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# Acute administration of 3,4-methylenedioxymethamphetamine (MDMA) induces oxidative stress, lipoperoxidation and TNFα-mediated apoptosis in rat liver

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ABSTRACT

Liver toxicity is one of the consequences of ecstasy (3,4-methylenedioxymethamphetamine MDMA) abuse and hepatocellular damage is reported after MDMA consumption. Various factors probably play a role in ecstasy-induced hepatotoxicity, namely its metabolism, the increased efflux of neurotransmitters, the oxidation of biogenic amines, and hyperthermia. MDMA undergoes extensive hepatic metabolism that involves the production of reactive metabolites which form adducts with intracellular nucleophilic sites. MDMA-induced-TNF-α can promote multiple mechanisms to initiate apoptosis in hepatocytes, activation of pro-apoptotic (BID, SMAC/DIABLO) and inhibition of anti-apoptotic (NF-κB, Bcl-2) proteins. The aim of the present study was to obtain evidence for the oxidative stress mechanism and apoptosis involved in ecstasy-induced hepatotoxicity in rat liver after a single 20 mg/kg, i.p. MDMA administration. Reduced and oxidized glutathione (GSH and GSSG), ascorbic acid (AA), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and malondialdehyde (MDA), an indicator of lipid peroxidation, were determined in rat liver after 3 and 6 h after MDMA treatment. The effect of a single MDMA treatment included decrease of GR and GPx activities (29% and 25%, respectively) and GSH/GSSG ratio (32%) with an increase of MDA (119%) after 3 h from ecstasy administration compared to control rats. Liver cytosolic level of AA was increased (32%) after 6 h MDMA treatment. Our results demonstrate a strong positive reaction for TNFα ($p < 0.001$) in hepatocytes and a diffuse apoptotic process in the liver specimens ($p < 0.001$). There was correlation between immunohistochemical results and Western blotting which were quantitatively measured by densitometry, confirming the strong positivity for TNF-α ($p < 0.001$) and NF-κB ($p < 0.001$); weak and intense positivity reactions was confirmed for Bcl-2, SMAC/DIABLO ($p < 0.001$) and BID reactions ($p < 0.001$).

The results obtained in the present study suggest that MDMA induces loss of GSH homeostasis, decreases antioxidant enzyme activities, and lipoperoxidation that causes an oxidative stress that accompaines the MDMA-induced apoptosis in liver cells.



*Abbreviations:* AA, ascorbic acid; α – Me Da, α – methyldopamine; A-SMase, acidic sphingomyelinase; ATP, adenosintriphosfato; Bax/Bak, BCL2-associated X protein/BCL2-antagonist/killer 1; Bcl-2, B cell lymphoma gene-2; BHT, butylhydroxytoluene; BID, BH3-interacting domain death agonist; BSA, bovine serum albumin; CAT, catalase; CYP450, cytochrome P450; DTNB, 5,5′-Dithio-bis (2-nitrobenzoic acid); GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione reduced; GSSG, glutathione oxidized (disulfide); GST, glutathione transferase; HSC, hepatic stellate cells; IAPs, inhibitor of apoptosis proteins; I-κBα, inhibitory protein of factor kappa B; IKK, IκB kinase; JNK, c-Jun N-terminal Kinase; LSAB, labeled streptavidin biotin; MAT1, methionine-adenosyl-transferase type I; MDA, malondialdehyde; MDMA, 3,4-Methylenedioxymethamphetamine; MPT, mitochondrial permeability transition; NADH, nicotinamide adenine dinucleotide (reduced form); NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; NF-κB, nuclear factor kappa B; N-Me-α-MeDA, N-methyl-α-methyldopamine; PBS/Tween-20, phosphate buffered saline with Tween 20 (PBST-20X); RIP, receptor interacting protein; RIPA buffer, radioimmunoprecipitation assay buffer; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS PAGE, sodium dodecyl sulphate – polyacrylamide gel electrophoresis; SMAC/DIABLO, second mitochondria-derived activator of caspases/direct IAP binding protein with low PI; SOD, superoxide dismutase; TCA, trichloroacetic acid; TdT, terminal deoxynucleotidyl transferase; TNB, 5-thionitrobenzoic acid; TNF-R1, tumor necrosis factor receptor; TNF-α, tumor necrosis factor α; TRADD, TNFRSF1A-associated via death domain/tumor necrosis factor receptor type 1-associated DEATH domain protein; TRAF-2, TNF receptor-associated factor 2; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

