

N-Methyl-3,4-methylenedioxyamphetamine-induced hepatotoxicity in rats: oxidative stress after acute and chronic administration

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Background. The underlying mechanisms of N-Methyl-3,4-methylenedioxyamphetamine-MDMA-induced hepatotoxicity are still unknown. The aim of this study was to evaluate hepatic oxido-reductive status in the rats liver after the single and repeated administration of MDMA. **Methods.** MDMA was dissolved in distilled water and administered in the doses of 5 mg, 10 mg, 20 mg, and 40 mg/kg. The animals from the acute experiment were treated per os with the single dose of the appropriate solution, through the orogastric tube. The animals from the chronic experiment were treated per os, with the doses of 5, 10, or 20 mg/kg of MDMA every day during 14 days. The control groups were treated with water only. Eight hours after the last dose, the animals were sacrificed, dissected, their livers were rapidly removed, frozen and stored at -70°C until the moment of analysis. The parameters of oxidative stress in the crude mitochondrial fractions of the livers were analyzed. **Results.** Superoxide dismutase (SOD) activity increased in the livers of the animals that were treated with single doses of MDMA. Chronically treated animals showed the increased SOD activity only after the highest dose (20 mg/kg). The content of reduced glutathione decreased in both groups, but the depletion was much more expressed after the single administration. Lipid peroxidation index increased in dose-dependent manner in both groups, being much higher after the single administration. **Conclusion.** The increased index of lipid peroxidation and the decreased reduced glutathione levels suggested that MDMA application induced the state of oxidative stress in the liver. These changes were much more expressed after the single administration of MDMA.

Key words: N-Methyl-3,4-methylenedioxyamphetamine; drug toxicity; liver; oxidative stress; lipid peroxidation; glutathione.

Introduction

N-Methyl-3,4-methylenedioxyamphetamine, MDMA ("Ecstasy") is a derivate of methamphetamine (known as "meth", "crystal", or "speed"). It is the third most used illicit drug after cannabis and amphetamines. It has become popular among the participants in "raves", because it enhances energy, endurance, sociability, and sexual arousal (1). Ecstasy also has serious toxic effects such as: hyperpyrexia, rhabdomyolysis, cardiac arrhythmias, cerebrovascular accidents, intravascular coagulopathy, and hepatic necrosis (2).

MDMA is almost always taken orally and is prepared for this purpose as a single-dose tablet (3). Experimental evidence showed that MDMA could act as a serotonergic neurotoxin. The increase in the net release of serotonin (and possibly dopamine) is the major mechanism of action underlying the changed mental effects of MDMA (4, 5). The increased release of noradrenaline is mainly responsible for its physical effects (6).

MDMA and the related drugs are largely metabolized in the liver by the cytochrome P450 variety designated CYP2D6. Demethylenation of MDMA is catalyzed by cytochrome P-450 2D6 (CYP2D6) and cytochrome P-450

2D1 (CYP2D1) in humans and rats, respectively, and is polymorphically expressed. It was suggested that CYP 2D6 deficiency might be the reason for the unexplained toxicity of MDMA (7).

The incidence of hepatotoxic effect of MDMA has been increasing over the last few years (8, 9). There is some evidence that ecstasy-induced hepatotoxicity is a result of the increased body temperature. High ambient temperature and hyperthermia as a deleterious condition might aggravate its direct toxic effects (10).

Since there were some indications that oxidative stress is involved in the first stage of MDMA-induced liver damage, the aim of this study was to evaluate hepatic oxidoreductive status after single and repeated administration of MDMA.

Methods

The adult rats of both genders (200–250 g) of the Wistar strain were used in the experiment. Until the experiment the animals were housed five per cage, under the conditions of controlled temperature and lighting, with food and water provided *ad libitum*. All the animals received care in strict accordance with Good Laboratory Practice and the Guidelines for the humane care of animals developed by Ethical Committee of the Military Medical Academy and other federal statutes. Ecstasy was obtained from police sources in accordance to the Official Register of the Federal Republic of Yugoslavia (11).

