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Zinc Mitigates MDMA-Prompted Apoptosis by Alteration of Cross Talk Among Bcl-2, Bax and P53 Genes Expression in Mouse Sertoli and Leydig Cell Lines

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Abstract

Background: 3,4-methylenedioxymethamphetamine (MDMA) affects the male reproductive system. We investigated the mRNA levels of bax, bcl-2, and p53 genes in MDMA-induced apoptosis in mouse Sertoli (TM4) and Leydig (TM3) cells before and after taking Zinc.

Methods: The TM3 and TM4 cells were cultured in four groups: I (untreated medium), II (medium with 5 mM MDMA), III (medium with 8 µM Zinc), and IV (medium with 8 µM Zinc prior to 5 mM MDMA administration). After 48 hours, total RNA was extracted from the samples, and cDNA was synthesized. The relative gene expression level was evaluated using the SYBR Green PCR kit.

Results: In the MDMA group, the relative amounts of bax and p53 gene expressions increased; conversely, the relative amount of bcl-2 gene expression decreased in TM3 and TM4 cell lines. In the MDMA+Zinc group, there were no statistically significant differences between this group and the control group regarding the mRNA levels of bax and p53 genes in the TM3 cell line, as well as the mRNA levels of bax and bcl-2 genes in the TM4 cell line. Statistically significant differences were observed between the MDMA+Zinc and MDMA groups regarding the relative expressions of bax and p53 genes in the TM3 cell line and bcl-2 and p53 genes in the TM4 cell line.

Conclusions: Zinc mitigates MDMA- induced apoptosis by altering the crosstalk among bcl-2, bax, and p53 gene expressions in the tested cell lines.

Keywords: Apoptosis, Ecstasy, Leydig cell line, Sertoli cell line, Zinc.

Introduction

The drug, 3,4-methylenedioxymethamphetamine (MDMA), a synthetic amphetamine drug (Ecstasy), has toxic effects on human organs such as the brain, liver, kidneys, heart, and testes. The use of psychotropic substances, anabolic androgenic steroids, marijuana, cocaine, and methamphetamine can be important risk factors for infertility and liver damage among young people under the age of 25 (1). This drug causes defects in cell functioning through the production of reactive oxygen species (ROS) and damage to intracellular organelles such as mitochondria and lysosomes; for this reason, such substances are classified as genotoxic and cytotoxic compounds (2). Sertoli and Leydig cells are somatic cells that provide testicular structure and function. Leydig cells are positioned adjacent to the seminiferous tubules within the spaces between the testes that contain Sertoli cells (3). Sertoli cells, as central regulators of spermatogenesis, are present in the seminiferous tubules. Sertoli cells determine

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sperm count, maturity, and motility (3). MDMA induces cell death in a wide range of cells by inhibiting the superoxide dismutase enzyme and oxidizing cytoplasmic proteins, thereby increasing oxidative stress (4). Although the mechanisms related to the effects of ecstasy on different cells are not very clear, reports indicate that such compounds affect the pituitary-gonadal axis. The abuse of MDMA leads to severe adverse effects, including cardiac hypertension, hyperthermia, arrhythmias, hyponatremia, serotonin syndrome, seizures, liver injury, coma, and even death (1, 5). Long-term consumption of MDMA increases the risk of DNA damage in sperm and modifies the histopathology of the testes (5). The mechanisms for these reactions are still to be clarified. MDMA reduces the activity of various enzymes such as aldehyde hydrogenase, tryptophan hydroxylase, thiolases, and ATP synthetase, causing a decrease in the levels of anti-apoptotic proteins. Subsequently, the release of cytochrome C from the mitochondria and the activation of the caspase-3 enzyme initiate the cell death pathway, leading to tissue destruction (2). Recent studies have indicated that amphetamines mainly influence testosterone production by interstitial tissue and spermatogenesis. The results show that this effect is induced by increasing the production of cyclic AMP and reducing the activity of calcium channels and enzymes related to this hormone 's production. MDMA prevents testosterone secretion by decreasing gonadotropin secretion, thereby causing infertility (6). Zinc has various impacts on cell death and survival. In this regard, Zinc depletion induces cell death via apoptosis (or necrosis if apoptotic pathways are blocked) in different cell lines and enhances apoptosis through death receptors. Conversely, adequate Zinc levels support the preservation of cell survival pathways, such as autophagy and ROS regulation (7). The consumption of antioxidants in men affects spermatogenesis and sperm health, preventing the harmful effects of ROS while simultaneously reducing testicular oxidative stress (8). We observed that Zinc protects against MDMA-induced apoptosis of Sertoli (TM4) and Leydig (TM3) cells in mice by attenuating caspase-3 gene expression (1, 9). Caspases, in association with another set of molecules by altering different signaling pathways, lead to apoptosis. In this study, we investigated the expression of bax, bcl-2, and p53 genes in cultured TM3 and TM4 cell lines that were treated with MDMA, Zinc, and MDMA+ Zinc, in the medium pre-treated with Zinc.

