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The Effects of Different Dose of Chronic Ritalin on the Brain of Prepubertal Female Balb/C Mice

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Abstract

Background

Methylphenidate (MPH) is commonly prescribed for children who have been diagnosed with attention deficit hyperactivity disorder (ADHD); however, the action mechanisms of methylphenidate have not been fully elucidated. Studies have shown a relationship between apoptosis signaling pathways and psychiatric disorders, as well as therapeutic targets for such disorders. So, we examined the effects of chronic methylphenidate administration on the brain of mice.

Materials and Methods

Animals were administered MPH at doses of 2, 5 and 10 mg/kg for 60 days. At the age of three months and in estrous phase, brian tissues were removed and washed in cold phosphate-buffered saline and some of them were frozen at -80ºC for Western blot analysis. We measured the levels of pro-apoptotic protein, Bax and anti-apoptoticprotein, Bcl-2, in the brain of neonate female Balb/c mice. The rest of the brains were fixed in formalin (10% phosphate-buffered, pH = 7.4). Then samples were embedded in paraffin according to routine histologic procedures.

Results: Our results showed that MPH with a dose of 10 mg/kg causes a considerable increase in the level of the Bax protein as compared with other groups. In contrast, in the partial cortex of female mice under treatment with high dose of MPH (10 mg/kg) could less Bcl2 levels as compared with 5 mg/kg MPH. However, 5 mg/kg MPH have a significant effect on Bcl2 levels compare with each of mentioned doses ($P<0.05$).

Conclusion

Our results suggest that long-term administration of MPH in the mouse brain had influence on the cascade of apoptosis and its effects, depends on dose rate.

Key Words: Apoptosis, Brain, Mice, Methylphenidate, Ritalin.

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1- INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is the most common neurodevelopmental disorder of childhood characterized by three core symptoms: hyperactivity, impulsiveness, and sustained inattention (1). The symptoms of ADHD affect cognitive, behavioral, emotional, and social functioning (2). It affects school performance and create social misbehavior in the patients (3). The ADHD incidence is from 3 to7 percent, with more prevalence in boys than girls (2). These patients often have serious impairments in academic, social and interpersonal functioning. ADHD is also associated with several comorbid conditions and disorders such as mood disorders, disruptive behavior disorders and learning disabilities (4). Children with ADHD are impaired in executive functions, particularly in fine temporal discrimination (TD), i.e., the discrimination of intervals that differ in millisecond range, which has been shown to be one of the best discriminatory measures for ADHD among a large battery of tasks (5). It is considered as a chronic disorder and approximately 30–50% of individuals diagnosed with ADHD during childhood continue to show its core symptoms into adulthood (1).

The biochemical etiology of ADHD has been related to low levels of catecholamines (namely epinephrine, norepinephrine, and dopamine) and serotonin in certain areas of the brain. These neurotransmitters are responsible for activating the areas of the brain needed for focus and concentration (2). Some recent studies have also shown evidence for abnormalities of glutamate/glutamine and creatine in the brain (6, 7). A disturbance in the interaction between the glutamatergic and dopaminergic systems has been proposed as a key pathogenetic factor in ADHD (6). However, more research needs to be done in this area (2).

One of the most frequently medication for ADHD is the stimulant methylphenidate (MPH). MPH blocks dopamine transporters in the striatum and norepinephrine transporters (NET) in NET-rich cortical regions, including prefrontal cortex, where it increases concentrations of both dopamine and norepinephrine. There is a strong association between dopamine, the striatum, and fine temporal processes (5). The striatal dopamine receptor agonist MPH has been shown to improve motor timing and time estimation deficits in children with ADHD in the millisecond (8, 9) and second ranges (9, 10).

Functional magnetic resonance imaging (FMRI) studies in ADHD patients have shown that single doses of MPH consistently upregulate and normalize frontostriatal activation during cognition (11-14). The only FMRI study investigating the influence of MPH during TD showed that a single dose of MPH significantly upregulated and normalized all under activations observed in ADHD patients relative to control subjects during placebo in dorsolateral prefrontal cortex, ventrolateral prefrontal cortex, anterior cingulate cortex and cerebellum (11).

