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Protective effect of pentoxifylline on male Wistar rat testicular germ cell apoptosis induced by 3,4-methylenedioxymeth amphetamine

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ABSTRACT

Objective(s): 3, 4-methylenedioxymethamphetamine (MDMA) one of the methamphetamine derivatives that affect the reproductive system, has not been well understood. Many young people are consumers of drugs such as MDMA that can affect their reproductive capability. Apoptosis is the main mechanism for male infertility. Pentoxifylline (PTX) increases cAMP intracellularly and reduces tumor necrosis factor-α.
This study aimed to investigate the protective effect of PTX administration in MDMA-induced apoptosis in testes of male Wistar rats.
Materials and Methods: Thirty male Wistar rats weighing 250–300 g were randomly divided into five groups: control group (without any intervention), group receiving 7.5 mg/kg MDMA three times every two hours for one day, first experimental group receiving 100 mg/kg PTX just at the time of third injection of MDMA, second experimental group receiving 100 mg/kg PTX a week before MDMA administration, and the vehicle group, which received MDMA+saline. Two weeks later, testes were removed and prepared for H&E staining, TUNEL and Western blot techniques.
Results: There was a significant decrease of the score in the MDMA group compared with the control group. In first and second experimental groups, the quality of seminiferous epithelium was improved compared with the MDMA group. The number of TUNEL-positive cells/tubule increased in MDMA and vehicle groups, which is decreased by administration of PTX before MDMA. Expression of active caspase-3 significantly increased in MDMA group, which is significantly decreased by administration of PTX before MDMA.
Conclusion: PTX can significantly reduce the severity of lesions in the testes following administration of MDMA.

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Introduction

Methamphetamine (MAMP) is one of the addictive stimulant drugs that stimulate the central nervous system and is also an important tonic drug in the field of reproduction because several studies have demonstrated its teratogenic and embryotoxic effects (1). 3, 4-methylenedioxymethamphetamine (MDMA or “ecstasy”) is one of the MAMP derivatives that has become very popular among young adults (2). The effect of MDMA on reproductive neuroendocrine function and the reproductive axis has not been well understood. It has been well known that the same neurotransmitter systems that control MDMA, stimulate central nervous system actions including serotonergic and dopaminergic pathways as well as regulating reproduction. Reproductive function in mammals is driven under control of gonadotropin-releasing hormone (GnRH) neurons in the preoptic area and hypothalamus of rodents, which in turn are regulated by afferent inputs from other brain regions. The hypothalamic GnRH cells can coordinate reproduction with other environmental and homeostatic conditions through this neural circuitry. (3, 4) Thus, GnRH neurons, through these central afferent inputs, are a potential target of MDMA (3).

Nowadays, a growing number of young people

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of reproductive age are consumers of addictive stimulants such as MDMA that can adversely affect their reproductive capability. MDMA can alter neuroendocrine control of reproductive functions through several neurological pathways and can also affect hypothalamic–pituitary–gonadal reproductive axis (3, 4). In addition to hypothetical conse-quences of neuroendocrine disturbances, other mechanisms of action with chronic exposure to MDMA upon male reproductive organs or functions are also possible. Some MDMA metabolites can interfere with redox cycle, produce reactive oxygen species (ROS) and arylate tissue macromolecules (5, 6). Therefore, MDMA metabolites can directly affect reproductive organs by unspecific mechanisms such as oxidative stress or genotoxicity.

Approximately 10% of couples are infertile among whom half are accompanied with male infertility. Apoptosis, which plays an important role in testicular germ cell development, is one of the main mechanisms for male infertility that is commonly caused by testicular exposure to elevated levels of temperature, toxicants, radiation, chemotherapy, and hormonal depletion (7). Pentoxifylline (PTX), a methylxanthine phosphodiesterase inhibitor, increases cyclic adeno-
sine 30:50 monophosphate (cAMP) intracellularly (8), reduces superoxide anions, and inhibits tumor necrosis factor-α (TNF-α). This factor is responsible for apoptosis or programmed cell death (9, 10). PTX can stimulate sperm motility, improve fertilizing ability (11), and also has a protective effect against cadmium toxicity in testes (12).

This drug has been used in clinical settings to scavenge reactive oxygen species and to reduce lipid peroxidation associated sperm membrane damage and apoptosis (9, 13).

The present study aimed to investigate the protective effect of PTX administration in MDMA-induced apoptosis in testes of adult male Wistar rats as a model for reproductive effects of ecstasy usage in humans.

