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Expression of *bax* and *bcl2* Genes in MDMA-induced Hepatotoxicity on Rat Liver Using Quantitative Real-Time PCR Method through Triggering Programmed Cell Death

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Abstract

Background: 3-4methylenedioxymethamphetamine (MDMA) is a synthetic and psychoactive drug, which is known popularly as Ecstasy and has toxic effects on human organs.

Objectives: Considering the potential toxic interaction, this study was performed to quantify the expression of *bax* and *bcl2* genes in MDMA-induced hepatotoxicity on rat liver. Subsequently, we evaluated pentoxifylline as a possible protective drug on hepatotoxicity.

Materials and Methods: Adult male Wistar rats weighting 250 - 300 grams were used in the study. The rats were equally distributed into four experimental groups (5 rat/group). MDMA was dissolved in PBS and injected intraperitoneally (IP) including untreated control, MDMA (MDMA dissolved in PBS), treated-1 (MDMA followed by PTX) and treated-2 (PTX followed by MDMA). All animals given MDMA received 3 doses of 7.5mg/kg with two hours gap between doses. Liver tissue was removed after anaesthetizing. Subsequently, RNA isolation, cDNA synthesis and Real-Time PCR were performed. Finally, data analyzed statistically to determine significantly differences between the groups (P value < 0.05).

Results: Using Real-Time quantitative PCR results, the gene expression ratio of *bcl2* were calculated 93.80±20.64, 340.45 ± 36.60 and 47.13 ± 5.84 fold in MDMA, treated-1 and treated-2 groups, respectively. Furthermore, this ratio for *bax* gene obtained 2.13±0.33 fold in MDMA, 1.55 ± 0.26 fold in treated-1 and 10.44 ± 1.56 fold in treated-2 groups.

Conclusions: The present study focused on molecular mechanism of MDMA in programmed cell death using gene expression quantification of a pro-apoptotic and anti-apoptoic gene in MDMA-induced hepatotoxocity. The results showed that MDMA prompted apoptosis in liver and pentoxifylline protected against hepatotoxicity before and after taking MDMA.

Keywords: Pentoxifylline, Anti-Apoptotic Gene, MDMA, Pro-Apoptotic Gene, qReal-Time PCR

1. Background

3-4methylenedioxymethamphetamine (MDMA) is a synthetic, psychoactive drug structurally similar to the stimulant methamphetamine and the hallucinogen mescaline (1). Although MDMA has been commonly considered as a safe drug, there are increasing evidences of its toxicity. MDMA is a neurotoxic substance for serotonergic and dopaminergic neurons and decreases 5-HT transporters (2). MDMA is known popularly as Ecstasy and affects human organs mainly brain, liver, kidney and heart. Liver is an organ of immense complexity that has fascinated mankind since antiquity (3). It comprises of multiple phenotypically distinct cell types. One type of polarized epithelial cell is the hepatocyte. Hepatocytes regulate intermediary metabolism and have an important role to detoxify endo- and xenobiotic, manufacture critical circulating proteins and generate bile acid dependent bile flow (3). According to the recent epidemiological data, the use of MDMA has increased almost in all parts of the world (4). Although many investigations reported MDMA-induced liver damage (5, 6), the fundamental mechanism accounting for hepatic toxicity is poorly understood.

Pentoxifylline (3, 7-Dihydro-3, 7-dimethyl-1-(5-oxohexyl)-1H-purine-2, 6-dione) has been classified as a haemorheological drug and a phosphodiesterase inhibitor. Pentoxifylline (PTX) is used for the treatment of intermittent claudication caused by peripheral artery disease. PTX significantly reduce blood viscosity in patients with

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peripheral arterial disorders and increase erythrocytes deformability in healthy subjects and patients with peripheral vascular disease (7).

Cell deaths have been classified by consensus agreement including apoptosis, necrosis, necroptosis, autophagy and cornification (8, 9). Apoptosis is a specific morphological aspect of cell death characterized by membrane blebbing, shrinkage of the cell, chromatin condensation and nuclear fragmentation (3). During apoptosis, pro- and anti-apoptotic genes interact with each other to control the integrity of the cell. The B-cell lymphoma-2 (Bcl-2) family is a group of proteins that regulate mitochondrial dysfunction (10). This family comprises anti-apoptotic (*bcl2*, *Bcl*-xL, *Bcl*-w, *Mcl*-1 and *A1*), pro-apoptotic multidomain effector proteins (*bax*, *Bak*, and *Bok*) and BH3-only pro-apoptotic members such as *Bid*, *Bim*, *Bad*, *Bik*, *Bmf*, *Hrk*, *Noxa* and *Puma* (3).

