5$) (each experiment was performed with cells obtained from a different mouse). Statistical comparisons were made with one-way ANOVA followed by Fisher's Protected Least Significant Difference (PLSD) test. The level of significance was set at $P < 0.05$.

Results

The increase in incubation temperature from 37 °C to 41 °C caused oxidative stress in the freshly isolated mouse hepatocytes, reflected as a time-dependent induction of lipid peroxidation (Fig. 1A) and consequent loss of cell viability (up to 40–45%) (Fig. 1B), although the variations in GSH and GSSG levels were similar to those observed under normothermic conditions (data not shown). On the other hand, MDMA (100, 200, 400, 800 and 1600 µ*M*) induced a concentration- and time-

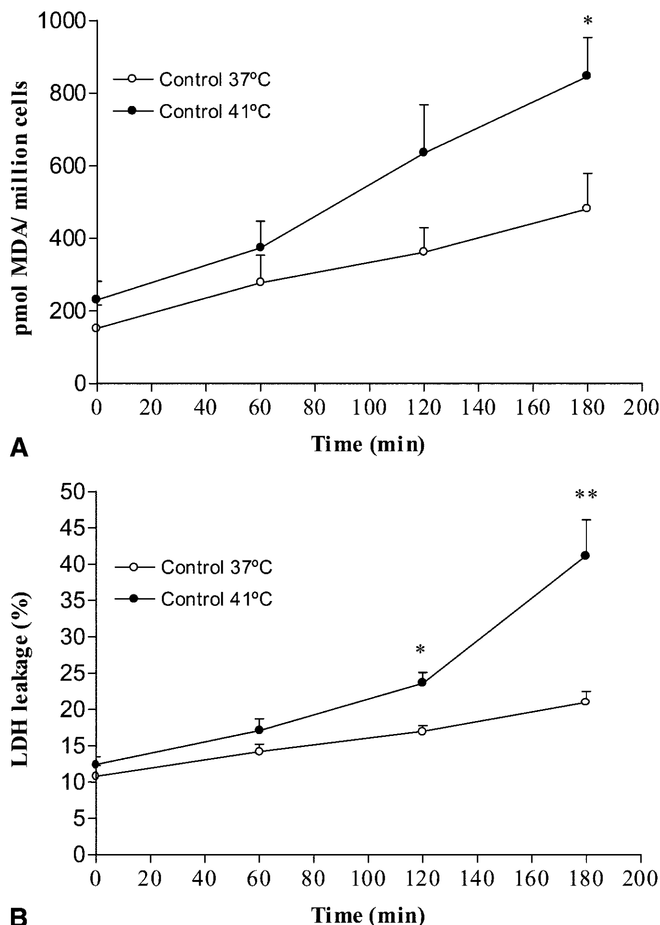


Fig. 1A, B Effect of different temperatures (37°C or 41°C) on TBARS formation (**A**) and LDH leakage (**B**) in freshly isolated mouse hepatocytes. Values are means ± SEM from five different experiments (*$P < 0.05$, **$P < 0.01$)