Materials and Methods

This study was conducted according to the principles and procedures of National Institute of Health Guidelines for Animal Experiments of Islamic Azad University. Briefly, male Wistar rats weighing 250–300 g were kept in special cages. The animals received food and water *ad libitum*. The temperature was maintained at 23±2 °C, in a 12 hr: 12 hr light/dark cycle. All procedures used in the study were approved by the Ethics Committee for the Use of Experimental Animals of Islamic Azad University Tehran Medical Branch. All chemicals were purchased from Sigma except PTX powder that was kindly donated by Amin Pharmaceutical Co. (Isfehan-Iran 2014).

Experimental groups and drug

Thirty male Wistar rats were randomly divided into five groups: control group (without any intervention), the group receiving 7.5 mg/kg MDMA three times, every two hours for one day (14), first experimental group receiving 100 mg/kg PTX just at the time of the third injection of MDMA, second experimental group receiving 100 mg/kg PTX a week before MDMA administration, and the vehicle group, which received MDMA+saline. The rats were sacrificed after 2 weeks from the beginning of all experiments. Right testis from each animal was fixed in paraformaldehyde 4% for more than 3 days. The testis was cut vertically into two pieces and embedded in paraffin and sectioned (3 and 5 µm) for histological assessment (hematoxylin and eosin (H&E] staining and TUNEL method). The left testis was rapidly snap-frozen in liquid nitrogen and kept at −80 °C for detecting caspase-3 activities.

Histologic examination of spermatogenesis

Thirty round tubular cross-sections (with H&E staining) were studied per section with regard to the quality of seminiferous epithelium. The seminiferous tubules were graded using the Johnsen score as: score 10 (complete spermatogenesis with regular tubules); score 9 (many sperms, irregular germinal epithet-lium); score 8 (few sperms); score 7 (no sperms, many spermatids); score 6 (few spermatids); score 5 (no sperm or spermatids); score 4 (few spermatocytes); score 3 (presence of spermatogonia); score 2 (presence of Sertoli's cells); or score 1 (no cells). The Johnsen score per tubule was expressed as mean±standard error for each group.

Evaluation of apoptosis

For detection of apoptosis, the deoxyuridine nick-end labeling (TUNEL) assay was performed using an In-Situ Cell Death Detection kit (POD kit, Roche, Germany) according to the manufacturer's protocol. Briefly, the sections were deparaffinized in xylol, rehydrated by successive series of alcohol, washed in phosphate-buffered saline (PBS) and deproteinized by proteinase K (20 µg/ml) for 30 min at room temperature. The sections were rinsed and incubated with 3% H_2O_2 in methanol for 10 min in dark to block endogenous peroxidase (POD) then, the sections were incubated in TUNEL reaction mixture for 60 min at 37 °C in humidified atmosphere and rinsed with PBS. Sections were visualized using converter-POD for 30 min at 37 °C in a humidified atmosphere in the dark and rinsed with PBS, and 50–100 µl DAB substrate [diaminobenzidine (DAB)] was added and rinsed with PBS. Thirty seminiferous tubules on circular cross-sections in each testis were evaluated. The number of TUNEL-positive nuclei per tubule was counted and expressed as mean±SEM for each group.

Table 1. Johnsen scores and the statistical analysis results

	Control group (N=6)	MDMA group (N=6)	Experimental 1 (N=6)	Experimental 2 (N=6)	Vehicle (N=6)
Johnsen score	9.7±0.20	4.6±1.09	7.16±0.32	7.28±0.36	4.08±1.05

Data were shown as mean±standard deviation

Caspase-3 activity

For Western blot analysis, part of testis tissue (100 mg) was homogenized in ice-cold lysis buffer containing tris-HCl (50 mM, pH 8.0), NaCl (150 mM), Nonidet P-40 (1%), glycerol (10%), phenylmethylsulfonyl fluoride (10 μl/ml), sodium deoxycholate (0.5%), and aprotinin (30 μl/ml), in addition to a protease inhibitor cocktail (Roche Applied Science). The homogenized testes were subjected to centrifugation at 12000 g for 20 min at 4 °C, and the supernatant collected. A total of 100 μg from the total protein of supernatant was loaded onto each lane and electrophoresed on SDS-PAGE gels (10%). Proteins were transferred onto nitrocellulose membranes for 1 hr at room temperature and blocked with PBS that contained non-fat dried milk powder (5%) for 2 hr. Membranes were washed using tris buffer that contained Tween 20 (1%), then probed with a monoclonal anti-cas3 antibody (1:1000; Abcam, St. Louis, MO, USA) overnight after which a secondary anti-rabbit akp-linked antibody (1:10000; Abcam,) was added for 1 hr at room temperature, then the membranes were stained with BCIP/NBT. β-actin served as a positive control for protein loading, and a high range molecular weight standard was used to determine protein sizes. Results were evaluated using the UVIdoc software (UVIdoc version 12.6 for Windows, 2004).