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## 1. Introduction

Hepatotoxicity is one of the medical consequences of 3,4-methylenedioxymethamphetamine (MDMA) consumption and hepatocellular damage has been reported after MDMA administration [1,2]. However, some aspects of the pathogenesis associated with MDMA elicited hepatic injury remain unclear [3]. Various factors probably play a role in MDMA-induced hepatotoxicity, namely its metabolism, the increased efflux of neurotransmitters, the oxidation of biogenic amines, and hyperthermia [4]. Potentiation of MDMA-toxicity upon freshly isolated mouse hepatocytes, elicited by hyperthermia MDMA-induced, has been reported by Carvalho [5]. Hyperthermia potentiated MDMA-induced depletion of GSH, production of lipid peroxidation and loss of cell viability up to 90–100%. Studies demonstrate that even the single administration of MDMA can significantly alter the cellular antioxidant defence system in such a way as to induce cardiac oxidative stress [3]. Beitia et al. [2] examined the effects of single and repeated administration of MDMA on rat liver. After acute MDMA administration, no significant changes in lipid peroxidation and hepatic GSH content and liver antioxidant enzymes (GPx, GR, SOD, GST, CAT) were observed whereas multiple MDMA administration produced some evidence of oxidative stress, namely, increased lipid peroxidation and decreased GSH content and GPx activity. In contrast Ninković et al. [6] showed an oxidative stress state much more expressed after the single administration of MDMA. Despite the well-established role of increased oxidative/nitrosative stress in MDMA-induced liver damage [7–11], the underlying mechanisms by which increased oxidative stress causes liver damage is still poorly understood. Investigations into the role of GSH in modulating apoptotic signaling suggest that cellular redox changes following enviromental stress induced by citotoxic agents may be modulated not only by the generation of ROS but also by the extrusion of GSH from cells [12]. There are reports suggesting that oxidative stress is involved in the induction of programmed cell death in some systems. Montiel-Duarte et al. [9] showed that MDMA induces apoptosis of primary rat hepatocytes and of a cell line of rat hepatic stellate cells (HSC) and the role played by oxidative stress in the apoptotic death of HSC elicited by MDMA. These authors concluded that MDMA induces programmed cell death on HSC and this effect is accompanied by oxidative stress. Carvalho et al. [13] evaluated the effects of two main MDMA metabolites, MDA and α-methyldopamine (α-MeDA) and the effect of MDMA and one of its metabolites N-methyl-α-methyldopamine (N-Me-α-MeDA) in freshly isolated rat hepatocytes. The results obtained in this study suggest that the metabolism of MDMA into highly reactive catechol-metabolites is one of the main causes of MDMA-induced hepatotoxicity in vivo. The authors also evaluated the ability of antioxidants, namely ascorbic acid and N-acetyl-L-cysteine, to prevent N-Me-α-MeDA-induced toxic injury, using freshly isolated rat hepatocytes The results showed that administration of antioxidants prevented N-Me-α-MeDA toxicity. Thus, it can be postulated that the metabolism of MDMA and 3,4-methylenedioxyamphetamine, resulting in the formation of the highly reactive compounds N-Me-α-MeDA and α-MeDA, respectively, is one of the main causes of their hepatotoxic effects. The toxic effects are characterized by a loss of GSH homeostasis due to conjugation of GSH with N-Me-α-MeDA and α-MeDA, decreases in the antioxidant enzyme activities, and cell death. Recent evidence has suggested apoptosis as contributing to MDMA toxicity, this raises two major possibilities, which are considered in this report: (1) MDMA might induce apoptosis by causing intracellular stress, and/or (2) MDMA might increase the susceptibility to apoptosis induced by the extrinsic pathway. One possible mechanism for the latter would be the depletion of reduced glutathione (GSH), which has been suggested in some studies to sensitize to tumor necrosis factor (TNF-α)-induced cell death [14]. In hepatocytes GSH depletion has been shown to induce apoptosis by itself or to sensitize the cells towards TNF-α-induced apoptosis, and the effect of some anti-apoptotic agents has been demonstrated to be mediated by stabilizing the GSH pool [9]. GSH depletion might influence other redox-sensitive steps in the apoptosis cascade, such as enhanced opening of the mitochondria permeability pore or increased ceramide production or other signaling steps.

In the present work, we studied the role played by oxidative stress in the apoptotic response caused by MDMA in rat liver after a single 20 mg/kg i.p. dose. Reduced and oxidized glutathione (GSH and GSSG), ascorbic acid (AA), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and malondialdehyde (MDA), an indicator of lipid peroxidation, were determined in rat liver after 3 and 6 h after MDMA treatment. MDMA-induced-TNF-α can promote multiple mechanisms to initiate apoptosis in hepatocytes, so we performed an immunoistochemical study and a Western blot analysis to evaluate cell apoptosis and to measure activation of pro-apoptotic (BID, SMAC/DIABLO) and inhibition of anti-apoptotic (NF-κB, Bcl-2) proteins [15].

## 2. Materials and methods

The experimental procedures followed the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1996) and were approved by the University of Siena Committee for animal experiments.

### 2.1. Animal model and experimental protocol

For the evaluation of oxidative stress, three groups of seven male albino rats (Wistar, Charles River) weighing 200–250 g were used to analyse the effect of MDMA administration (20 mg/kg, i.p.) on rat's liver. After treatment, animals were sacrificed by decapitation at the following times: group I, 3 h; group II, 6 h; group III, control group. Liver samples were used to determine the biochemical parameters of oxidative stress. Animal model and experimental protocol for the evaluation of histopathological examination: 50 male albino rats (Wistar; Charles River) weighing 200–250 g were used to analyse the effect of MDMA administration (20 mg/kg, i.p.) on rat liver. After treatment, animals were sacrificed by decapitation at the following times: group I (14 rats) after 24 h of which 2 died spontaneously within 4 h after administration of MDMA, group II (14 rats) after 16 h, group III (14 rats) after 6 h and control group of eight animals was treated with saline i.p.; control group was sacrificed at the following times: 2 rats 6 h after treatment, 2 rats 16 h after treatment, and 4 rats 24 h after treatment. The plasma concentration of MDMA and the metabolite methylenedioxyamphetamine was carried out on 25 rats, each weighing 200–250 g, divided into three groups of seven animals each treated with MDMA 20 mg/kg, i.p.: group I sacrificed 6 h after treatment; group II sacrificed 16 h after treatment; and group III sacrificed 24 h after treatment of which 2 died spontaneously within 4 h after administration of MDMA. One control group of four animals was treated with saline i.p. and sacrificed: 1 rat 6 h after treatment, 1 rat 16 h after treatment, and 2 rats 24 h after treatment were used for toxicological analysis. Plasma samples obtained after the treatments were stored at −80 °C until gas chromatography/mass spectroscopy (GC–MS) analysis.

### 2.2. Biochemical analysis

#### 2.2.1. Oxidative stress evaluation

The livers of the treated and control animals were immediately dissected and then frozen at −80 °C. Concentrations of GSH and