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Article

Study the effect of 3,4-Methylenedioxy methamphetamine on cytochrome P450 2E1 activity

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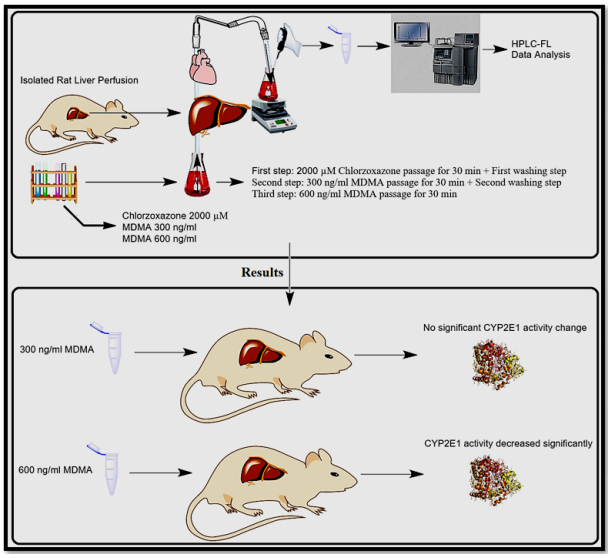
Evaluating the effects of ecstasy on CYP2E1 activity is of great concern, mainly due to growing trends in abuse and co-administration of MDMA with ethanol and the dominant role of this isoenzyme on ethanol metabolism. This study aimed to evaluate the effects of MDMA on CYP2E1 activity. A total of 24 male rats were selected and divided into three groups. The first and second groups consisted of 12 rats and were employed to optimize the perfusion method, and the third group was employed for studying the alteration of CYP2E1 activity after liver exposure to MDMA (300 and 600 ng/ml). The amount of chlorzoxazone and 6-hydroxy chlorzoxazone in a sample obtained from liver perfusion before and after exposure to a buffer containing MDMA was determined by HPLC-FL. The enzymatic activity of rat CYP2E1 decreased after liver perfusion with a buffer containing 600 ng/ml of MDMA. However, no significant changes were observed in chlorzoxazone and 6-hydroxy chlorzoxazone concentration in perfusate before and after liver perfusion with a buffer containing 300 ng/ml of MDMA. Our findings suggest that the activity of CYP2E1 in rats might decrease only after administration of MDMA at a lethal dose. However, further animal and human studies are needed to confirm our assumption.

Keywords: MDMA. Ecstasy. Chlorzoxazone. Isolated perfused rat liver (IPRL). Rat CYP2E1.

Graphical Abstract



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INTRODUCTION

MDMA (3, 4-methylenedioxymethamphetamine), also known as ecstasy, is recognized as one of the most significant trends in drug abuse among young people (Palamar, Kamboukos, 2014). According to the World Drug Report published by United Nations Office on Drug and Crime in 2017 (Han, Parker, 2017), the number of ecstasy users was estimated to be around 21.6 million.