Statistical analysis

Data were expressed as mean±SD. Significant difference was analyzed by a one-way ANOVA test followed by Tukey's Multiple Comparison test. $P < 0.05$ was considered statistically significant in primary tests.

Results

Histological assessments

According to the Johnsen testicular biopsy scoring, there was a significant decrease of the score in the MDMA group compared with the control group. In first and second experimental groups, the means of testicular biopsy scores increased significantly compared with the MDMA group (Figure 1). In the control group, testes had a normal testicular architecture and seminiferous tubular morphology with normal spermatogenesis, including primary and secondary spermatocytes, spermatids, and spermatozoa. Loose intertubular connective tissue, which contained fibroblasts, blood vessels, and interstitial or Leydig cells was seen between seminiferous tubules. These cells were large and polyhedral with euchromatic nuclei, containing nucleoli. The cytoplasm was scanty and poorly stained. The capillaries were infiltrated among the clumps of Leydig cells (Figure 1A). In MDMA and saline groups marked morphological changes were observed in testes with severe distortion of tubules.
Some tubules contained a few primary and secondary spermatocytes while other tubules had few germinal cells with pyknotic nuclei and extensive disorganization (Figures 1C and 1E). In the MDMA group, there was a marked reduction in spermatogenesis. The architecture of testes was maintained, but the germinal epithelium showed disorganization as well as marked degenerative changes. The cell membrane of Leydig cells was poorly defined, with cytoplasmic vacuolations in many cells. The nuclei were circular to oval in shape and were smaller as compared to control groups.

In the first experimental group, disorder and disorganization were revealed in all cells of the germinal epithelium, but this disturbance was reduced compared with the group receiving ecstasy. Interstitial space was increased in some parts, but normal Leydig cells with intact nuclei and nucleoli were seen in these spaces. Vascular congestion in some parts was indicated. Degeneration of seminiferous tubules was also lower than in the ecstasy group (Figure 1B).

In the second experimental group, seminiferous tubule degeneration was detected. Disturbance and disorganization were reduced in germinal epithelium and increased interstitial space was seen. A number of seminiferous tubules seemed furrowed. Leydig cells appeared normal, but many of them were observed in the tissues. In most cases, spermatogonia, primary and secondary spermatocytes, spermatids, and spermatozoa were observed, but their numbers were reduced (Figure 1D).

In histopathological examination of the testicular tissue, there was statistically significant difference between the control group and the other groups in terms of mean Johnsen score (Table 1). However, PTX usage caused nonsignificant improvement in mean Johnsen score in comparison to MDMA and vehicle groups (Table 1).

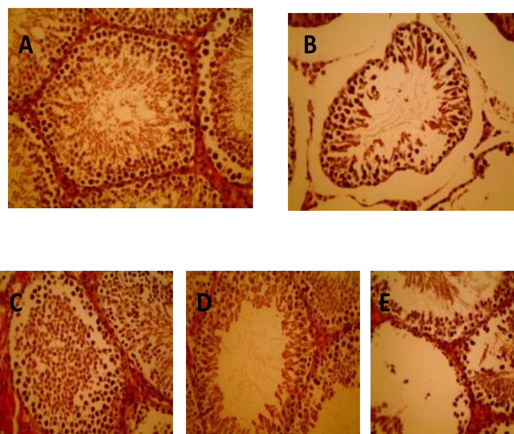

Figure 1.(A):Photomicrograph of testis tissue section of a control group: normal seminiferous tubular morphology with loose intertubular connective tissue (score 10), (B):experimental group1 (MDMA+PTX): seminiferous tubule with few germinal cells and pyknotic nuclei and extensive disorganization (score 6), (C): MDMA group: tubules had few germinal cells with pyknotic nuclei and extensive disorganization, intertubular space has been increased (score 5) (D) : experimental group2(PTX+MDMA): degeneration of seminiferous tubules is lower than the other groups except control group (score 8), (E): Vehicle group: damaged tubule with few germinal cells has showen (score 4). (H&E, X: 400)

Assessment of germ cell apoptosis

The results revealed a number of TUNEL-positive germ cells/tubule as reported in Figure 2. Apoptotic cells were detected using TUNEL assay in germ cells of seminiferous tubules in the control group. However, few apoptotic cells were found in this group (P<0.05) (Figure 2A). Apoptosis mainly involved primary spermatocytes and occasionally round spermatids (P< 0.05), but the number of TUNEL-positive cells/tubule significantly increased in MDMA and saline groups (Figures 2B and 2E).

