Ekatys-induced toxicity in rat liver

Beitia G, Cobreros A, Sainz L, Cenarruzabeitia E. Ekatys-induced toxicity in rat liver.

Abstract: Background/aims: The aim of the present study was to examine the effects of single and repeated administration of 3,4-methylenedioxymethamphetamine (MDMA, “ekatys”) on rat liver. Methods: Animals were given an acute (20 mg/kg) and repeated (20 mg/kg, b.i.d., for 4 consecutive days) intraperitoneal dose of MDMA, and at various times after administration the hepatic and serum determinations were made. Results: The effect of acute MDMA administration included increased triglyceride and cholesterol levels and an increase in all enzyme activities 6 h post administration. The toxic effect of MDMA was also observed in other hepatic processes. Glycogen content showed a marked decrease, which was accompanied by a decrease in serum glucose levels. No significant changes in lipid peroxidation and hepatic GSH content were observed. In contrast, multiple MDMA administration produced some evidence of oxidative stress, namely, increased MDA content and decreased GSH content, a small decrease in liver glycogen at 3 h recovering 6 h post dose, no effect on blood glucose and increased AST and ALP activities but no effects on ALT activity. Seven days after the last MDMA injection a tendency towards recovery was shown. Conclusion: Our results show that the liver toxicity caused by MDMA administration involves several mechanisms.

Materials and methods

Drugs used

The MDMA was obtained from the Audiencia Provincial de Navarra. All other reagents were of the highest grade commercially available and were obtained from E. Merck (Darmstadt, Germany) and Sigma Chemical Company (St. Louis, MO, U.S.A.). Catalase, glutathione reductase, glutathione peroxidase, glutathione-S-transferase and superoxide dismutase were purchased from Randox Laboratories Ltd. (Crumlin, UK).
Animals and treatments

Male Wistar rats (200–220 g) were housed in plastic cages in a room with a controlled temperature (22°C) and maintained on a 12 h light-dark cycle with free access to food and water. Two different schedules of treatment with MDMA were used in this study. Acute treatment consisted of a single dose of MDMA (20 mg/kg, i.p.), the rats being sacrificed 3 h and 6 h later. Repeated treatment consisted of the same dose of MDMA (20 mg/kg, i.p.) which was given b.i.d. for 4 consecutive days, the rats being sacrificed 3 h, 6 h and 7 days later.

The dose of MDMA (20 mg/kg, i.p.) was chosen because it is in a similar range of doses (10–20 mg/kg) that have been shown to produce neurotoxicity in rats (17). MDMA dose refers to the hydrochloride. The time points (3 h, 6 h and 7 days) were selected because of the reported neurotoxic effects (18).

Measurement of hepatic reduced glutathione (GSH) levels

Hepatic GSH content was estimated by a colorimetric method as previously described by Ellman (21). Samples of liver (0.3 g) were homogenised in 6 ml of 10% Trychlooroacetic acid, containing 5 mM sodium ethylenediamine tetraacetic acid (EDTA), and later centrifuged at 3000×g for 15 min. The volume of the supernatants was fixed in 10 ml by addition of phosphate buffer (pH 7.4). Aliquots of 1.0 ml were taken and diluted to 10 ml with phosphate buffer. Then, 3.750 ml of these dilutions was mixed with a solution of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (0.08mg/ml in phosphate buffer) and incubated in a bath at 37°C for 30 min. After cooling, the absorbance of each sample was determined at 412 nm, and the GSH concentration of these samples was calculated with a standard curve using a dissolution of GSH (0.12 mg/ml) as standard.

Measurement of antioxidant enzymatic activities:

superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR) and catalase (CAT)

Animals were sacrificed by decapitation and their livers quickly removed and placed in homogenisation medium (250 mM mannitol, 70 mM sucrose, 1 mM EDTA adjusted with Tris to pH 7.4). One part of the liver was immediately homogenised in a Potter-Elvehjem glass homogeniser (1 g of tissue plus 10 ml homogenisation medium). Unbroken cells, cell debris, and nuclei were sedimated at 800×g for 10 min, and the supernatant was used as homogenate. This was kept on ice while being sonicated at 300 w for 30 seconds in three 10 seconds intervals and centrifuged at 20000×g for 10 min. Aliquots (1.0 ml) of the resulting supernatant were pipetted into plastic tubes, stoppered, and stored at −70°C until assayed. The enzyme activities of SOD, GR, GPx, GST and CAT were measured using a Cobas MIRA clinical analyser (Roche Diagnostic Systems, Inc., Basel, Switzerland). Analyses, and calculations were performed automatically according to the programmed instructions.