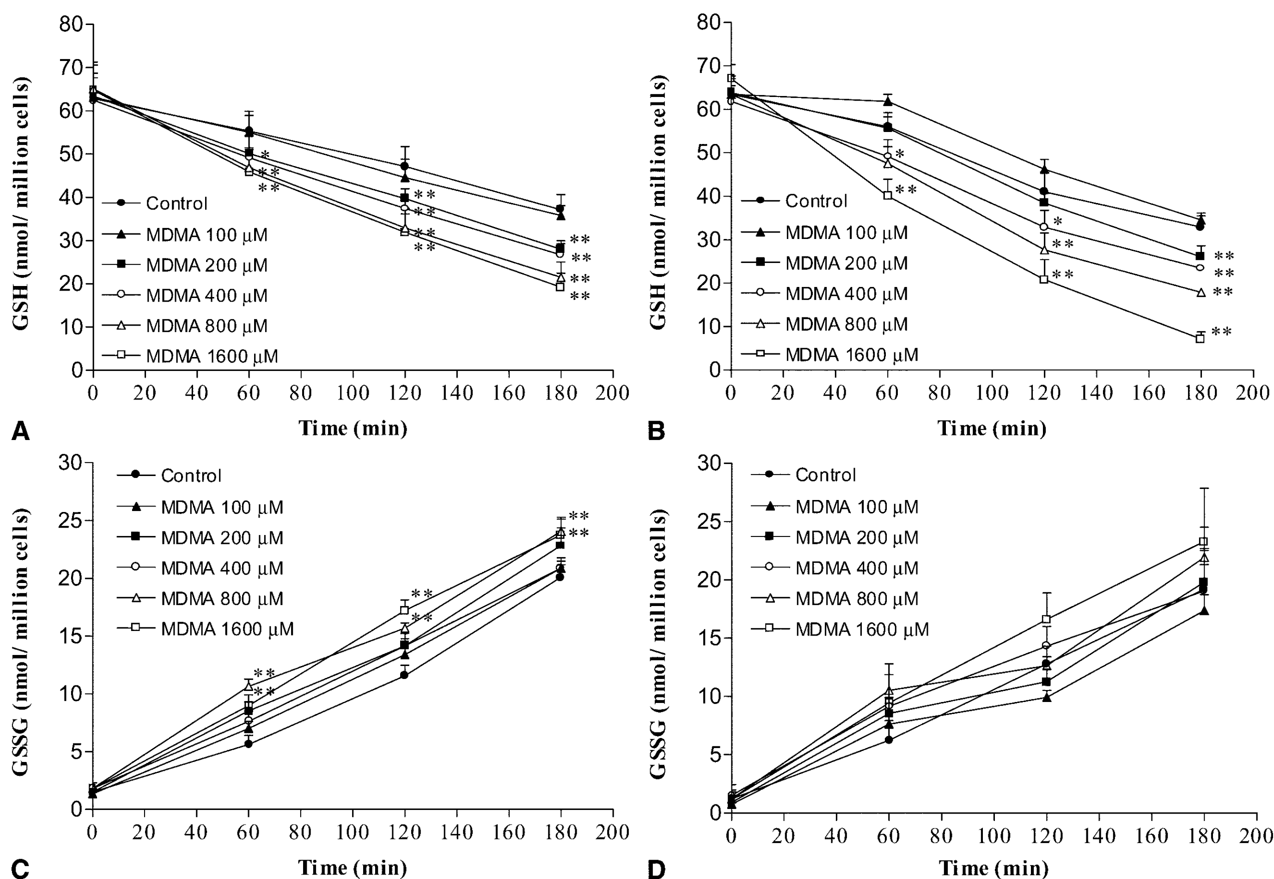
dependent GSH depletion at 37 °C (Fig. 2A) but had a negligible effect on lipid peroxidation (Fig. 3A) or cell viability (Fig. 4A) at this temperature. It is noteworthy that hyperthermic conditions (41 °C) heightened MDMA-induced depletion of GSH (Fig. 2B), production of lipid peroxidation (Fig. 3B) and loss of cell viability (Fig. 4B) (up to 90–100%). It is also important to note that the GSH depletion induced by MDMA was not due to GSH oxidation and that the observed potentiation of MDMA-induced GSH depletion by hyperthermia was still not due to an increase of GSSG formation (Fig. 2A–D). Unquestionably, the dramatic damage observed in the cells exposed to the drug at 41 °C gives a clear picture of the potentiation of MDMA toxicity by hyperthermia.

Discussion

The observed synergistic toxicity of MDMA and hyperthermia against isolated mouse hepatocytes has never been reported before, despite the fact that it may help to understand why this drug of abuse is hepatotoxic to some abusers. MDMA causes hyperthermia in both animals and humans. However, while the MDMA-induced hyperthermia in experimental animals is dose-related and dependent on ambient temperature (O'Shea et al. 1998), in humans, this effect is not always related to the amount of ingested drug (Henry et al. 1992). If someone takes MDMA in sufficient amounts, an increase in body temperature is usually observed (Fineshi and Masti 1996; Walubo and Seger 1999). Although the mechanism of MDMA-induced hyperthermia is still a matter of debate, anecdotal evidence indicates that it is augmented by high environmental temperatures, dehydration and excessive physical exertion (e.g. at rave parties) (Walubo and Seger 1999), which may explain the different susceptibility among humans.

MDMA-induced hyperthermia is provoked by an increase in brain serotonin levels, thus exciting the thermal control regions in the anterior hypothalamus/preoptic area, possibly by increasing the temperature set point, which stimulates the sympathetic center and increases sympathetic discharge. Catecholamines are released and these stimulate alpha or beta adrenergic receptors (depending on the particular tissue's receptor reserve), which increases mitochondrial metabolism and heat generation. Also, muscular contractions and peripheral vasoconstriction contribute to body temperature elevation (Walubo and Seger 1999). A pre-existing metabolic myopathy or genetic predisposition similar to that seen in patients with malignant hyperthermia

Fig. 2A–D Effect of MDMA on GSH and GSSG levels in freshly isolated mouse hepatocytes. Cells were incubated at 37°C (**A**, **C**) or 41°C (**B**, **D**) with MDMA (100, 200, 400, 800 and 1600 μ*M*). Values are means ± SEM from five different experiments (*$P < 0.05$, **$P < 0.01$)