The liver is the prime target for MDMA injury since this organ is the major site for the metabolism and bioactivation of MDMA (Rouini M *et al*., 2020). Several mechanisms have been proposed to explain the toxicities associated with the MDMA abuse, including increased outflow of neurotransmitters, impairment of mitochondrial function, oxidation of biogenic amines, drug-drug interactions from polydrug abuse, metabolic bioactivation, and hyperthermia (Baumann, Rothman, 2009; Docherty, Green, 2010). Unfortunately, estimating the MDMA-induced liver damage is complicated since the initiation and extent of the liver damage associated with MDMA abuse are not proportional to the amount and interval of the drug ingestion. Besides, abusers tend to have different polydrug abuse habits, and the MDMA content of illicit tablets varies considerably (Henry, Jeffreys, Dawling, 1992; Morefield *et al*., 2011). It has been clearly shown that biotransformation of parent compound has a profound role in MDMA-related hepatotoxicity because of the formation of active redox metabolites, among the other mechanism proposed for MDMA toxicities (Antolino-Lobo *et al*., 2010; Carvalho *et al*., 2010). Phase I metabolism of MDMA involves two main routes, including N-demethylation to 3,4-methylenedioxy amphetamine (MDA) catalyzed mainly by CYP1A2 and to a lesser extent by CYP2B6 and CYP2C19, and/or O-demethylation of the ring substitute of both MDMA and MDA into the highly reactive catechol metabolites of 3,4-dihydroxymethamphetamine (HHMA) and 3,4-dihydroxyamphetamine (HHA) predominantly by CYP2D6 and to a less extent by CYP3A4 and CYP2C19 isoenzymes (Meyer, Peters, Maurer, 2008; Van *et al*., 2007). The two latter could be further oxidized to the corresponding ortho-quinones, which can enter in a redox cycling leading to the formation of reactive oxygen and nitrogen species, which has been proposed to be the reason for cytotoxicity in several tissues such as liver, brain, kidney, and heart (Antolino-Lobo *et al*., 2010; Carvalho *et al*., 2002). While several studies have focused on the interaction of MDMA with CYP2D6 and CYP3A4 enzymes (Antolino-Lobo *et al*., 2011b; Downey, Daly, O'Boyle, 2014; Rietjens *et al*., 2012), we believe that studying the damage to the other CYP450 isoenzymes not involved in MDMA biotransformation would also be important.

MDMA is mainly metabolized in the liver by several cytochrome P450 isoenzymes, including CYP2D6, CYP3A4, CYP1A2, CYP2B6, and CYP2C19. Several studies elucidated the inhibitory effect of MDMA on the enzyme involved in its metabolism, which are CYP2D6, CYP3A4, and CYP1A2 (Antolino-Lobo *et al*., 2011b; Meyer *et al*., 2010).

Simultaneous use of MDMA with other substances should be of great concern since MDMA is generally taken with other agents to enhance psychological effects; there is also likely the deliberate co-administration of MDMA with other drugs among ecstasy abuser in order to attenuate the aversive effects (Gouzoulis-Mayfrank, Daumann, 2006; Yuki *et al*., 2013). Thus, it is feasible to propose that exposure to MDMA may increase the potential for drug-drug interaction adverse effects through alteration in the activity of the enzymes which are not involved in this drug metabolism. To evaluate the extent of activity alteration for enzymes that are not involved in MDMA metabolism, we focused on investigating the activity of Cytochrome P450 2E1 upon exposure to MDMA in a series of ex-vivo experiments using the isolated perfused rat liver (IPRL) method. Liver perfusion has been extensively used in pharmacological and toxicological studies in recent years (Neyshaburinezhad *et al*., 2021; Neyshaburinezhad *et al*., 2020a; Neyshaburinezhad *et al*., 2020b; Rezai *et al*., 2020).

As the fatal toxicity associated with the simultaneous use of ethanol and ecstasy has been frequently reported, the study of the potential increase in MDMA-induced hepatotoxicity caused by ethanol is of great clinical importance (Pontes *et al*., 2008; Upreti *et al*., 2009).

CYP2E1 is involved in the biotransformation of ethanol and a miscellaneous group of drugs, including chlorzoxazone, acetaminophen, and volatile anesthetics. It is also reported that CYP2E1 is important for the inactivation of the group of environmental carcinogens

such as nitrosamines, benzene, and styrene. Among these drugs, chlorzoxazone (CZX) has been extensively used as an appropriate probe substrate in most surveys to determine enzyme activity in both in vitro and in vivo studies (Ernstgard, Warholm, Johanson, 2004; Muzeeb *et al*., 2005). Reports indicated that rats are the best animal models for human CYP2E1 (Martignoni, Groothuis, de Kanter, 2006), and therefore rat liver was employed in this study. It was demonstrated that the formation of 6-OH-chlorzoxazone (6-OH-CZX) is more specific for CYP2E1 activity at high substrate concentrations (500 μM) in contrast to low substrate concentrations (10 μM) (Yuan *et al*., 2002).