On the day of the experiment, the rats were randomized into the groups for the acute and the chronic experiment. MDMA was dissolved in distilled water and administered in the doses of 5 mg, 10 mg, 20 mg, and 40 mg/kg. The animals from the “acute experiment” were treated *per os* with the single dose of the appropriate solution (in the volume of 0.5 ml, using orogastric tube). The animals from the “chronic experiment” were treated *per os* with the dose of 5, 10, or 20 mg/kg every day for 14 days. The control groups for the acute and chronic experiments were treated with 0.5 ml of water in the same procedure (single or repeated administration).

Rectal temperatures of the rats were taken using rectal sonde thermometer (Tupe du3s ellab a-s, Copenhagen). Temperature was measured per hour, until the moment of decapitation of acutely treated animals. In chronically treated groups rectal temperature was measured daily, 8 hours after the administration of MDMA, every hour in the same way. Eight hours after the last dose the animals were decapitated and dissected, their livers were rapidly removed, frozen, and stored at -70°C until the moment of analysis.

Liver samples were homogenized on ice in cold buffered sucrose 0.25 mol sucrose; (Serva, Feinbiochemica, Heidelberg, New York), 10 mmol phosphate buffer pH 7.0 and 1 mmol EDTA (Sigma chem. co. St. Louis, USA). The homogenates were centrifuged at 2 000 g for 15 min at 4°C .

Crude sediments were redissolved in sucrose medium and centrifuged again. The supernatants were centrifuged at 3 200 g for 30 minutes at 4°C , and the obtained sediments were redissolved in deionized water. After one hour of incubation, the samples were centrifuged at 3 000x g for 15 minutes at 4°C , and supernatants (crude mitochondrial fractions) were stored at -70°C (12). Proteins were determined by the Lowry method, using bovine serum albumin as a standard (13).

Superoxide dismutase (EC 1. 15. 1. 1.; SOD) activity was measured spectrophotometrically, as an inhibition of epinephrine autooxidation at 480 nm. The analysis was performed in sodium carbonate buffer (50 mmol, pH 10.2; Serva, Feinbiochemica, Heidelberg, New York), containing 0.1 mmol EDTA (Sigma, St. Louis, USA), after the addition of 10 mmol epinephrine (Sigma, St. Louis, USA) (14).

Reduced glutathione (GSH) was determined by using 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 36.9 mg in 10 ml of methanol), which had reacted with aliphatic thiol compounds in Tris-HCl buffer (0.4 mol, pH-8.9), thus making yellow colored p-nitrophenol anion. Color intensity was used for spectrophotometric measuring of GSH concentration at 412 nm. Liver tissue was prepared in 10% sulfosalicylic acid (15).

Lipid peroxidation index was measured as a quantity of produced malon-dialdehyde, after the stimulation of peroxidation with the addition of ferrosulphate (Merck, Darmstadt), and 0.5 mmol ascorbic acid (Serva, Heidelberg). After the stimulation of lipid peroxidation, thiobarbituric acid reagent (TBAR-15% trichloroacetic acid (Merck, Darmstadt) + 0.375% TBA + 0.25 mol HCl) reacted with malondialdehyde, which was made from polysaturated fatty acids in the process of peroxidation. The product of the reaction – malondialdehyde was measured spectrophotometrically at 533 nm (16).

Descriptive data were expressed as the mean $\pm$ standard error (SE). Statistical analysis was performed on PC Pentium, using a statistical software program (Statistica 5.0 for Windows). The groups were compared by Student's t-test and the analysis of variance. Differences were considered statistically significant at $p < 0.05$.

Results

After the single administration of MDMA the animals were very thirsty, compared to the animals from the control group. These signs were less expressed after a few hours. Behavioral changes in the repeatedly treated animals were similar to the acutely treated ones.

Body temperature of the rats increased markedly, reaching its maximum approximately one hour after the administration of MDMA, and remained elevated for more than 6 h. Hyperthermic response was dose-dependent, with the largest change at the highest dose administered. Also, hyperthermic reactions were highest after the first dose (Figures 1 and 2).