Assay of serum enzymatic activities and glucose serum levels

In order to determine serum transaminase and alkaline phosphatase activities and glucose serum levels, animals were anaesthetised using sodium pentobarbital, 50–60 mg/kg body weight, and blood samples were removed from the retro-orbital sinus. The enzymatic activities and glucose levels were measured by automated enzymatic assays.
using Technicon-Bayer kits for each enzyme (Technicon R.A.1000 autoanalyser).

Measurement of hepatic glycogen content

Hepatic glycogen content was determined according to the method of McGarry and Kawajima (22). Hepatic samples (0.3 g) were digested with 5 M KOH and were precipitated with ethanol. Later, samples were centrifuged at 1000×g for 15 min. An aliquot of the precipitate was mixed with 5 ml of anthrone reagent (0.05% w/v anthrone, 1% w/v thiourea in sulfuric acid 72%, v/v). The colour was developed by placing the tubes in a boiling bath for 10 min. The absorbance of each sample was determined at 620 nm and the glycogen concentration of these samples was calculated with a standard curve using a glycogen kit (E. Merck, Darmstadt, Germany).

Histology

Immediately after the sacrifice of the animals, liver sections were excised and immersed in 10% buffered neutral formalin for 24 h. Fixed portions were then processed, embedded in paraffin blocks, sectioned at 5 μm, mounted on glass slides, and stained with hematoxylin-eosin and Masson stains.

Statistical analysis

Data were reported as mean±SD and were analysed using the one-way analysis of the variance test (ANOVA), followed by the Scheffe test. The minimum number of animals used to determine mean values and statistical differences was eight for each treatment.

Results

Effect of MDMA on lipid peroxidation

A single dose of MDMA (20 mg/kg, i.p.) did not cause any significant change in lipid peroxidation. However, MDMA (20 mg/kg, i.p., b.i.d., for 4 consecutive days) produced an increase in lipid peroxidation, the effect being significant 3 h (18%) and 6 h (26%) after last injection. Seven days later, MDA levels returned to control values (Fig. 1).

Effect of MDMA on hepatic triglycerides and cholesterol levels

A series of experiments was performed to evaluate hepatic levels of triglycerides and cholesterol after acute and repeated MDMA administration. Acute MDMA treatment caused a significant increase in hepatic triglycerides and cholesterol content, the effect being significant 6 h after drug injection. Repeated administration of MDMA produced a significant increase in cholesterol levels, both 3 h and 6 h after drug administration, which returned to control values seven days later. However, no significant change in hepatic triglycerides content was observed after repeated MDMA treatment at any time of sacrifice (Table 1).

Effect of MDMA on reduced glutathione (GSH) hepatic content

A single dose of MDMA did not cause any significant change in hepatic GSH content. The effect

Table 1. Effect of single and repeated administration of MDMA on cholesterol and triglycerides content in rat liver

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival time</th>
<th>Cholesterol (mg/g liver)</th>
<th>Triglycerides (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td>3 h MDMA</td>
<td>1.06±0.10</td>
<td>9.97±0.41</td>
</tr>
<tr>
<td></td>
<td>6 h MDMA</td>
<td>1.40±0.10*</td>
<td>11.12±0.50*</td>
</tr>
<tr>
<td>Repeated</td>
<td>3 h Control</td>
<td>0.80±0.09</td>
<td>9.43±0.29</td>
</tr>
<tr>
<td></td>
<td>3 h MDMA</td>
<td>1.11±0.05**</td>
<td>10.24±0.64</td>
</tr>
<tr>
<td></td>
<td>6 h MDMA</td>
<td>1.40±0.05***</td>
<td>8.78±0.88</td>
</tr>
<tr>
<td></td>
<td>7 days MDMA</td>
<td>0.85±0.04</td>
<td>7.19±0.26</td>
</tr>
</tbody>
</table>