The present study was carried out to investigate the alteration in enzymatic activity of the hepatic CYP2E1 by using CZX as a probe drug with isolated perfused rat liver (IPRL) to different inlet concentrations of MDMA.

MATERIAL AND METHODS

Material

CZX and 6-OH-CZX were obtained from Sigma–Aldrich (Poole, Dorset, UK). Standards of MDMA, MDA, HMA, and HHMA were synthesized in the Medicinal Chemistry Department, Faculty of Pharmacy, Tehran University of Medical Sciences. The structure of the compound was confirmed by IR, 1H-NMR, and 13C-NMR spectra. All other chemicals were supplied by Merck (Darmstadt, Germany). The water used in all experiments was of Direct-Q® quality (Millipore, France). Xylazine 2% and ketamine 10% (Alfasan, Holand) were acquired from a local supplier.

Animal studies

Animals

Twenty-four male Sprague Dawley rats (weighing 250–300 g) were maintained in a clean room with 12 hours light-dark cycle, controlled temperature (20-23 ˚c), relative humidity of 50%, and free access to standard laboratory chow and water for this experiment. Rats were divided into three groups.

In the first group, eight rats were divided into four subgroups and given CZX-containing medium (100, 500, 1000, and 2000 μM) in a single-pass experiment (n=2 in each study) to justify the probe substrate concentration and the time of perfusion.

In the second group, other four rats were employed and split into two subgroups to determine the time required for the first washing step (after liver perfusion with 2000 μM CZX) and second washing step (after liver perfusion with 600 ng/ml of MDMA) in order to minimize the total time of perfusion (n=2 in each study).

In the third group, the remaining 12 rats were divided into two subgroups and received a medium containing MDMA (300 and 600 ng/ml) by liver perfusion to estimate the alteration in CYP2E1 activity.

The animals in the first and second groups were employed to optimize the perfusion method, while the animals in the third group were utilized to study the alteration of the CYP2E1 enzymatic activity.

This study was approved by the Institutional Review Board of Pharmaceutical Research Centre of Tehran University of Medical Sciences [Project Code: 93-02-33-21634].

Liver perfusion

The rats were anesthetized using an intraperitoneal injection of xylazine (15mg/kg) and ketamine (75 mg/kg) mixture. The portal vein and superior vena cava (as input and output of the perfusion medium) were catheterized using intravenous 16- and 18-gauge catheters, respectively. Five hundred units of heparin were injected into the inferior vena cava. The perfusion medium was freshly prepared Krebs-Henseleit buffer in a total volume of about 200 ml reservoir. The perfusion medium was passed through the portal vein with a constant flow rate of 10 ml/min using a peristaltic pump.

The temperature (37°±0.5˚C), pH (7.4±0.2), and perfusion pressure (14 mmHg) were intermittently monitored and maintained throughout the experiment. Liver viability was proven by the overall macroscopic appearance of the liver (wet liver weights of < 4% of body weight at the end of perfusion) and monitoring the liver enzyme activities (AST and ALT) throughout

the perfusion at different time intervals (Jamshidfar *et al*., 2017).

Experimental protocols

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology Policy for Experimental and Clinical studies (Tveden-Nyborg, Bergmann, Lykkesfeldt, 2018).

To reduce between-subject variations, this study was designed such that any alterations in CYP2E1 activity before and after MDMA exposure could be measurable in all rat perfused livers. Hence, any changes in probe substrate metabolism before and after liver perfusion with MDMA-containing medium encountering of each perfused liver with MDMA were calculated to demonstrate any induction or inhibition on CYP2E1 activity

Five experimental protocols were planned based on evaluations to address different concerns during the course of experiments for this investigation, and all initial results were used to justify the final MDMA and probe substrate concentrations used in this study along with the minimum time need for each designed step.