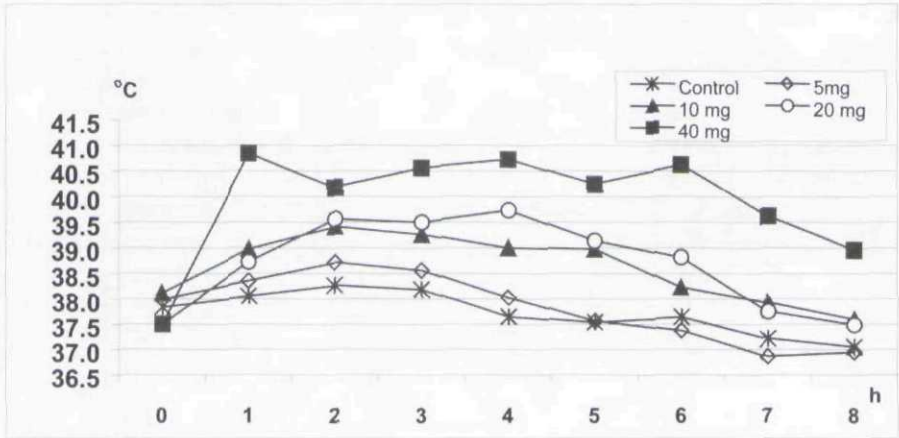


Fig. 1 – Effects of acute administration of MDMA (5, 10, 20, 40 mg/ kg *per os*) on mean values of rat rectal temperature.

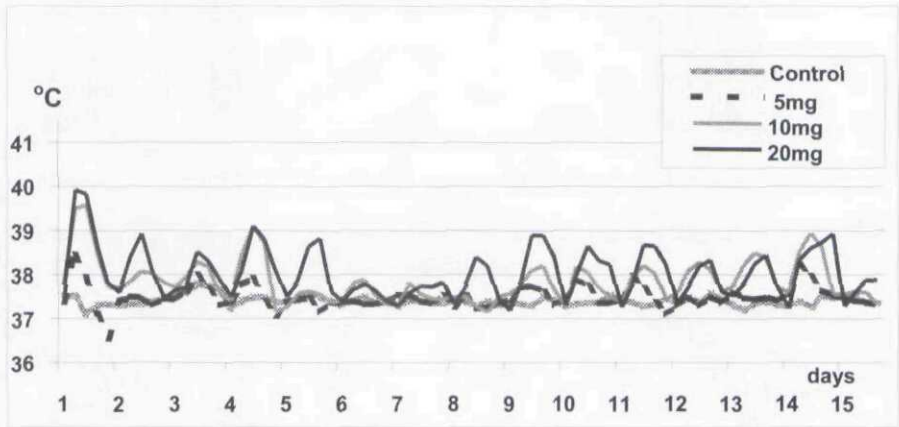


Fig. 2 – Effects of repeated administration of MDMA (5, 10, 20 mg/kg *per os*) on mean values of rat rectal temperature.

Analyzing crude mitochondrial fractions of the liver tissues, the following results were obtained:
Superoxide dismutase activity (SOD) in the livers increased in the acutely treated animals, but this increase was

much more expressed in the groups treated with the lower doses. In the chronically treated animals there was a significant increase of SOD only in the group treated with 20 mg/kg, compared to the control group (Figure 3).

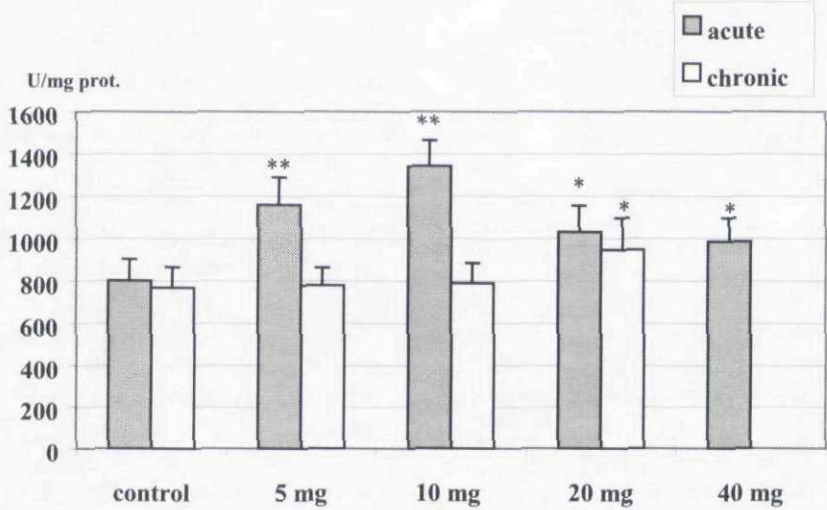


Fig. 3 – Activity of SOD in the livers of acutely and chronically treated Wistar rats with MDMA.
* $p < 0.05$, ** $p < 0.01$.