2. Objectives

Considering the potential toxic interaction, this study was performed to evaluate hepatotoxic effects of MDMA on adult rat hepatocytes. Subsequently, we investigated the effect of pentoxifylline as possible protective drug on hepatotoxicity befor and after taking MDMA.

3. Materials and Methods

3.1. Animal and Drug Administration

Sample size and power in each group were calculated with 95% confidence level, 5% confidence interval and 10 rats, respectively. In this experimental study, adult male Wistar rats weighting 250 - 300 grams (Cellular and molecular research center, Iran university of Medical Sciences, Tehran, Iran) were used. The animals were housed under reversed 12:12 hours light and dark cycles at constant room temperature (22 ± 2°C). All procedures were performed in accordance with ethics committee of cellular and molecular research center, Iran university of medical sciences and European communities council directive of November 1986 (86/609/EEC).

All chemicals were purchased from Sigma except PTX powder, which was gifted kindly by the Amin Pharmaceutical Co. (Esfahan-Iran). However, pure MDMA was gifted by Dr. Foroumadi, faculty of pharmacy and pharmaceutical sciences research center, Tehran university of medical sciences.

The rats were equally distributed into four experimental groups (5 rat/group) including untreated control, MDMA (MDMA dissolved in phosphate buffered saline), treated-1 (MDMA followed by PTX) and treated-2 (PTX followed by MDMA). MDMA was dissolved in PBS and injected intraperitoneally (IP). All animals given MDMA received 3 doses of 7.5 mg/kg with two hours gap between doses. In treated-1 group, pentoxyfilline (100 mg/kg) was injected simoultanously with third dose of MDMA. In treated-2, MDMA was given one week after pentoxyfilline.

3.2. Tissue Collecting

Two weeks after the first drug administration, all rats were anaesthetized by Ketanamine and xylazine before killed. The liver was removed, dissected and frozen immediately in liquid nitrogen to store at -80°C.

3.3. Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from 5 mg liver tissue using High Pure RNA isolation kit (Roche, Germany). According to the handbook kit, the samples were treated with DNase I enzyme supported in the kit to avoid DNA contamination. Finally, Optical Density (A_{260}/A_{280} and A_{260}/A_{230}) and concentration were measured using Nanophotometer 2000c (Thermo Science, USA).

Reverse transcription reaction was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania). Reaction volume was 20 µL and components were used including 1 µg of total RNA as template (11 µL), RevertAid RT 200 U/µL (1 µL), RiboLock RNase Inhibitor 20 U/µL (1 µL), Random Hexamer primer (1 µL), dNTP Mix 10 mM (2 µL) and Reaction Buffer 5x (4 µL). Then, samples were incubated for 10 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 75°C.

3.4. Oligonucleotide Set Design

In the present study, *bcl2*, *bax* and Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) genes were selected as targets and internal reference gene, respectively. The sequences of interest genes were obtained from NCBI database and primer sets were designed via GeneRunner and Primer Express software v.3.0 (Applied Biosystems, Foster City, USA) and analyzed in Basic Local Alignment Search Tool to avoid secondry structure and homology with other genom region. Oligonucleotid sequences are shown in Table 1.

Table 1. Oligonucleotide Sequences for Interest and Reference Genes

Oligo Name	Sequence (5′ → 3′)	Amplicon, bp
***gapdh*-F**	AAGTTCAACGGCACAGTCAAGG	22
***gapdh*-R**	CATACTCAGCACCAGCATCACC	22
***bax*-F**	AGGGTGGCTGGGAAGGC	17
***bax*-R**	TGAGCGAGGCGGTGAGG	17
***bcl2*-F**	ATCGCTCTGTGGATGACTGAGTAC	24
***bcl2*-R**	AGAGACAGCCAGGAGAAATCAAAC	24

3.5. qReal-Time PCR Data Analysis

Real-Time PCR is a high-technique monitoring amplification process step by step. In the assay, SYBR Green I was used as reporter dye. Real-Time PCR reactions were performed via Rotor-Gene Q apparatus (Qiagen, Germeny). Total PCR reaction volume was 25 μL including 12 μL of SYBR Green I PCR Master Mix (TaKaRa, Japan), 1μL of forward and reverse oligonucleotide (400 nM), cDNA template (300 ng) and ddH_2O. Thermal cycling programme was performed for 30 seconds at 95°C (first denaturation), following 5 seconds at 95°C and 25 seconds at 60°C for 40 cycles and melting curve analysis ramping from 65°C to 95°C and rinsing 1°C each step.