Time and Concentration dependent metabolism of probe substrate (CZX) in isolated perfused rat liver (Pre-exposure to MDMA)

In cytochrome P450 probe reaction studies, special care must be taken with the substrate concentrations, and the measured enzyme-substrate affinity data should be provided. For this reason, selecting a saturated substrate concentration with respect to Michaelis–Menten constant (Km) for the test system is recommended in common microsomal or cell culture studies to activate mainly the considered CYP450 pathway and maintain high selectivity.

As there are few enzyme kinetics studies in isolated perfused rat liver conditions, this experiment was designed to calculate the appropriate probe substrate concentration (CZX) that should be used in this study. After pre-stabilization of the perfused liver by the drug-free medium for 10 min, the perfusion was continued with the CZX -containing medium (100, 500, 1000, and 2000 μM) in a single-pass experiment (n=2 in each study). Samples were taken for 30 minutes from the start of the perfusion at 5 minutes intervals.

All samples were centrifuged and refrigerated at −70 °C until assayed. Additionally, the inlet concentrations remained relatively constant during the entire perfusion time for all mentioned conditions.

In order to determine the time required for reaching the steady-state, the medium containing CZX (100, 500, 1000, and 2000 μM) was perfused through the liver for 1 hour (n=2 in each study), and samples were collected at a 5-minute interval.

Formation of the 6-OH-CZX by CYP2E1 increased when a higher concentration of probe drug was used in perfusion. However, the rise in the amount of 6-OH-CZX is not proportional to the increase in the concentration of CZX in the medium. Hence, we can assume that maximum enzyme-substrate affinity is reached with 2000 μM concentration of probe drug. The changes in the area under the curve ($AUC_{(0-30)}$) for CZX and 6-OH-CZX after liver perfusion with the medium containing 100, 500, 1000, and 2000 μM and the corresponding metabolic ratios.

The obtained results indicated that an increase in the AUC of the CZX and 6-OH- CZX is not proportional to the increase of the CZX concentration in the perfusion medium. Besides, the mean metabolic ratio and extraction value declined by increasing the CZX concentration in the perfusion medium. Thus the 2000 μM probe concentration in rat perfusion medium was used as a saturation concentration to study the alteration in CYP2E1 activity and chosen as appropriate probe concentration for rat liver perfusion.

Both CZX and metabolite reached relatively steady concentrations in the outlet soon after the start of perfusion. Therefore, the time for liver perfusion with a medium with CZX as a probe substrate (pre-and post-exposure to MDMA) was reduced to 30 minutes to decrease the total time of perfusion.

First washing step

In order to determine the time required for the first washing step, liver perfused with medium with the highest amount of the CZX employed in this study (2000 μM) for 30 minutes. Consequently, a drug-free medium was passed through the perfused liver for a

further 60 minutes, and samples were collected at 5 min time intervals to determine the minimum time required for the first washing step to achieve minimum CZX concentration (below 5% of initial CZX concentration).

Analysis of samples collected in the first washing step indicated that after 20 minutes of liver perfusion with the drug-free medium, the CZX concentration decreased to below 5% of its initial concentration after applying the highest probe substrate concentration (2000 μM).

Hence, after the liver exposure to the medium with CZX in the first phase, the perfusion was continued for a further 20 min with a drug-free medium to ensure removing of CZX effect on MDMA metabolism in all experiments.

Liver exposure to MDMA

Since the maximum plasma concentration after a common recreational dose of MDMA (100 mg) was reported between 200-300 ng/ml and the reported plasma concentrations of this compound in the mortality associated with MDMA was between 600-2800 ng/mL, we decided to study the effect of MDMA on other metabolism pathways in two definite concentrations of 300 and 600 ng/ml of MDMA.