The content of reduced glutathione in the livers decreased in both groups, but much more in the acutely treated animals, compared to the control. The decreases of GSH content in the livers of repeatedly treated animals showed the signs of dose dependence (Figure 4).

Lipid peroxidation index increased in both groups in the dose-dependent way, but such an increase was more expressed in the acutely treated animals (Figure 5).

chondrial metabolism directly, as well as indirectly, stimulating hyperreactivity and muscular contractions (19).

Hyperthermia and the consequent dehydration might be the reasons for tissue acidosis. It is well known that severe acidosis aggravates cell damage. Several mechanisms might be involved in the escalation of lipid peroxidation during acidosis: the increase of H^+ ion concentration; the acceleration of the dismutation rate of superoxide anion (O_2^-).

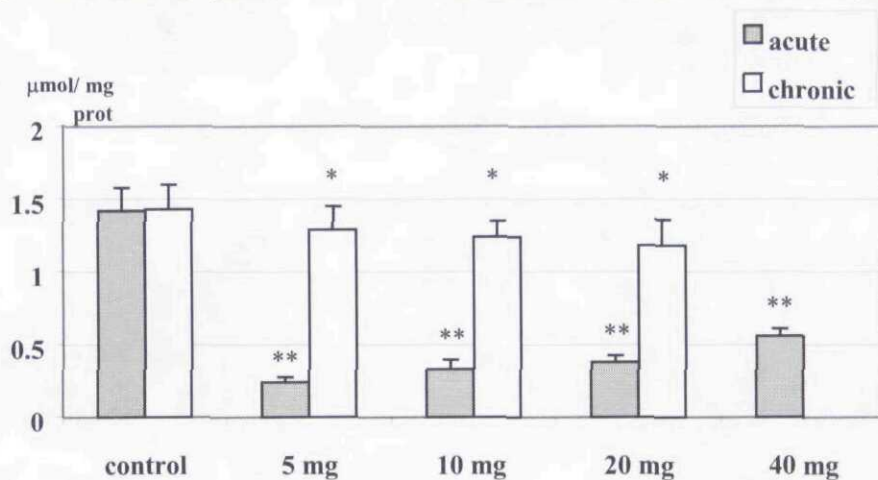


Fig. 4 – Reduced glutathione content in the livers of the rats acutely and chronically treated with MDMA.

* $p < 0.05$, ** $p < 0.01$.

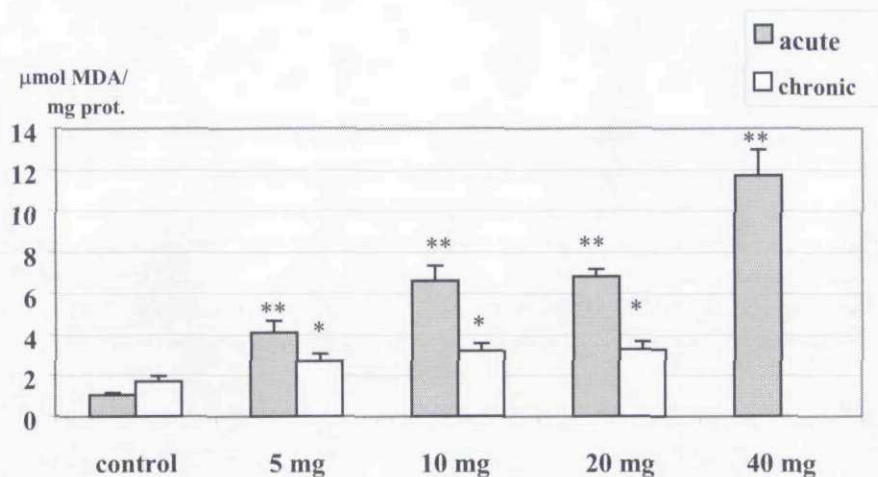


Fig. 5 – Lipid peroxidation index in the livers of the rats acutely and chronically treated with MDMA.