Materials and Methods

In this study, the resource equation approach was used to determine the sample size. Based on this approach, the acceptable degree of freedom (DF) in a one-way analysis of variance is between 10 and 20. With this description, the minimum and maximum number of tests in each group can be calculated based on the following formulas: Minimum n=10/k+1 and Maximum n=20/k+1. Regarding the number of groups (K) in the present study, that is equal to 4, the minimum and maximum number of tests in each group is calculated as 2 and 4, respectively. In the present study, finally, 3 tests in each group performed (10). In present investigation, two mouse testicular somatic cell lines: TM4 and TM3 cells were cultured in DMEM/F12 (ATOCEL, Austria) with 10% FBS (ATOCEL, Austria) and 1% penicillin-streptomycin (ATOCEL, Austria). The TM3 and TM4 cells were cultured in four groups: group I (untreated medium as control), group II (medium with 5 mM ecstasy), group III (medium with 8 µM Zinc), and group IV (medium with 8 µM Zinc prior to 5 mM MDMA administration). Based on the results from cell survival assay by Lozeie et al (9), the effective concentration was 5 mM for MDMA and 8 µM for Zinc. In group IV, medium pre-treated with 8 µM Zinc 24h prior to 5 mM MDMA-treatment. After 48h, total RNA was extracted from the samples in each group using the RNX Plus Solution Kit (SinaClon, Iran) (Catalog Number: RN7713C). The quality of isolated total RNA was confirmed. Then, first strand complementary DNA (cDNA) was

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synthesized from RNA samples via Thermo Scientific RevertAid First Strand cDNA Synthesis (Thermo Scientific #K1622, United States). The cDNA was efficiently synthesized at 42 °C for 60 min. Gene, Oligonucleotide primers, length of PCR products (bp) and PCR program used for real - time-PCR analysis in this study are summarized in Table 1 (11). Relative gene expression level was evaluated by SYBR Green PCR kit. Real-time PCR was performed on a Mic qPCR cycler (Bio Molecular Systems, Australia) using 2x qPCRBIO SyGreen (Biosystems, UK). The relative gene expression levels were normalized to endogenous GAPDH. The mRNA expression level of bax, bcl-2 and p53 genes were determined by Livak method ($2^{-\Delta\Delta CT}$) (12).

Table 1. Gene, Oligonucleotide primers (TAG Copenhagen A/S, Danmark), Length of PCR products (bp) and PCR program used for real-time PCR analysis in this study.

Gene	Primers (5'-3')		PCR products (bp)	PCR program
bcl-2 (NC_000067)	Forward	gtggatgactgagtacct	118	-94 °C for 3 min (one cycle); -94 °C for 30 sec, 62 °C for 30 sec ---72 °C for 45 sec (35 cycles); -72 °C for 5 min (one cycle)
	Reverse	ccaggagaaatcaaacagag		
bax (NC_000073)	Forward	ctacagggtttcatccag	133	
	Reverse	ccagttcatctccaattcg		
p53 (NC_000077)	Forward	gtatttcaccctcaagatcc	84	
	Reverse	tgggcatcctttaactcta		
GAPDH (NC_000072)	Forward	gagaaacctgccaagtatg	123	
	Reverse	ggagttgctgttgaagtc		

Statistical methods

To analyze the data, the REST 2009 software and the Statistical Package for the Social Sciences, version 20, SPSS Inc., Chicago, Illinois, USA (SPSS) were used. Kruskal-Wallis analysis was employed to compare the means of gene expression among three groups, and Tukey's post hoc test was applied for pairwise comparisons. A *P*-value less than 0.05 was considered statistically significant.