Our unpublished data on the inhibition of CYP2D6 by MDMA in rat perfused liver indicated that the maximum changes in the outlet concentration of this drug occurred in the first 30 minutes of the perfusion, and after this time, the MDMA and its main metabolites (MDA, HMA, and HHMA) reached relatively steady concentrations in the outlet perfusates. Hence, after the first washing step, the MDMA-containing medium (300 and 600 ng/ml) was passed through the liver for 30 min, and samples were collected at 5 min time intervals. All collected samples were centrifuged and refrigerated at −70 °C until analysis.

Effect of MDMA on the metabolism of CZX (second washing step)

In order to determine the time required for the second washing step, liver perfused with medium containing the highest amount of the MDMA employed in this study (600 ng/ml) for 30 minutes. Consequently, a drug-free medium was passed through the perfused liver for a further 60 min, and samples were collected at 5 min intervals to determine the minimum time required for the washing step to achieve minimum MDMA concentration (below 5% of initial MDMA concentration).

Analysis of samples collected in the second washing step indicated that after 20 minutes of liver perfusion with the drug-free medium, the MDMA concentration declined to below 5% of its initial concentration even after applying the highest concentration of MDMA employed in this study (600 ng/ml).

Thus, after liver perfusion with a medium containing MDMA, the perfusion was continued for a further 20 min with a drug-free medium to ensure removal of MDMA residue on perfusate.

Metabolism of CZX after exposure to MDMA (post-exposure step)

To evaluate any alteration in enzymatic activity after liver perfusion with Krebs-Henseleit buffer containing MDMA, the perfusion was continued with the medium containing CZX (2000 μM) for 30 minutes, and samples were collected at 5 min intervals. All collected samples were centrifuged and refrigerated at −70 °C until analysis.

HPLC methods

MDMA

MDMA concentration in the samples was determined by a previously developed HPLC-Fluorescence method (Jamali *et al*., 2017).

CZX and 5-OH- CZX

The HPLC method used to determine the determination of the CZX and 6-OH- CZX in the samples was previously described by Leclercq et al. with some modifications (Leclercq, Horsmans, Desager, 1998).

Pharmacokinetic analysis

The mean concentration at steady state

The mean concentration of CZX and 6-OH-CZX at the steady-state was the first pharmacokinetic parameter used in this study; hence the mean CZX and 6-OH-CZX concentration in outlet perfusate for the last three sampling time (20, 25, and 30 minutes) before and after liver exposure to 300 and 600 ng/ml calculated.

Metabolic ratio

The metabolic ratio was the second parameter employed in our study using the following formula:

$$\text{Metabolic ratio \% (MR)} = \frac{\text{Metabolite concentration at a specific time point}}{\text{Chlorzoxazone concentration at the same time point}} \times 100$$

The concentration CZX and 6-OH-CZX at the steady-state were used in these formulas.

Statistical Analysis

A paired t-test was employed using SPSS 20 to determine whether the changes in pharmacokinetic parameters before and after liver exposure to MDMA were statistically significant. A P-value of less than 0.05 was considered statistically significant. The data were expressed as mean ± SD.

RESULTS

Effect of rat liver perfusion with 300 ng/ml of MDMA on the CYP2E1 activity

The changes in CZX and 6-OH- CZX metabolic ratios for each sampling time before and after liver exposure to 300 ng/ml of MDMA are given in Figure 1A.

The mean CZX concentration decreased slightly after MDMA exposure from 1580.7 ± 77 to 1545 ± 65.7 μM while the 6-OH-CZX concentration increased from 9.1 ± 1 to 9.73 ± 0.9 μM after MDMA exposure. Therefore, the metabolic ratio increased from 0.58% ± 0.07 before MDMA exposure to 0.63% ± 0.05 after liver exposure to MDMA (Figure. 2). None of these changes were statistically significant (P-value > 0.05).