Contrarily, the number of apoptotic cells and seminiferous tubules in the second experimental group were reduced (P<0.05) (Figure 2D). A significant difference in the mean number of TUNEL-positive cells was found among rats treated with PTX before ecstasy administration and the MDMA group (P<0.05). Figure 3 shows the relationship of PTX treatment on the mean number of apoptotic cells in testicular sections in all groups.

Western blotting for active caspase-3 expression

Western blotting for active caspase-3 expression in the testes of all groups was performed (Figure 4). There was no significant difference in active caspase-3 expression between the control and second experimental groups. In the MDMA group, relative expression level of active caspase-3 increased compared to the control group (P< 0.05). Moreover, expression of active caspase-3 significantly decreased in the second experimental group.

Discussion

Based on the data, the acute administration of MDMA causes marked morphological changes in testicular tissues such as tubular degeneration and decreased number of Leydig cells, interstitial edema, and injured germ layer. In agreement with our finding, Barneys *et al* observed mild tubular degeneration and interstitial edema in chronic exposure to MDMA in rats (15). In another research performed by Barneys *et al* on the effect of chronic exposure to MDMA on testes of rat offsprings during the fetal period, the results showed a significantly higher incidence of sperm DNA damage and interstitial edema in testes (16). Apoptosis can occur in a considerable fraction of seminiferous tubules.

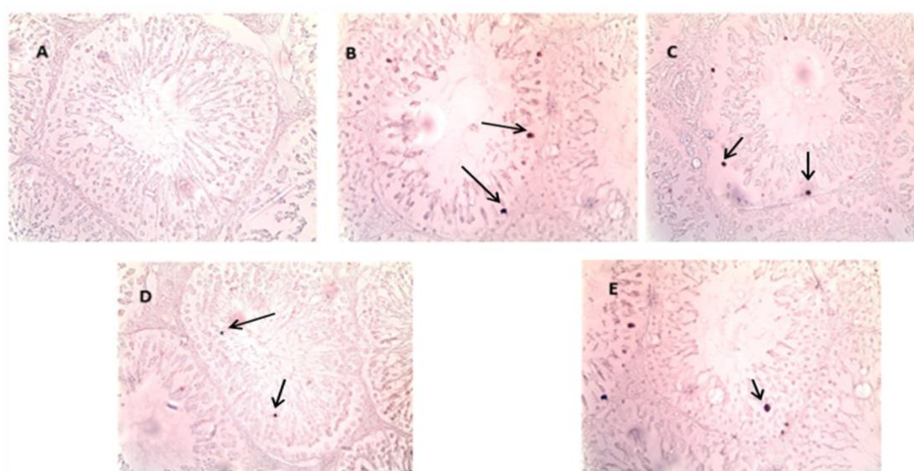

Figure 2. (A) Testis from the control group. No TUNEL-positive cells are observed in the seminiferous epithelium. (B) Testis from the MDMA group. Apoptotic cells are frequently found in the seminiferous epithelium. (C) Testis from the MDMA+ PTX group. A few Apoptotic cells are found in the seminiferous epithelium. (D) Testis from the PTX+ MDMA group. Apoptotic cells are significantly decreased compared to the MDMA group. (E) Testis from the vehicle group. As in the MDMA group, apoptotic cells are frequently found in the seminiferous epithelium. Arrows show the apoptotic cells. (Tunel, X: 400)

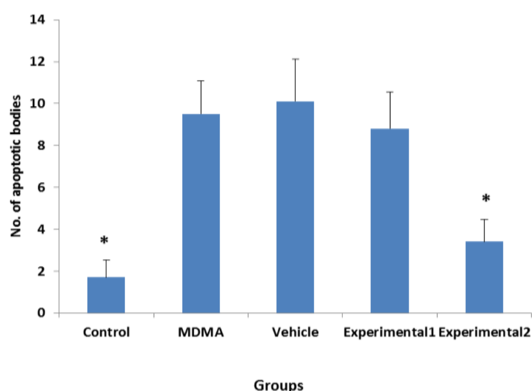

Figure 3. Bar graph shows the effect of PTXon MDMA-induced, germinal epithelium cell death (apoptotic body) in various groups