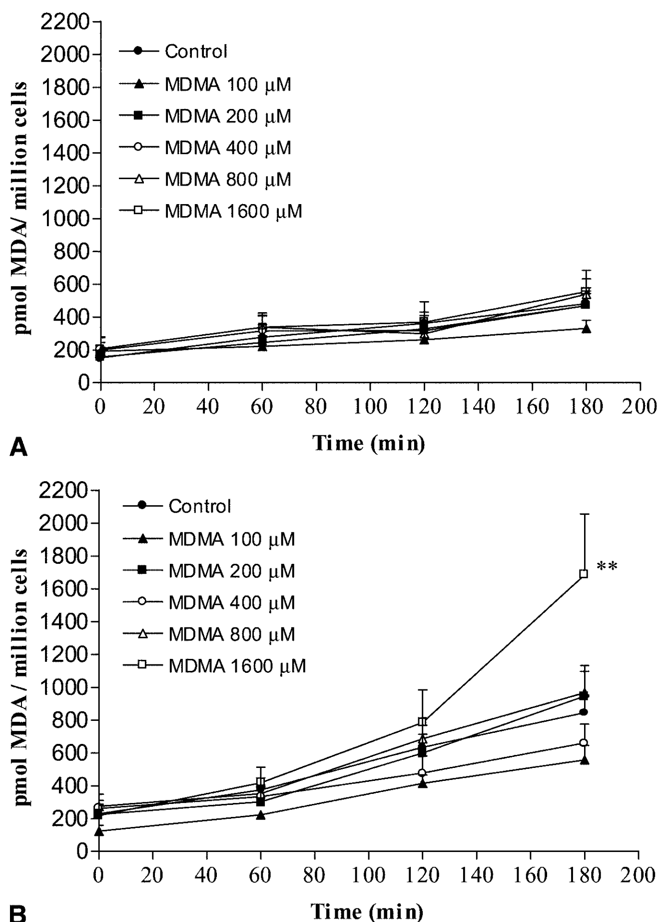


Fig. 3A, B Effect of MDMA on TBARS formation in freshly isolated mouse hepatocytes. Cells were incubated at 37°C (**A**) or 41°C (**B**) with MDMA (100, 200, 400, 800 and 1600 μ*M*). Values are means ± SEM from five different experiments (**P* < 0.05, ***P* < 0.01)

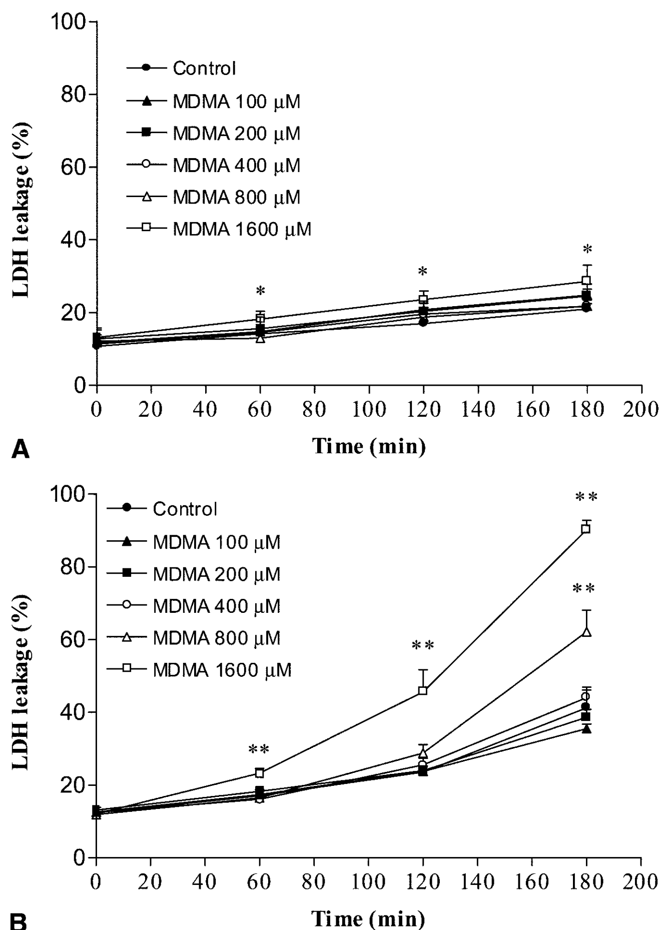


Fig. 4A, B Effect of MDMA on LDH leakage from freshly isolated mouse hepatocytes. Hepatocytes were incubated at 37°C (**A**) or 41°C (**B**) with MDMA (100, 200, 400, 800 and 1600 μ*M*). Values are means ± SEM from five different experiments (**P* < 0.05, ***P* < 0.01)

remains a possibility. Survivors of MDMA toxicity need to be evaluated for this if our understanding of this phenomenon is to be increased (Dar and McBrien 1996). Interestingly, in a reported case of 3,4-methylenedioxy-ethamphetamine-induced hyperthermia, subsequent in vitro testing for malignant hyperthermia proved to be negative (Tehan et al. 1993).