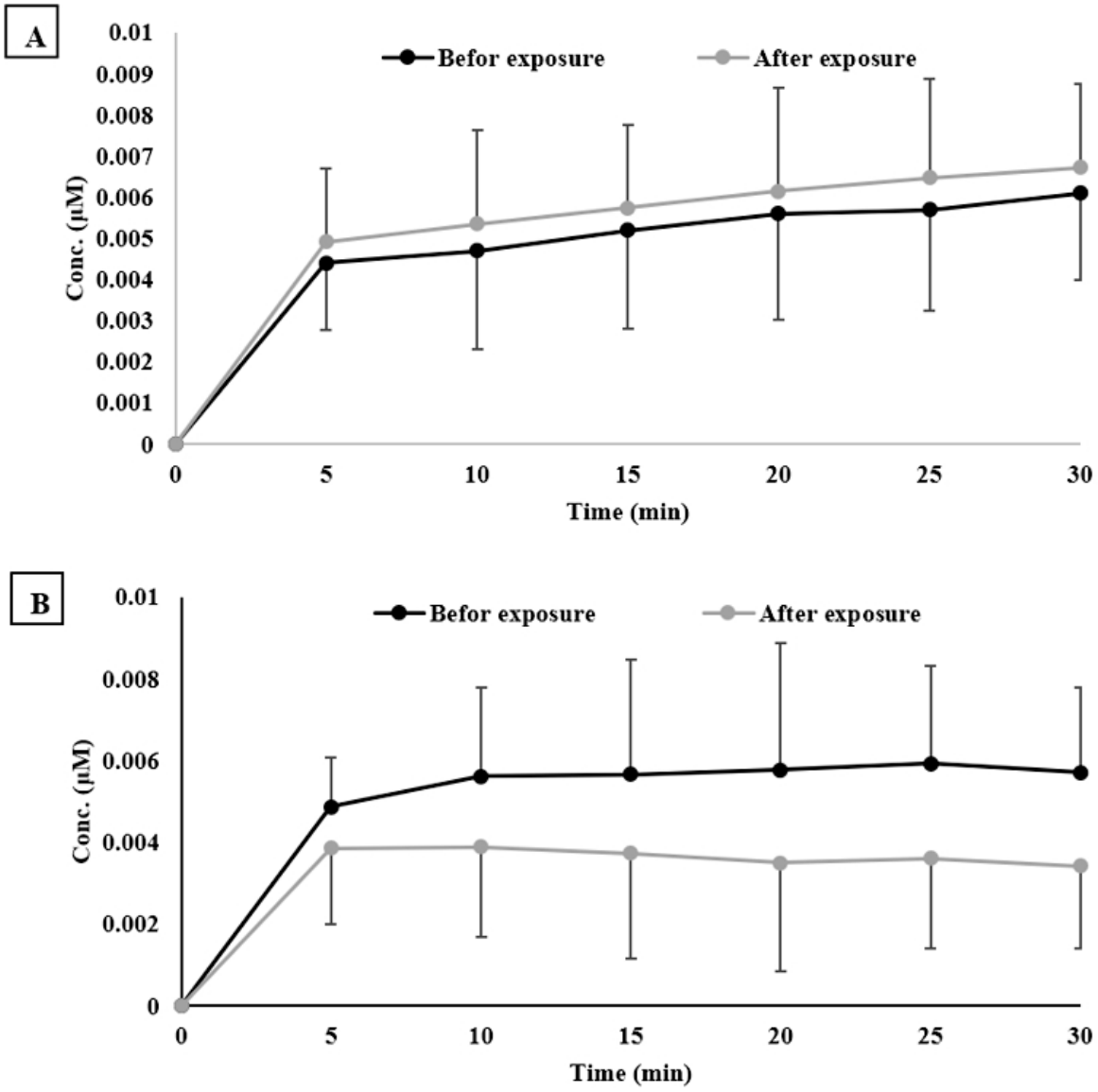

FIGURE 1 - Perfusate concentration-time profile of mean metabolic ratios before and after exposure of rats' liver to 300 ng/ml **(A)** and 600 ng/ml **(B)** of MDMA (Ecstasy) following the passage of the perfusion buffer containing 2000 µM chlorzoxazone through the portal vein.