Results

Here, we investigated the level of bax, bcl-2 and p53 mRNA in two mouse testicular somatic cultured cell lines (TM3 and TM4) before and after taking Zinc. Three genes (bax, bcl-2 and p53) were quantified from the four groups (I, II, III, and IV) by real-time PCR (Table 2).

In MDMA group, the relative amount of bax and p53 genes expression have been increased; and inversely, the relative amount of bcl-2 gene expression has been decreased in TM3 and TM4 cell lines (*P*-value<0.05). In the Zinc group, our findings imply that there were no statistically significant differences between Zinc and control groups regarding bax, bcl-2 and p53 genes expression level (*P*-value >0.05). In MDMA+Zinc group, there were no statistically significant differences between this group and control group regarding bax gene expression in TM3 and TM4 cell lines, p53 gene in TM3 cell line and bcl-2 in TM4 cell line (*P*-value >0.05). The relative amount of bcl-2 and p53 genes expression has been decreased in MDMA+Zinc group (vs. control group) regarding TM3 and TM4 cell lines, respectively (*P*-value<0.05). In a comparison regarding MDMA+Zinc and MDMA groups in TM3 cell line, we found that the relative amount of bax and p53 genes expression has been decreased significantly in MDMA+Zinc group (*P*-value<0.05); and, in the case of bcl-2, significant differences were not found between two groups (*P*-value>0.05). In TM4 cell line, the relative amount of p53 gene expression has been decreased significantly in MDMA+Zinc group (*P*-value<0.05); but the

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relative amount of bcl-2 gene expression has been increased significantly in MDMA+Zinc group (*P*-value<0.05) (Figs. 1 & 2).
Accordingly, in the case of bax gene expression, significant differences were not found between MDMA+Zinc and MDMA groups in TM4 cell line (*P*-value>0.05). Our findings showed that Zinc protects against MDMA-prompted apoptosis of the cells by a mechanism related to reduction of the relative amount of bax and p53 genes expression and up-regulating the relative amount of bcl-2 gene expression (Fig. 3).

Table 2. Relative gene expression (Fold change) of bax, bcl-2 and p53 in TM3 and TM4 cell lines regarding examined groups. *: *P*-value<0.05.

Cell Line	Gene	MDMA vs. control	Zinc vs. control	MDMA+ Zinc vs. control	MDMA+ Zinc vs. MDMA
TM3	Bax	12.62±0.83*	1.74±0.14	1.1±0.12	0.07±0.02*
	bcl-2	0.3±0.1*	1.86±0.4	0.41±0.1*	1.41±0.2
	p53	24.91±6.6*	1.46±0.08	1.31±0.47	0.04±0.01*
TM4	Bax	2.32±0.06*	0.46±0.1	1.99±0.61	1.65±0.34
	bcl-2	0.07±0.01*	1.73±0.4	1.63±0.3	24.21±5.44*
	p53	2.23±0.37*	0.87±0.08	0.51±0.09*	0.18±0.05*