Although the bioavailability of MPH is believed to be low because of extensive first-pass metabolism, studies with prepubertal rats show that drug uptake is high in the brain, with the maximal concentration occurring in the striatum (15). The density of dopamine transporters in the striatum is significantly reduced for a long time, even after termination of treatment (16, 17). These findings clearly suggest that MPH has short-term and long-term effects (18). Although there are several reports investigating the effects of the acute administration of MPH on animals reared under differing conditions (19, 20), the effects of chronic treatment remain largely unexplored (21).

However, there are few reports about the effects of MPH in apoptosis signaling pathways. So, the present study was aimed to evaluate the effects of chronic administration of MPH in pro-apoptotic protein, Bax, anti-apoptotic protein, Bcl-2, in the brain cortex of female balb/c mice.

2- MATERIALS AND METHODS

2-1. Animal study

Forty female BALB/c mice weighing 10-12 gr at the age of three weeks were used in this study. All animals were housed in a standard light–dark cycle animal room with ambient temperature and humidity. Mice were acclimated to their home cages for at least 10 days before testing. All animal manipulations were carried out according to the Ethical Committee for the use and care of laboratory animals of Iran University of Medical Sciences (IUMS). All efforts were made to minimize the number of animals and their suffering. Mice were divided into four groups of 10 animals each. Animals in the treated group were administered MPH (2, 5 and 10 mg/kg/day were dissolved in 0.2 ml Sodium Chloride 0.9 %, gavage) whereas those in the control group received normal physiological saline. Treatment was continued for 60 days.

The mice eat breast milk for up to three weeks. After breastfeeding, they received MPH 60 days before puberty. The death rate was 2%. At the end of the experimental period, the animals (at the age of three months and in estrous phase) were weighted and euthanized. Brian tissues were removed and washed in cold phosphate-buffered saline and some of them were frozen at -80ºC for Western blot analysis. The rest of the brains were fixed in formalin (10% phosphate-buffered, pH = 7.4). Then samples were embedded in paraffin according to routine histologic procedures.

2-2. Western blot analysis

Collected cortical brain tissues were homogenized in an ice-cold Ripa buffer and protease inhibitor cocktail tablets (Roche, Germany) for 1 h and were then centrifuged (Eppendrof, Hamburg, Germany) at 12,000 g for 20 min at 4 °C. The supernatant was removed and the protein concentration was determined with a BioRad assay system (Bio-Rad, San Francisco, CA, USA). The protein extracts (10 μg) were run on a 10 % SDS–PAGE, and electroblotted on nitrocellulose membranes (Millipore, USA).

The membranes were then stained with a washable Ponceau S solution to confirm equal protein loading. After washing the membranes with distilled water,they were blocked with Tris-buffered saline containing 0.02 % Tween-20 and 5 % of nonfat milk. Antibodies for Bax (mouse monoclonal, 1:1000 dilution; Beyotime Biotech), and Bcl-2 (mouse monoclonal, 1:1000 dilution; Beyotime Biotech) were applied at 4 °C. The blots were then washed and incubated with respective alkaline phosphatase-coupled secondary antibodies (Bio-Rad) at 1:10,000 dilutions. The substrate used to reveal Alkaline phosphatase activity was nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate complex (NBT/BCI) in 100 mM Tris–HCl, 100 mM NaCl, and 5 mM MgCl2 (pH 9.5). The reaction was stopped in 20 mM Tris–HCl and 5 mM EDTA (pH 8.0). Blots were analyzed with UVIdoc (Houston, Texas, USA) software.

2-3. Nissl Staining

Nissl staining detected surviving neurons. For Nissl staining, the paraffin slices were stained with 0.5% Nissl dye solution at room temperature for 15 min until they reached the desired depth of staining. After being rinsed in distilled water and dehydrated in graded serried of ethanol, the sections were immersed in xylene, mounted in neutral balsam, and then cover slipped. Only the neurons with Nissl Body

and the intact morphology were counted as surviving. Cell counting was performed on five randomly selected non-overlapping fields in the four squares subfields (c1-c4) of the parietal cortex per slide. The survival index was defined thusly: surviving index (%) =100* (number of surviving neurons/total number of neurons).

2-4. Statistical analysis

Data were expressed as mean ± standard error (SE). All the statistical analyses were carried out by SPSS software version 16.0. The comparisons among groups were performed using one-way ANOVA and posttest tukey. P-value less than 0.05 were considered statistically significant (df= 3 and F= 294.7 and 3.2 for Bax and bcl2, respectively).