Animals received saline (control group), MDMA (20 mg/kg, i.p.) or MDMA (20 mg/kg, i.p., b.i.d. for 4 days) for single or repeated treatment respectively. Rats were sacrificed 3 h, 6 h and 7 days after the last MDMA injection. Values are mean±SEM from 8–10 rats. *p<0.05, **p<0.01, ***p<0.001 vs. control group using one-way analysis of the variance followed by Scheffe test.
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tasy-induced injury

Liver enzymes behaved differently. With the exception of GPx, the catalytic activities of the antioxidant enzymes in the liver did not show any significant change between different groups (data not shown). However, MDMA (20 mg/kg, i.p.) caused a slight decrease in glutathione peroxidase (GPx) activity, the effect being significant 7 days after repeated MDMA treatment (Fig. 2B).

Effect of MDMA on serum transaminase and alkaline phosphatase activities

The effect of MDMA on serum ALT, AST and ALP activities is shown in Table 2. AST activity showed an increasing tendency after acute MDMA treatment, but the significant increase became evident 6 h after drug administration. Repeated MDMA treatment caused a significant increase in

![Graph A](image)

**Fig. 2.** Effect of single (20 mg/kg, i.p.) and repeated (20 mg/kg, i.p., b.i.d., 4 consecutive days) administration of MDMA on GSH content (A) and on glutathione peroxidase activity (B) in rat liver. Animals were sacrificed 3 h, 6 h, and 7 days after the last MDMA administration. Data are the mean±SEM of 8-10 rats. *p<0.05, **p<0.01 vs. to control group (one-way analysis of the variance followed by Scheffe test).

Of repeated MDMA treatment was more marked. A significant reduction of approximately 22% and 27% in GSH hepatic content was found 3 h and 6 h respectively after the last injection. Seven days later, hepatic glutathione levels returned to control values (Fig. 2A).

**Effect of MDMA on antioxidant hepatic enzyme activities**

To establish the mechanism of toxicity as oxygen radical-mediated, there are a number of direct and indirect methods that can be employed. Direct methods include the measurement of superoxide hydrogen peroxide, or hydroxyl radical. These species are very reactive and their quantitation can be difficult. Therefore, indirect methods of study are often used. One such method is the measurement of changes in endogenous antioxidant enzyme activity. In this sense, the five principal antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase in liver were determined in rats after single or repeated MDMA administration.

![Graph B](image)

**Fig. 3.** Effect of single (20 mg/kg, i.p.) and repeated (20 mg/kg, i.p., b.i.d., 4 consecutive days) administration of MDMA on hepatic glycogen content (A) and on glucose serum levels (B) in rat. Animals were sacrificed 3 h, 6 h, and 7 days after the last MDMA administration. Data are the mean±SEM of 8-10 rats. *p<0.05, **p<0.01 vs. to control group (one-way analysis of the variance followed by Scheffe test).
Table 2: Effect of single and repeated administration of MDMA on serum ALT, AST and ALP activities in rat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td>Control</td>
<td>28.66 ± 2.20</td>
<td>83.92 ± 5.89</td>
<td>251.67 ± 3.67</td>
</tr>
<tr>
<td></td>
<td>MDMA 3h</td>
<td>29.33 ± 1.51</td>
<td>86.70 ± 4.43</td>
<td>361.17 ± 11.76</td>
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<tr>
<td></td>
<td>MDMA 6h</td>
<td>44.34 ± 5.37**</td>
<td>133.97 ± 14.78**</td>
<td>384.50 ± 35.59**</td>
</tr>
<tr>
<td>Repeated</td>
<td>Control</td>
<td>32.00 ± 1.25</td>
<td>79.83 ± 1.45</td>
<td>250.43 ± 10.05</td>
</tr>
<tr>
<td></td>
<td>MDMA 3h</td>
<td>34.80 ± 2.04</td>
<td>102.00 ± 2.91***</td>
<td>337.38 ± 76.99</td>
</tr>
<tr>
<td></td>
<td>MDMA 6h</td>
<td>31.00 ± 3.21</td>
<td>98.00 ± 2.55**</td>
<td>336.66 ± 2.91**</td>
</tr>
<tr>
<td></td>
<td>MDMA 7days</td>
<td>33.83 ± 1.26</td>
<td>101.43 ± 3.67**</td>
<td>385.29 ± 32.14**</td>
</tr>
</tbody>
</table>