Amplification efficiency for target and reference genes validated using 4-fold dilution series of control cDNA template as 2000, 200, 20 and 2 ng. Then, standard curve was drawn by plotting the logarithmic input cDNA concentration versus mean CT and the slope was determined. PCR reaction efficiency was calculated via the following formula;

$$E = \left(10^{\frac{-1}{\text{slope}}}\right) - 1 \quad (1)$$

Expression level of target genes were calculated using comparative threshold cycle formula. The expression level of target genes to reference gene in treated samples compared to controls was calculated using the following formula:

$$\text{Expression level of target gene} = 2 - \Delta\Delta\text{CT} \quad (2)$$

where Δ ΔCT is calculated by the following equation (11):

Equation 3.

$\Delta\Delta CT = [(mCT_{target} - mCT_{reference})_{test\ sample} - (mCT_{target} - mCT_{reference})_{control\ sample}]$

3.6. Statistical Analysis

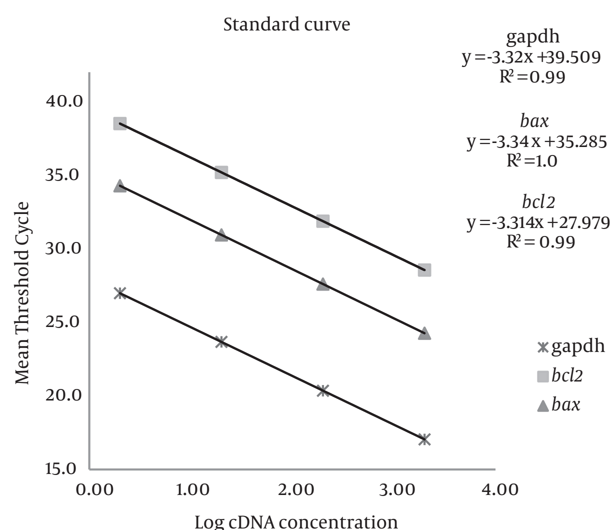
In this study, all mathematical procedures were calculated using Statistical Package for the Social Sciences software (SPSS Inc v. 22). The statistical operations included mean ratio (M), Standard Deviation (SD), Determination coefficient (R2), Confidence Intervals (95% CI) and Standard Error of Mean (SEM). Furthermore, to determine differences between gene expression of interest groups, One-way ANOVA, non-parametric analysis (independent sample Kruskal-Wallis test) and then, Tukey (Post hoc test) were performed. P-value < 0.05 was considered statistically significant.

4. Results

4.1. Real-Time RT-PCR

The slope of standard curve for targets and reference genes were calculated from -3.316 to -3.344 (Figure 1). PCR efficiencies, additionally for all interest genes were obtained between 97.0 and 99.0%. the amplicons of *gapdh*, *bcl2*, and *bax* were melted at 85.5°C, 86.2°C and 89.3°C, respectively (Figure 2). Melting curve analysis confirmed the specific amplification of fragments and no primer dimmers formation.

Figure 1. Standard Curves: Mean Threshold Cycle Versus Log cDNA Concentration



Y-intercept and Determination coefficient for interest genes; *bax* (y = -3.34x + 35.285 and R^2 = 0.99), *bcl2* (y = -3.314x + 27.979 and R^2 = 1.00) and *gapdh* (y = -3.32x + 39.509 and R^2 = 0.99).

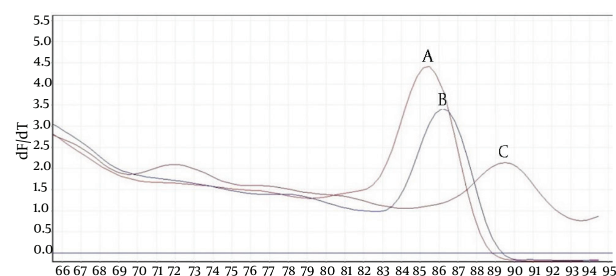


Figure 2. Melting Curve Analysis of the Target and Reference Genes for PCR Products. A: *gapdh*, B: *bcl2*, C: *bax*.