3- RESULTS

3-1. Body weight

Comparison of mean body weight between control and MPH treated groups was done. Reductions in body weight gains were seen only for the 2 and 10 mg/kg dose. In the 5 mg/kg dose group, there was no significant difference in body weight between control and MPH treated groups (**Table.1**).

Table-1: Body weight and dimension of ovaries in control and MPH treated groups.

Groups	Control	Group 1 2 mg/kg/day	Group 2 5 mg/kg/day	Group 3 10 mg/kg/day
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Difference of primary and secondary body weight	20.0286±0.84	14.9250±1.45	19.0625±133	14.5425±1.80*

SE: Standard error.

As you can see in the **Table.1,** reductions in body weight gains were seen only for the 2 and 10 mg/kg dose. In the 5 mg/kg dose group, there was no significant difference in body weight between control and MPH treated groups. The data are shown as means ± SEM. The number of rats in each group was 10. The data were analyzed by one-way analysis of variance followed by Tukey.

3-2. Involvement of Bcl-2 and Bax proteins in the cell apoptosis-induced MDMA

The initiation and execution of apoptosis are mediated by Bcl-2 family proteins. Bcl-2 exerts anti-apoptotic effects and Bax has pro-apoptotic effect. The expression of Bcl-2 protein was increased in the 5 and 2 mg/kg compared to control group. However there was the highest increase of Bcl-2 protein expression in 5 mg/kg. The expression of Bax protein was increased in the 10 mg/kg compared to control group (**Figure.1**). The expression of Bcl-2 protein was increased in the 5 and 2 mg/kg compared to control group. However there was the highest increase of Bcl-2 protein expression in 5 mg/kg. The expression of Bax protein was increased in the 10 mg/kg compared to control group.*compared to control mice, # compared to MPH (2 mg/kg), ($^{*,\#}$ $P < 0.05$).

3-3. Nissl staining analysis

After final injection, the mice were decapitated and underwent Nissl staining. There were no statistical differences in the percentage of viable neurons between the different groups (**Figure.2**).

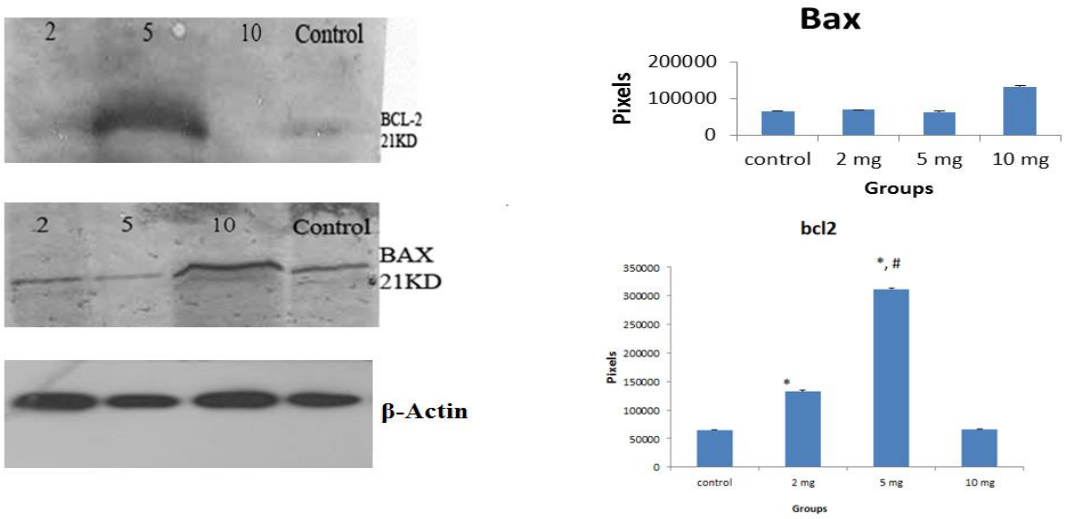

Fig.1: Involvement of Bcl-2 and Bax proteins in the cell apoptosis-induced MPH. MPH: Methylphenidate.