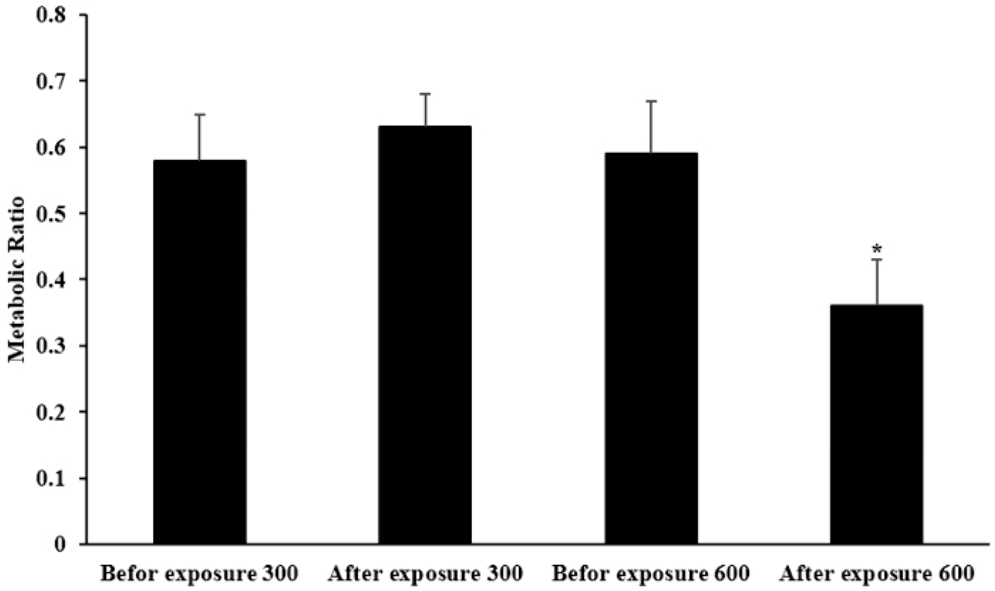

FIGURE 2 - Perfusate mean metabolic ratio profile (3 end-points), before and after exposure of rats' liver to 300 ng/ml and 600 ng/ml of MDMA (Ecstasy), following the passage of the perfusion buffer containing 2000 µM chlorzoxazone through the portal vein (n= 12, mean ± SEM). Each experiment was repeated independently three times in triplicate tests and data are shown as mean ± SEM. *P≤ 0.05.

Effect of rat liver perfusion with 600 ng/ml of MDMA on the CYP2E1 activity

The concentration profiles of CZX, 6-OH-CZX metabolic ratios before and after liver exposure to 600 ng/ml of MDMA are shown in Figure 1B.

The mean CZX concentration in perfusate at steady-state raised significantly from 1384 ± 21.8 to 1657.1 ± 74.2 µM after liver perfusion with a medium containing 600 ng/ml MDMA. The mean 6-OH-CZX concentration for the last three sampling times in perfusate before and after liver exposure to MDMA (600 ng/ml) was determined to be 8.1 ± 0.9 and 5.9 ± 1.2 µM, respectively, and the corresponding metabolic ratios decreased from 0.59% ± 0.08 to 0.36% ± 0.07 after liver exposure to MDMA (Figure. 2). This change was statistically significant (P-value = 0.03).

DISCUSSION

As mentioned before, studying the effects of MDMA on CYP2E1 isoenzyme is of great importance for several reasons.

Firstly, CYP2E1 is involved in the metabolism of ethanol, and the reports indicated the high frequency of polydrug use among MDMA abusers, notably ethanol users (Barrett, Darredeau, Pihl, 2006; Lieber, 1997). The extensive co-administration of these two substances increases the risk of hepatotoxicity and neurotoxicity associated with MDMA abuse (Vollenweider *et al*., 2002).