It has been shown that hyperthermia causes lipid peroxidative damage in perfused rat liver (Skibba et al. 1991) and in freshly isolated rat hepatocytes (Carvalho et al. 1997). The mechanisms responsible for the oxidative stress resulting from hyperthermia are still under study, but it is known that they involve oxygen-centered free radical formation, namely superoxide (Powers et al. 1992) and hydroxyl radicals (Flanagan et al. 1998). These radicals can be generated after the hyperthermia-induced conversion of xanthine dehydrogenase to xanthine oxidase in the presence of free iron (released from ferritin by superoxide radical) as the catalyst in the Fenton reaction (Powers et al. 1992). In the present work, lipid peroxidation occurred despite the maintenance of GSH content within the normothermia control levels. Strikingly, in a previous work, the same hyperthermic conditions applied to freshly isolated rat hepatocytes not only increased lipid peroxidation and cell mortality but also caused severe GSH oxidation (Carvalho et al. 1997). The reason for this different effect on GSH may be due to a better capacity to recover to basal GSH levels in mouse hepatocytes, or to a different intracellular localization of the reactive oxygen species (ROS) formation (e.g. within the lipidic domain). This interspecies variability in response to MDMA exposure is interesting and leads to the suggestion that the putative different toxicological pathways in rat and mouse hepatocytes should be studied in detail and compared with those in human hepatocytes in order to find the best animal model from which results may be extrapolated to humans.

The metabolic activation to a reactive metabolite capable of conjugating with GSH has been shown for 3,4-methylenedioxymethamphetamine (Hiramatsu et al. 1990). The resulting GSH depletion has already been demonstrated in isolated rat hepatocytes (Beitia et al. 1999) and was confirmed in the present study with a different rodent species. Since GSH has an important

protective role involving its oxidant neutralizing and its lipid peroxidase and/or tocopheryl radical-regenerating activities (Di Mascio et al. 1991), its depletion may render the cells more exposed to the deleterious effects of the ROS being formed within the cells. Thus, in a heatstroke caused by MDMA, the depletion of GSH may have primordial importance in the susceptibility of cells to oxidative injury. This putative toxicological mechanism is reinforced by the results obtained in the present study.

The metabolism of MDMA involves its conversion to α-methyldopamine (α-MeDA) by cytochromes P450 2D, 2B and 3A (Bai et al. 1999; Kreth et al. 2000). α-MeDA undergoes oxidation to the corresponding *o*-quinone, which is efficiently scavenged by GSH to form 5-(glutathion-S-yl)-α-methyldopamine (Hiramatsu et al. 1990). It is noteworthy that the conjugate can still be converted into a glutathione-*o*-quinone (Miller et al. 1997). The observed potentiation of MDMA-induced GSH depletion by hyperthermia may be due to an increase in metabolism, although this needs further study. The reactive *o*-quinone intermediates are Michael acceptors, and cellular damage can occur through alkylation of crucial cellular proteins and/or DNA. Alternatively, quinones are highly redox-active molecules which can enter a redox cycle with their semiquinone radicals, leading to the formation of ROS, including superoxide, hydrogen peroxide, and ultimately the hydroxyl radical (see Bolton et al. 2000). Thus, the metabolism of MDMA leads to the formation of highly aggressive agents that may cause irreversible cell damage, which will be potentiated by the depletion of antioxidant defense levels. This weakened state can be brought about by hyperthermia.

In the present study, the concomitant aggression of freshly isolated mouse hepatocytes by MDMA and hyperthermia proved to be synergistically toxic. Since hyperthermia and MDMA metabolism occur in vivo, it seems reasonable to surmise that these situations are potentially lethal and most certainly contribute to the hepatotoxicity that has been reported in humans. Although the concomitant effect of MDMA in people taking it, namely (1) an increase in body temperature and (2) a direct effect of the drug on GSH levels by the formation of adducts has not yet been reported, it is most likely that these effects occur in humans since they express the enzymes involved in the above-mentioned metabolism (Kreth et al. 2000).

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