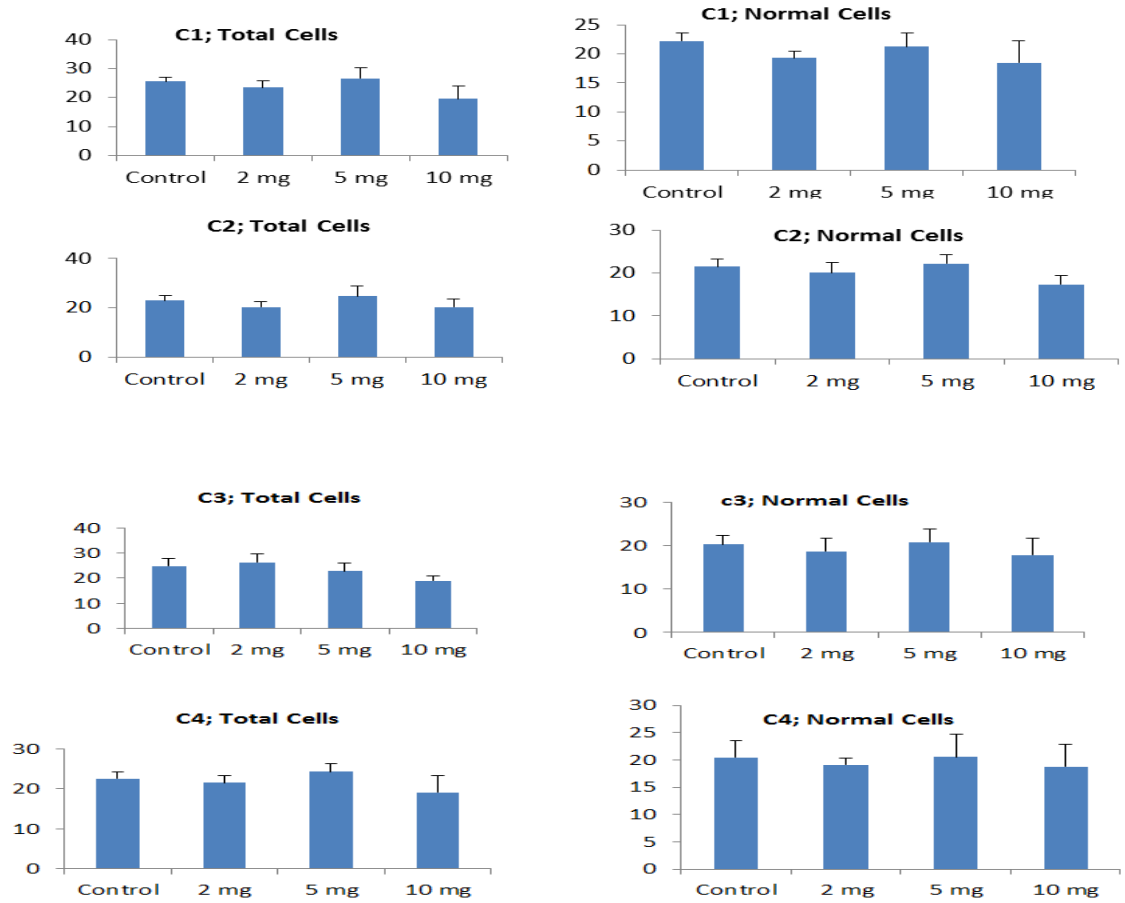

Fig. 2A: The vertical axises are cell counting, and horizontal axises are groups. The total and normal number of cells was counted and compared. There was no difference between them in different groups ($P<0.05$).