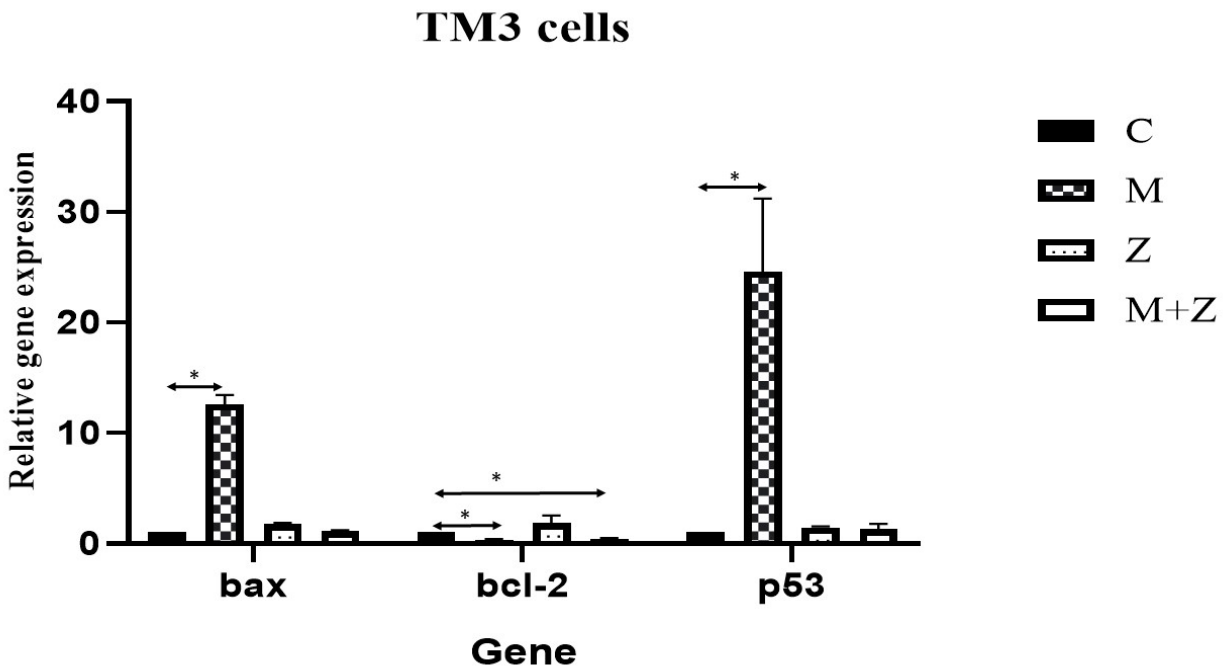

Fig. 1. Fold change (relative gene expression) of bax, bcl-2 and p53 genes in TM3 cell line regarding Control (C), MDMA (M), (Z), and MDMA+Zinc (M+Z) groups. The relative amount of bax and p53 genes expression has been decreased in MDMA+Zinc group (vs. MDMA group). *: *P-value*<0.05.

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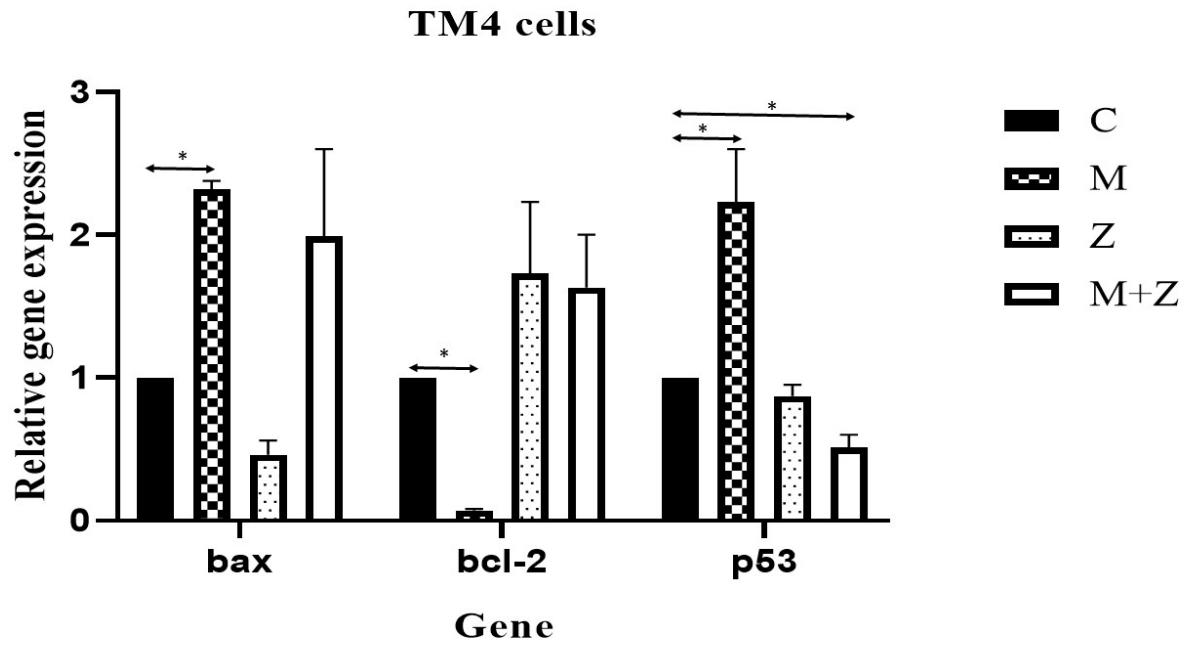