Real-Time quantitative PCR results revealed that gene expression ratio of anti-apoptetic gene, *bcl2* were 93.80 ± 20.64, 340.45 ± 36.60 and 47.13 ± 5.84 fold in MDMA, treated-1 and treated-2 groups, respectively (Figure 3). Moreover, this ratio for *bax*, pro-apoptotic gene were 2.13 ± 0.33 fold in MDMA, 1.55 ± 0.26 fold in treated-1 and 10.44 ± 1.56 fold in treated-2 groups (Figure 3, Table 2).

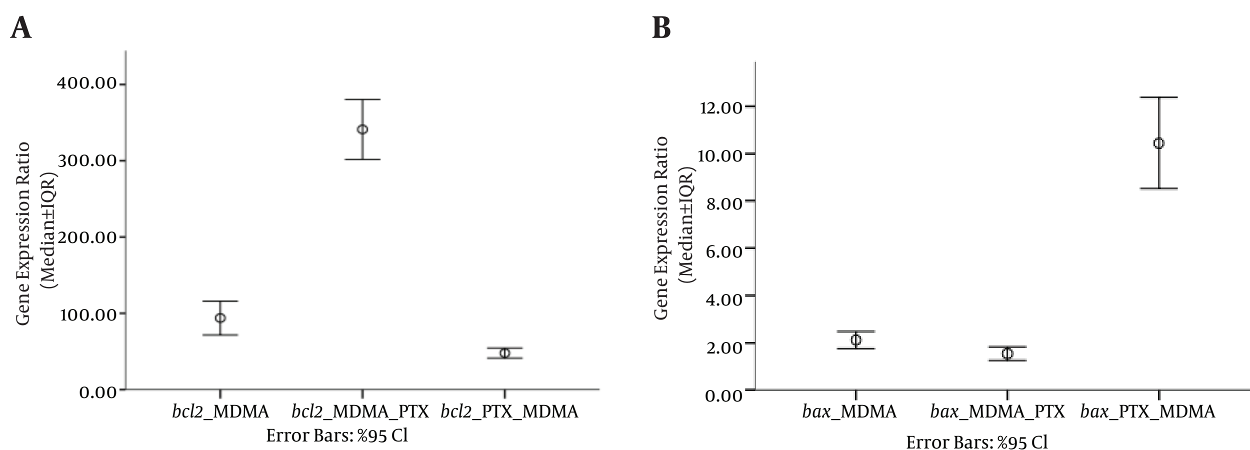


Figure 3. Gene Expression Ratio (Median ± IQR) of (A) *bcl2* and (B) *bax* in Experimental Groups Compared to Normal Control.

Table 2. The Expression Ratio of *bax* and *bcl2* in Experimental Groups Compared to Normal Control[a]

Target Genes	Experimental Groups			P-Value
	MDMA	MDMA + PTX	PTX + MDMA	
***bax* gene**	2.13 ± 0.33	1.55 ± 0.26	10.44 ± 1.56	< 0.001
***bcl2* gene**	93.80 ± 20.64	340.45 ± 36.60	47.13 ± 5.84	< 0.001

[a]Abbreviations: MDMA, 3-4methylenedioxymethamphetamine; MDMA + PTX, MDMA followed by PTX; PTX, Pentoxifylline; PTX + MDMA, PTX followed by MDMA, SD, Standard Deviation.

Statistical analysis showed significant difference for *bcl2* expression between experimental groups. The expression of this gene was increased in treated-1 compared to MDMA and treated-2 (P-value < 0.001) and between MDMA and treated-2 group (P value < 0.01). Moreover, *bax* gene expression significantly increased in treated-2 compared to MDMA and treated-1 group (P-value < 0.001) and no significant difference between MDMA and treated-1 group (P-value > 0.05).

The ratio of *bax* to *bcl2* (*bax*/*bcl2*) for MDMA, treated-1 and treated-2 calculated as 0.02, 0.005 and 0.222, respectively.