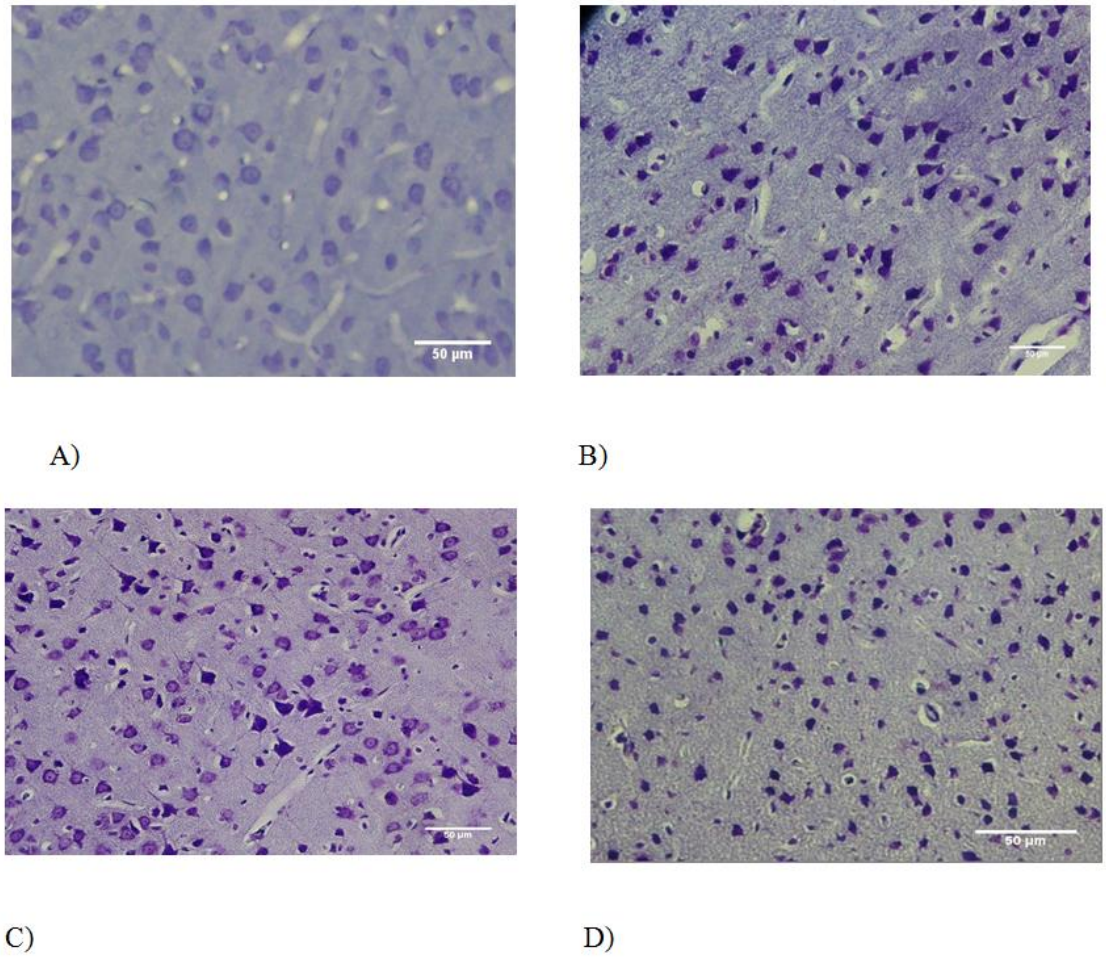

Fig. 2B: A: Identification of neuronal survival by Nissl-staining in the parietal cells treated with the long-term different concentrations of MPH. Control, B: 2 mg/kg, C: 5 mg/kg D: 10 mg/kg. There were no statistical differences in the percentage of viable neurons between the different groups. MPH: Methylphenidate.

4- DISCUSSION

MPH or Ritalin is used extensively in the treatment of ADHD and narcolepsy. Although this drug therapy was introduced about 50 years ago, it is still considered a mainstay of pharmacotherapy for the treatment of ADHD, especially in children (22). As the use of this drug has dramatically increased in the last years, so, investigations are necessary to explore the potential toxicity of MPH. In the present study we reported that the chronic treatment with MPH (2.0, 5.0 or 10.0 mg/kg) in the brain of young or adult rats led to alterations of body weight and protein involved with apoptosis signaling pathways. Our study showed that chronic MPH administration in female mice during adolescence, significantly decreased body weight. Teo et al. showed that significant body weight loss and reduction in food consumption were observed in males dogs that were orally dosed twice a day for total daily doses of 1, 3, 10 and 20 mg/kg/day MPH for 90 days, followed by a 30-day recovery period (23). Manjanatha et al. showed that a significant loss in average body weights of mice fed 4,000 ppm of MPH compared to mice fed the control diet was detected on week's 2–4 that probably associated with less food consumption (22). Panos et al. indicated

that MPH treatment had mild effects on offspring pre-weaning body weight (24). Despite the fact that methylphenidate has become the most prescribed drug to treat attention deficit hyperactivity disorder in the past years, and the consequence of long-term use of MPD are unclear, therefore, research on side effects and potential toxicity of this drug is very necessary (25-27). As we know, the main effect of methylphenidate is elevating dopamine and noradrenaline concentration in the synaptic cleft of the extracellular by inhibiting their reuptake to the presynaptic terminal in cortical brain regions (26, 28).