Fig. 2. Fold change (relative gene expression) of bax, bcl-2 and p53 genes in TM4 cell line regarding Control (C), MDMA (M), (Z), and MDMA+Zinc (M+Z) groups. The relative amount of p53 gene expression has been decreased in MDMA+Zinc group (vs. Control/MDMA group); but in the case of bcl-2, the relative amount of bcl-2 gene expression has been increased (vs. MDMA group). *: *P-value*<0.05.

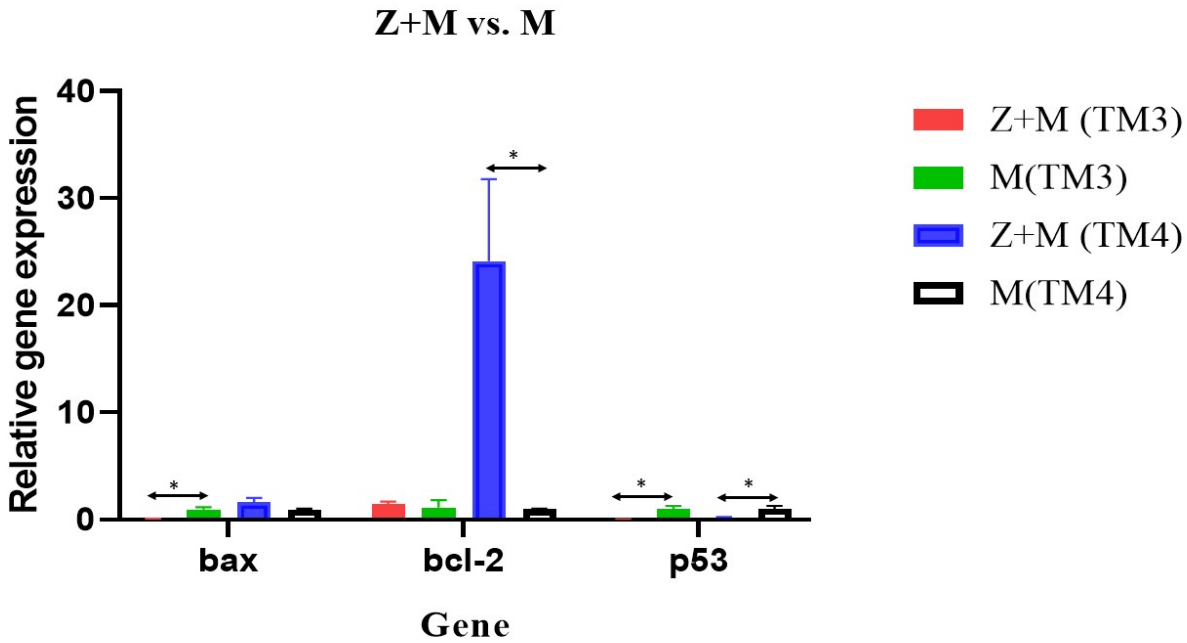

Fig. 3. Fold change (relative gene expression) of bax, bcl-2 and p53 genes in TM3 and TM4 cell lines regarding MDMA (M), and MDMA+Zinc (M+Z) groups. Our results indicated that Zinc protects against MDMA-induced apoptosis by attenuation of bax and p53 genes expression and induction of bcl-2 gene expression. *: *P-value*<0.05.