Animals received saline (control group), MDMA (20 mg/kg, i.p.) or MDMA (20 mg/kg, i.p., b.i.d. for 4 days) for single or repeated treatment respectively. Blood samples were taken 3 h, 6 h and 7 days after the last MDMA injection. Values are mean ± SEM from 8–10 rats. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control group using one-way analysis of the variance followed by Scheffe test.

AST activity. Seven days later AST activity remained significantly increased.

A single injection of MDMA significantly increased ALT activity 6 h after drug administration. Repeated MDMA treatment did not elicit any significant change in ALT activity at any time of sacrifice.

MDMA caused a significant increase in ALP activity at both, 3 h and 6 h after single or repeated drug administration. Furthermore, seven days later, ALP activity remained significantly increased.

Effect of MDMA on glycogen hepatic levels and glucose serum levels

MDMA caused a significant decrease in glycogen hepatic content, the effect being more marked after acute treatment. Seven days after repeated MDMA treatment, glycogen hepatic levels returned to control values (Fig. 3A).

Serum glucose levels after single or multiple administration of MDMA are shown in Fig. 3B. MDMA caused a substantial reduction in the glucose levels 6 h after a single injection. However, no significant effect on glucose serum levels after repeated MDMA treatment was observed.

Histological examination

Figure 4A shows the liver section of a control rat showing, in the centre of the photograph, the portal tract. Striking changes were identified in the liver. Acute MDMA treatment caused cell necrosis particularly in portal areas with inflammatory infiltrate consisting in lymphocytes and macrophages following administration of MDMA. Animal received saline (control group) (A) or MDMA (20 mg/kg, i.p.) (B) being sacrificed 6 hours later.
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Fig. 5. Liver histology showing the hepatic vein in the centre of the photograph. Hepatic necrosis with inflammatory infiltrate around hepatic vein following administration of MDMA. Animal received saline (control group) (A) or MDMA (20 mg/kg, i.p., b.i.d., for 4 days) (B) being sacrificed 6 hours later.

phages denser in portal tracts, 6 h post administration (Fig. 4B). Figure 5A shows the liver section of a control rat showing, in the centre of the photograph, the hepatic vein. Repeated injection produced cell necrosis and inflammatory infiltrate around the hepatic vein. These effects were more marked 6 h after the last administration (Fig. 5B) but seven days later no changes were observed.

Discussion

Hapatotoxicity is one of the medical consequences of MDMA consumption (14). Jaundice, hepatomegaly, centrilobular necrosis and hepatitis are some of the MDMA-induced liver conditions (11, 14). The most reported form of liver injury is hepatitis (15-17, 23).

The mechanism of MDMA-induced hepatic injury is unclear but a spectrum of severity seems to exist as assessed by histological changes varying from mild to moderate lobular hepatitis and to features of massive hepatic parenchymal collapse with areas of nodular regeneration. The severity of liver damage does not seem to correlate with either the amount or frequency of MDMA ingested suggesting an idiosyncratic type of reaction (13).

In the present study, the major biochemical events indicative of liver injury after acute and repeated MDMA administration to rats, were investigated. The attack of reactive oxygen species on polyunsaturated fatty acids, essential constituents of biological membranes, has been shown to result in peroxidative damage of these lipids (24). Malondialdehyde, the reaction product of lipid peroxidation usually determined in experiments, exerts several biological effects including cross-linking of proteins and nucleic acids, and is presumably mutagenic and carcinogenic. Lipid peroxidation and subsequent cellular damage are regarded as an important mechanism underlying the toxicity of several xenobiotics (25).