Also it causes dopamine release (26, 29, 30). Although many of the key apoptotic proteins have been involved with neurodegenerative diseases and mood disorders, they are an excellent target candidate for therapeutic approaches (25, 31, 32). Some of investigations have showed the role of apoptosis signaling pathways in ADHD or with some of the drugs that related to this disease such as methylphenidate (25). Therefore, in the present finding we evaluated the effects of chronic administration of MPH on apoptosis signaling protein such as Bax and Bcl-2 levels, in the cortex of adolescent female mice after the last injection. It is well known that Bax and Bcl-2 are two apoptotic proteins that together regulate the process of apoptosis.

So, our report showed that chronic treatment of different concentration of methylphenidate could alter the apoptosis signaling pathways in partial cortex of adolescence female mice. Treatment with MPH at dose 2 and 5 mg/kg on the partial cortex of adolescent female mice was no significant difference in Bax levels in comparison with negative control group. Although MPH with a dose of 10 mg/kg cause a considerably increase in level of the Bax protein as compare with other groups. In contrast, in partial cortex of adolescent female mice under treatment by high dose of MPH (10 mg/kg) could less Bcl2 levels as compare with 5 and 2 mg/kg MPH. However, 5 mg/kg MPH have highest effect on Bcl2 levels compare with each of mentioned doses. According to the results indicated that chronic treatment with 10 mg/kg MPH elevated levels of Bax when compared to other groups suggesting that MPH could cause neurotoxic effects. However, MPH at the dose of 5 mg/kg increased Bcl-2 and not any effect at Bax levels on the parietal cortex of adolescent female mice compare with control group. These finding suggested that long-term administration of MPH in different doses could effect on neurons of cortex. In other words MPH at the dose of 5 mg/kg could neuroprotective effect and at the dose of 10 mg/kg could neurotoxin effect in parietal cortex of adolescent female mice. Thus, our results supported the idea that high dose of MPH (10 mg/kg) might be caused neurotoxicity in parietal cortex neurons of adolescent female mice. Conversely, MPH at the dose of 5 mg/kg could neuroprotective effect. These results are consistent with results from another study that demonstrated (30).

MPH has both neuroprotective and pro-apoptotic actions in neonatal brain of rodents that caused normal and abnormal aspects of brain development that can be followed by another action mechanism beyond its effects on the dopaminergic system. Indeed, changing in apoptosis signaling pathway are not due dopaminergic neurotransmission alone (25, 33). In this regard Dela Peña et al. (29), showed that the long-term effects of MPH on the brain of spontaneous hypertensive rats (SHR) that changed the expression of gene related with apoptosis, suggesting that in preclinical studies these alteration could effect on cognitive impairment when MPH is administered chronically. On the other hand Gislaine et al. (25) showed that MPH at all doses (1, 2 and 10 mg/kg) increased Bax in the cortex; however, with

the exception of MPH treatment at the dose of 1 mg/kg which elevated the Bcl-2 in the cortex of young and adult rats, all other doses of MPH (2 and 10 mg/kg) reduced with MPH. As we showed, treated the parietal cells to long-term different concentrations of MPH did not significantly change in the C1, C2, C3 and C4 regions in total and normal cells count. Due to their young age of mice and their ability to repair cellular changes, we did not have significant cell death, and we did not see any significant cellular reductions. Or perhaps cell death signaling has stopped at a certain stage. This needs to be further investigation.

4-1. Suggestion

It is recommended that this work be investigated in different species and in different doses.

5- CONCLUSION

The present study focused on molecular mechanisms of MDMA in programmed cell death using gene expression quantification of a pro-apoptotic and anti-apoptoic gene in MDMA-induced neurotoxocity. The results showed that MDMA prompted apoptosis in brain in higher dose. According to our study, the best dose is 5 mg/kg.

6- CONFLICT OF INTEREST: None.

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