* $p < 0.05$, ** $p < 0.01$.

Discussion

The etiology of MDMA-induced hepatotoxicity is probably multifactorial, but the influence of hyperthermia which occurs after the ingestion of MDMA shouldn't be neglected (17). Hyperpyretic reaction occurs due to the increased brain serotonin level, which stimulates thermoregulation regions in the anterior hypothalamus/preoptic area. Temperature set-point increase stimulates the sympathetic centre, and consequently releases catecholamines (18). The activation of adrenergic receptors stimulates mito-

to hydrogen peroxide, which can react with Fe^{2+} and generate highly toxic hydroxy radical; conversion of O_2^- to more reactive and more lipid soluble hydroperoxy radical. Thus, a possible effect of acidosis might be to stimulate free radical generation. It was shown that the production of lipid peroxides by tissue homogenates was significantly enhanced by lowering pH from 7 to 6 (20).

In intact cells the balance between free radical generation and neutralization is upheld by complex mechanisms, which include the maintenance of spatial separation between radical formation sites and vulnerable biomolecules, together with the

presence of enzymatic and antioxidant system. Peroxy radical, and particularly hydroxy radical, can vigorously abstract hydrogen atoms from unsaturated fatty acids to form lipid radicals, being the initial step in the lipid peroxidation. In the lipid bilayers this chain process initiates cascades of free-radicals, generating reactions that disrupt membrane integrity and facilitate the entry of calcium ions into the cells. Lipid peroxidation is also known as lipid autooxidation, because in the presence of oxygen lipid the radical is spontaneously peroxidized (molecular oxygen is added directly). Hydrogen peroxide is produced by the dismutation of superoxide anions, a reaction catalyzed by the enzyme SOD (21, 22). Although there is some evidence of the minor role of nitric oxide (NO) in the mechanisms of MDMA action, its relationship with reactive oxygen species should be taken in account. Nitric oxide has much more affinity for the reaction with superoxide, producing highly toxic peroxynitrite, than there exists the affinity of SOD for superoxide in the reaction of dismutation (23, 24). This could be a reason of smaller increase of SOD activity in the animals acutely treated with higher doses.

The depletion of GSH found in this experiment, could result in oxidative tissue damage in which mitochondria are particularly affected. Mitochondria are the primary target of oxidative damage if the balance between the production and the removal of reactive oxygen species is disturbed (25). The electron transport chain of mitochondria is the main contributor to the ongoing production of small amounts of free radicals in normal cells. The overflow of redundant calcium, as a result of disrupted redox balance, generates a cellular crisis that leads to cell death (26). It is also known that MDMA secondary products are highly reactive with glutathione, probably causing such a decrease in reduced glutathione (27, 28). The metabolism of MDMA involves the production of reactive metabolites which can conjugate with glutathione. Alpha-methyldopamine, which is the result of MDMA-demethylation by cytochromes P450, is oxidized to o-quinone (29). It is known that quinones, with their semiquinone radicals might be a source of production of reactive oxygen species (30). The depletion of GSH may allow hydrogen peroxide to accumulate to toxic levels. In this experiment there were some differences between the acute and chronic changes in the content of reduced glutathione. After a single dose of MDMA, the decrease of GSH was prompt in all the groups. After the chronic administration of MDMA the differences were much less pronounced. Cellular glutathione can be depleted by the release of the

oxidized form, a process accelerated under the conditions of oxidative stress. The transport of oxidized glutathione from the cell has a defensive role. Such a process abolishes oxidation of the essential thiol groups, which occurs at the membrane. Glutathione content was also reported to decrease under some conditions through the irreversible formation of protein-glutathione mixed disulphides (31). The reduction of hydroperoxide in water in the presence of glutathione peroxidase is a reaction that counteracts the accumulation of superoxide anion. In that reaction the reduced glutathione is e^- donor and it oxidizes to oxidized glutathione. Except in the conditions of the extreme production of hydroperoxide, oxidized glutathione converts very fast to a reduced form in the presence of glutathione reductase and NADPH (32). In that way, the tissue score remains balanced. The decreased levels of reduced glutathione were found in both groups because the experiment was performed in the extreme conditions, in which the oxidized form could not be transformed into a reduced one.