Secondly, several studies have indicated that MDMA administration leads to the induction or inhibition of an enzyme involved in its own mechanism and may affect the enzymatic activity of other isoenzymes in the same manner (Nilchi et al., 2018; Yang *et al*., 2006). In this research, we conducted an ex-vivo study on the CYP2E1 enzymatic activity alteration of MDMA by using CZX as a well-established probe drug with the aim of isolated perfused rat liver method.

Finally, CYP2E1 is essential for the inactivation of a group of carcinogens, namely nitrosamines, benzene, and styrene; hence studying the alteration in the activity of this isozyme is of great concern (Lieber, 1997).

The MDMA plasma level after common recreational use and lethal dose reported to be 300 ng/ml and 600

ng/ml, respectively (Milroy, 2011). Thus, in this study, animals were subjected to Krebs-Henseleit buffer containing 300ng/ml and 600ng/ml MDMA.

The technique used in this study was the isolated perfused rat liver since we wanted to study the enzymatic activity alteration of CYP2E1 in a rat's liver. In order to minimize the within-subject variation, the CYP2E1 activity was estimated before and after exposure to MDMA in the same rat with two washing steps.

The data obtained from this study demonstrate that CYP2E1 activity after liver exposure to 600ng/ml MDMA decreased; however after liver perfusion with 300 ng/ml MDMA, no significant change in CYP2E1 activity was observed (Figure 1 and 2).

The reduction in CYP2E1 activity after perfusion with 600 ng/ml of MDMA was indicated by a significant increase in CZX concentration, while an insignificant decrease in 6-OH-CZX concentration in the last three sampling times was observed. The rise in CZX concentration simultaneously with the reduction of 6-OH-CZX after liver perfusion with 600 ng/ml of MDMA could also demonstrate the reduction in the CYP2E1 enzymatic activity.

There are several explanations for the variation between CYP2E1 enzymatic activities after liver exposure to 300 ng/ml and 600 ng/ml.

First of all, as we stated earlier, CZX is metabolized primarily to 6-OH-CZX in the presence of the hepatic CYP2E1; it is then rapidly glucuronidated and eliminated by the kidney (Conney, Burns, 1960; Mishin *et al*., 1998). In this study, we estimated the amount of 6-OH-CZX, which is known as a free metabolite in perfusate, and did not assess the amount of conjugated metabolite. Since phase II metabolism occurs rapidly, the estimation of the conjugated and unconjugated metabolite together might be more reliable for the assessment of CYP2E1 activity.

Secondly, it looks as if MDMA could change the activity of the enzymes involved in phase II metabolism in addition to alteration the activity of phase I enzymes (Antolino-Lobo *et al*., 2011a). According to the result of this study, reduction in the activity of phase II enzymes is more probable even with lower doses of MDMA in comparison to phase I enzymes (CYP2E1) since the apparent increase of 6-OH-CZX with negligible changes in CZX concentration was occurred after liver perfusion with 300 ng/ml of the compound. However, after liver perfusion with a buffer containing 600 ng/ml MDMA, the CZX concentration increased significantly, and the 6-OH-CZX concentration reduced, which could be explained by a reduction in the activity of both phase I and II enzymes at this concentration. Further animal studies are required to confirm this hypothesis.

CONCLUSION

The data obtained from this study indicated that MDMA could not reduce the activity of the CYP2E1 at recreational doses (300 ng/ml), while at the lethal doses (600 ng/ml), it could reduce the enzyme activity. We can extrapolate the data from this study to humans since CZX is a well-established probe drug for CYP2E1 and reports indicated that hepatic rat's CYP2E1 is a major enzyme involved in the hydroxylation of CZX. Thus, we can presume that at a higher dose, MDMA may reduce CYP2E1 activity and alter the ethanol metabolism in humans since rat CYP2E1 is the best animal model for human CYP2E1. Although to support our hypothesis, further human and animal studies are required.

DECALRATION OF INTEREST

The authors report no conflicts of interest.

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