Discussion

In this study, the expression of bax, bcl-2, and p53 genes in MDMA- induced apoptosis in two mouse testicular somatic cell lines before and after taking Zinc was examined. Our findings indicated that 1) the relative gene expressions of bax and p53 increased, while the relative gene expression of bcl-2 decreased in the MDMA group concerning both cell lines. 2) There were no statistically significant differences between the MDMA+Zinc and

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control groups regarding the bax gene in TM3 and TM4 cell lines, the p53 gene in TM3 cell lines, and bcl-2 in TM4 cell lines. Our findings show that pre-treatment with 8 µM Zinc protects against MDMA-induced apoptosis by alteration of cross talk among bcl-2, bax and p53 genes expression in two cell lines. Our results are in agreement with others (1, 7, 9,13-16). It has been demonstrated that the abused MDMA induces apoptosis in human serotonergic cells (17). In this way, chronic exposure to MDMA alters the cell cycle and induces caspase-dependent apoptosis by increasing G2/M phase arrest and DNA damage, impairing cellular defenses, enhancing lipid peroxidation, and modifying testes histopathology (18,19). These effects lead to the production of ROS and reduced levels of antioxidants (20). ROS accumulation results in intracellular oxidative stress, destruction of cellular macromolecules, and necrotic cell death (20). Taghizadeh et al. (2016) demonstrated a high amount of ROS production, collapse of mitochondrial membrane potential, mitochondrial dysfunction, membrane impairment, and cytochrome c release in brain mitochondria isolated from rats treated with MDMA (21). A high level of ROS molecules causes oxidative stress and inflammation in various body tissues. Oxidative stress stimulates the intrinsic pathway of apoptosis by activating p53, bax, and subsequently the caspase cascade (20). Mechanisms of p53-associated apoptosis are currently under study. P53 decreases the expression of the apoptosis-suppressing gene bcl-2, which promotes cell survival, while simultaneously increasing the expression of the bax gene, which promotes cell death, acting as a gene that inhibits the expression of the bcl-2 protein (22-24). A large number of studies have been designed and implemented to investigate the effect of Zinc on different cellular processes (25,26). Zinc sulfate attenuates oxidative stress and apoptosis in the testicular cells of male Wistar rats (27).

MDMA (at least 5 µM) is toxic to these cells and leads to a decreased number of normal cells and testosterone production. The concentration of Zinc in organs such as the prostate, testes, and seminal fluid is high, indicating the importance of Zinc in the male reproductive system. Additionally, Zinc is vital for testosterone production by the Leydig cells (28,29). In human semen, Zinc deficiency causes testicular failure, Leydig cell damage, and apoptosis (29). Apoptosis occurs in mammalian systems via 1) an extrinsic pathway triggered by death receptors, 2) an intrinsic pathway that occurs through the mitochondria, and 3) other apoptosis pathways that exist, such as apoptosis mediated by a family of serine proteases. The extrinsic pathway is governed by the binding of exogenous death ligands at the cell surface, while the intrinsic pathway responds to signals from within the cell, including radiation and chemotherapeutic agents (30). Lastly, the anti-apoptotic proteins bcl-2, bcl-x, bcl-w, and bcl-b as well as the pro-apoptotic proteins bax, bak, bcl-xs, and bok play critical roles in the intrinsic pathway. The Tumor Necrosis Factor Receptor Superfamily (TNFRSF) exists in the extrinsic pathway. These two pathways converge at the activation of caspases in apoptosis (30). Based on the information provided, there are many signaling transmission pathways that remain unknown and must be evaluated to determine their role and importance in cell survival and apoptosis. Our findings imply that the relative gene expressions of bax and p53 have been increased; however, in the case of bcl-2 gene, the relative gene expression has been decreased in the MDMA group. Additionally, regarding the lack of statistically significant differences between the MDMA+Zinc and control groups concerning the bax and p53 genes, it is concluded that Zinc mitigates MDMA- induced apoptosis by altering the crosstalk among bcl-2, bax, and p53 gene expression in TM3 and TM4 cell lines.

Ethical Consideration

Ethics committee of Urmia University of Medical Sciences approved all stage of this study (IR.UMSU.REC.1397.448).

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Conflict of interest
The authors declare that they have no conflicts of interest.

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