Lipid peroxidation, as a measure of tissue destruction, increased in all the groups treated with MDMA. Superoxide dismutase activity showed an increasing trend in the groups acutely treated with MDMA, but no dose dependence was found. It was obvious that some mechanisms of defence were activated in the chronically treated animals. Also, lipid peroxidation index was increased more in the acutely treated than in the chronically treated groups, and these increases exhibited dose dependence. These biochemical results were in accordance with the changes in body temperature (33, 34). Warm environment at "rave parties", where MDMA is often consumed, may exacerbate the toxic effect of ecstasy. Ecstasy promotes energy crisis directly, or via thermo-disregulation. Reactive oxygen species can be a part of the mechanism of destruction, as well as the result of energy disregulation. It was shown that hyperthermia potentiated MDMA-induced toxicity in freshly isolated mouse hepatocytes. Hyperthermia itself was proven as an important cause of cell toxicity (35, 36).

Conclusion

Acute as well as chronic administration of MDMA produced oxidative stress in the rat livers, expressed as GSH depletion, and the increased lipid peroxidation. The temperature changes and the investigated parameters of oxidative status were more pronounced after the single administration of MDMA.

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- The paper was received on June 10, 2003.

Apstrakt

Ninković M, Maličević Ž, Selaković V, Simić I, Vasiljević I. *Vojnosanit Pregl* 2004; 61(2): 125–131.

HEPATOTOKSIČNOST IZAZVANA N-METIL-3,4-METILENDIOKSAMFETAMINOM KOD PACOVA: OKSIDATIVNI STRES NAKON AKUTNOG I HRONIČNOG DAVANJA

Uvod. Mehanizam koji se nalazi u osnovi hepatotoksičnosti izazvane N-Metil-3,4-metilendioksiamfetaminom (MDMA) je još uvek nepoznat. Cilj ove studije bio je da se ispita oksido-reduktivni status u jetri pacova nakon pojedinačnog i višekratnog davanja MDMA. **Metode.** MDMA je rastvoren u destilovanoj vodi i davan u dozi od 5 mg, 10 mg, 20 mg i 40 mg/kg. Životinje iz akutno tretiranih grupa primile su *per os* jednokratnu dozu odgovarajućeg rastvora, pomoću orogastrične sonde. Životinje iz hroničnog eksperimenta bile su tretirane svakodnevno 14 dana, *per os*, dozom od 5 mg, 10 mg i 20 mg/kg. Kontrolne grupe dobijale su samo vodu. Osam sati nakon poslednje doze, životinje su žrtvovane, obdukovane, jetre su brzo izvađene, zamrznute i čuvane na –70 °C do trenutka analiza. Analizovani su parametri oksidativnog stresa u grubo prečišćenoj mitohondrijskoj frakciji jetre. **Rezultati.** Aktivnost superoksid dizmutaze (SOD) povećala se u jetri životinja koje su primile jednokratne doze MDMA. Hronično tretirane životinje pokazale su povećanje aktivnosti SOD samo kod najveće doze (20 mg/kg). Redukovani glutation smanjio se u obema grupama, ali ovo smanjenje bilo je izraženije nakon jednokratnog davanja. Indeks lipidne peroksidacije se povećao na dozno-zavisani način u obema grupama i to mnogo više nakon pojedinačnog davanja. **Zaključak.** Povećanje indeksa lipidne peroksidacije i smanjenje redukovaniog glutatona sugerišu da davanje MDMA izaziva stanje oksidativnog stresa u jetri. Ove promene bile su mnogo izraženije nakon pojedinačnog davanja MDMA.

Ključne reči:

N-Metil-3,4-metilendioksiamfetamin; lekovi, toksičnost; jetra; stres, oksidativni; lipidi, peroksidacija, glutation.