Living organisms contain various free radical scavenging systems for the clarification of the pathologic role of free radicals, it is therefore essential to estimate the changes in both the generation and the scavenging of free radicals. The reduced form of glutathione (GSH), the major intracellular thiol, neutralizes many kinds of radicals, either directly or in association with glutathione peroxidase (26).

Depletion of GSH, especially in the liver, can cause irreversible damage and cell death. GSH plays an important role in protecting cells against reactive O₂ intermediates and free radicals. Glutathione deficiency worsens free radical-induced toxicity, while free radical production depletes glutathione. Decreased glutathione is an early marker of free radical insult and is evident before overt cell death occurs (27).

A single dose of MDMA did not change glutathione levels, and free radical production from MDMA was not quantitatively significant enough to deplete glutathione and allow uncontrolled reaction with vital cellular components. In contrast, re-
peated MDMA injection produced a sufficient quantity of free radicals to deplete glutathione. Furthermore, glutathione peroxidase activity showed a tendency to decrease after MDMA injection. In addition, not only GSH itself but also the GSH redox cycle, catalysed by the enzyme glutathione peroxidase, could contribute to radical scavenging.

Numerous studies have documented that decreases in glutathione precede lethal cell injury from free radical generators (28). The leakage of intracellular enzymes, suggests irreversible damage. In this sense, ALT and AST serum activities as indicators of liver injury were determined. Three hours after a single dose of MDMA, no significant changes were observed. In contrast, a significant increase in ALT and AST activities, 6 h after drug administration were obtained. Repeated MDMA treatment produced a significant increase in AST activity but no effect on ALT activity was observed. In all cases, MDMA produced a disproportionate increase in AST activity compared with ALT activity. This effect is frequently an index of important cell necrosis. Furthermore, ALP activity as indicative of cholestasis was determined. MDMA caused an increase in ALP activity, the effect being more marked after a single dose. Seven days after repeated MDMA treatment, ALP activity remained increased. These effects resemble those seen in humans in which increases in ALT, AST and ALP activities have been reported (13, 15, 29, 30). These findings were corroborated with histological examination which, according to the post-mortem changes was reported in deaths associated with MDMA intake (12–17). Hepatic necrosis and inflammatory infiltrate were evident and overall findings were consistent with a drug related hepatitis.

There appears to be a relationship between glutathione deficiency and glycogen metabolism. Recently, it has been reported that glutathione depletion caused by various drugs stimulates glycogen breakdown (31). Our results show that repeated administration of MDMA (20 mg/kg, i.p.) causes a significant decrease in hepatic glycogen content which is not accompanied by a modification in serum glucose levels. In this sense, the effect on hepatic glycogen content could be explained by the significant GSH depletion observed after repeated MDMA treatment.

On the other hand, 3 h after a single dose of MDMA a 60% reduction of liver glycogen content, without additional effect on serum glucose levels was observed. A larger reduction of glycogen content (approximately 83%) and a significant decrease of plasma glucose levels were found 6 h after MDMA.

By contrast glutathione levels did not change after acute MDMA treatment. It has been recently suggested that MDMA could be regarded as a chemical stressor as MDMA-induced neurochemical, behavioural and endocrine alterations closely resemble those elicited by exposure to acute stress (32). McGuinness et al. established that during chronic stress hormone infusion the rise in epinephrine exerts potent stimulatory effects on glucose production principally by enhancing hepatic glycogenolysis (33). Thus, these results suggest that MDMA-induced stimulation of glycogenolysis could be associated with the sympathomimetic effects observed after acute MDMA administration (34).

Taken together with the present results, the liver toxicity caused by acute MDMA treatment involves several mechanisms which cause increased lipid levels, increased liver enzymes and impaired gluconeogenesis leading to a fall in blood glucose and depletion of liver glycogen. In contrast repeated treatment produced some evidence of oxidative stress, namely, increased MDA content and decreased GSH content, but no evidence of hepatocyte damage. As far as we know, these results could be the first major step for research in liver injury. Furthermore, according to the sympathomimetic effects reported after acute MDMA injection (34), these results could be explained by the acute effect of the last dose rather than any long-term chronic effects.

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References

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