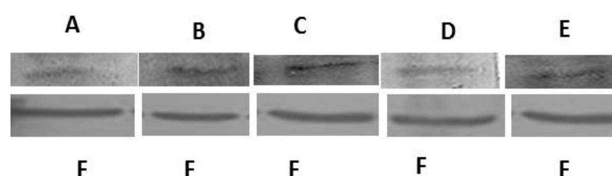

Figure 4. Effect of PTX on MDMA-Induced germinal epithelium cell apoptosis. Western blotting analysis in experimental groups indicated that the PTX treatment could decrease the expression of caspase 3 after MDMA exposure. According to the weight of protein (caspase 3) and protein marker, the blocking is done within the target band.
A: Control; B: MDMA; C: Experimental group 1; D: Experimental group 2; E: Vehicle; F: β actin

These histological injuries are the outcome of general toxicity of MDMA, and several mechanisms may be involved including hormonal disturbance, direct toxic action of MDMA or its metabolites, and the alteration of 5-HT activity in testes. Chronic exposure to MDMA also increases DNA damage in sperm and alters testes histopathology in young male rats (17, 18). Amphetamines can induce apoptosis by endonucleosomal DNA cleavage and nuclear breakdown as well as differential expression of proapoptotic caspase-3 proteins belonging to caspase family (19). It also showed that 5-HT plays a key role in the male reproductive system. It decreases testicular blood flow and vasomotion and is also important for the establishment of spermatogenesis in developing testes. Researchers described p-chloroamphetamine, a 5-HT synthesis inhibitor, can disrupt spermatogenesis (20). Thus, methamphetamine derivatives can disrupt spermatogenesis in seminiferous tubules by altering 5-HT concentration.

MDMA can alter the local temperature, nutrient availability, the vasomotor function or can even act directly on sperm tails (15).

The use of ecstasy can produce oxygen active forms, activate lipid peroxidation and also oxidative stress development (21, 22). Oxidative stress, in turn, can damage all intracellular macromolecules (glutathione, DNA, RNA, proteins, lipids, and ATP). Any change in the levels of these substances strongly affects cell viability, and many cause cell damage and death (23, 24). Various concentrations of reactive oxygen species (ROS) can be beneficial or harmful to cells and tissues. High levels of ROS induce oxidative modification of cellular macromolecules, inhibit protein function, and promote cell death (25). They have a central role in testes physiology such as sperm maturation and capacitation (26). Many studies showed that ROS in semen is an indicator of sperm quality and male fertility status (27, 28).

The present study agreed with Tian and his colleagues (29) who observed that methamphetamine decreased the proliferation of sperm, increased apoptosis of these cells, and disrupted mitochondrial networks. Our data also demonstrated that the injection of 200 mg/kg PTX before the administration of MDMA would decrease apoptosis and caspase-3 in testicular tissue whereas PTX injection after the administration of MDMA did not have such protective effect. PTX (1-9[5-oxohexyl] -3-7-dimethylxanthine), as a methylxan-thine phosphodiesterase inhibitor, has been reported to stimulate sperm motility (13, 30). The beneficial effects of this drug may be due to various mechanisms such as inhibition of phosphodiesterases, increased cAMP levels, and down-regulation of TNF-α, IL-1, IL-6, transforming growth factor-beta (TGF-β), interferon-gamma (IFN-γ), reducing superoxide anion, and reactive oxygen species (ROS), which damage DNA (31-34). Recently, the use of PTX has been considered for its role in the improvement of sperm functions. cAMP is involved in the control of sperm motility and in the regulation of the acrosome reaction (12). Many studies have shown the beneficial effects of PTX on sperm function and positive outcomes of male factor infertility, (9-35, 36). On the contrary, there are other studies that have not shown beneficial effects of PTX on sperm function and semen parameters (37). It seems that some problems such as inadequate sample size, using different questionnaires, failure to detect confounding risk factors, poor selection of study subjects, etc. influenced study results (38). Pretreatment of asthenozoospermia with PTX has been shown to improve *in vitro* fertilization outcome (39). It is well known that PTX has protective effects on DNA. Pretreating sperm with PTX also showed less DNA damage in specific genes after heat shock treatment. PTX is also a sperm stimulator because of its antioxidant property that acts as an ROS scavenger (40). In our study, the PTX effects on apoptotic cells and caspase-3 protein activity were likely due to its antioxidant activity.

Conclusion

The results of this study indicated that abuse of methamphetamines such as MDMA can change normal seminiferous epithelium via decreased cell

proliferation, increased cell death, and caspase3 activity resulting in poor fertility. These results also showed that using PTX can decrease these changes and may be an important new strategy in protection against ecstasy-related reproductive disturbances in men who have decided to quit using these materials.

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