5. Discussion

Although MDMA alone or in combination with other abused substances can damage various organs (such as liver, heart, brain and kidney), its use has been increased over the past decade. Many researches performed to figure out the mechanism of MDMA-mediated neurotoxicity (2, 12-14), but little research is performed about the mechanisms of MDMA-induced hepatotoxicity (2, 15, 16). As Liver is a target organ for MDMA toxicity, the present study focused on molecular mechanism of MDMA in apoptosis using gene expression quantification of a pro-apoptotic and an anti-apoptoic gene in hepatotoxocity. Data analysis showed that MDMA induced programmed cell death in liver switching pro-apoptotic gene on. This is demonstrated by upregulation of *bax* gene in treated-2 group approximately 7-fold than treated-1 (MDMA followed by PTX). The expression of *bax* increased in MDMA and treated-2 compared to normal controls (P value < 0.001). An investigation on apoptotic mechanisms triggered by MDMA (alone or combined) in immortalized human HepG2 cells showed down-regulation of BCL-2 and *bcl* $-_{XL}$ mRNA levels with concurrent upregulation of *BAX* and *BAD* genes (17).

Cristina Montiel-Duarte et al. reported that MDMA caused apoptosis in freshly isolated rat hepatocytes and cell line of hepatic stellate cells (HSC), as shown by chromatin condensation of the nuclei and accumulation of oligonucleosomal fragments in the cytoplasm. In both cell types, apoptosis correlated with decreased levels of *BCL*-$_{XL}$, release of cytochrome C from the mitochondria and activation of caspase-3 (2). These results suggested that liver cells apoptosis could be involved in hepatotoxicity of MDMA. Beitia et al. (18) demonestrated that MDMA significantly increased ALT, AST and ALP enzymes and reduced the number of hepatocytes in male Wistar rats. In another study, Kwan-Hoon Moon et al. showed that MDMA causes oxidative inactivation of key mitochondrial enzymes, which contributes to mitochondrial dysfunction and subsequent liver damage in MDMA-exposed animals (19).

Pentoxifylline (PTX) is a tri-substituted xanthine derivative designated chemically as 1-(5- oxohexyl)-3, 7-dimethylxanthine and a hemorrheologic agent, i.e. an agent that affects blood viscosity (20). However, the precise mechanism of Pentoxifylline and its effects in clinical improvement are still unknown. Recently, PTX with combined anti-inflammatory (TNF-α inhibition) and anti-fibrogenic properties has been found to be useful in patients with acute alcoholic hepatitis (21, 22). In the present study, PTX considered as a protective drug on MDMA-induced hepatotoxicity. The findings showed upregulation of *bcl2* gene in treated-1 compared to MDMA and treated-2 groups (P value < 0.001). Recent evidences showed that PTX act against MDMA through increasing expression of anti-apoptotic genes. In patients with severe alcoholic hepatitis, prednisolone and pentoxifylline are the first line therapy (23). Sharifi et al. reported that the number of apoptotic bodies significantly decreased in non-simultaneous injection of MDMA and PTX group than the other groups except control (15). In the parallel analysis, Movassaghi et al. performed chromosome staining in rat liver to investigate apoptotic bodies using TUNEL (Terminal deoxynuclotidyl transfrase dUTP nickend labeling) assay (16). They showed that number of TUNEL-positive cells in rat liver was significantly decreased in MDMA followed PTX group compared to MDMA and vehicle groups. Besides, they published that apoptotic bodies were significantly decreased by PTX in the liver and there was no significant difference between the number of apoptotic bodies in MDMA followed PTX group and control groups (16).

The ratio of *bax* to *bcl2* in interested groups showed pro-apoptotic gene to anti-apoptotic gene expression. In the present study, this ratio (*bax*/*bcl2*) decreased in treated-1 group (finally received PTX) compared to other groups. Moreover, this ratio increased in treated-2 and MDMA compared to treated-1 (P value < 0.001).

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Footnotes

Authors' Contribution: Mitra Behroozaghdam: performed laboratory operation, manuscript preparation extensively. Mehrdad Hashemi: design of the study, provided technical support, data analysis and revised the paper. Reza Mahdian: analysis of data and provided technical support. Gholamreza Javadi: editing the manuscript and provided technical support. Mansoureh Soleimani: performed the statistical analysis and editing the manuscript.

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