



Effect of Mephedrone Exposure During Gestation on the Morphometrical Parameters of the Cerebellum in the Balb/C Mouse Offsprings

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Abstract

Background: Addiction, particularly to synthetic stimulants like mephedrone, poses significant challenges for both treatment and societal health. Mephedrone, a cathinone derivative, is increasingly misused and has been shown to affect brain function. While the neurotoxic effects of mephedrone are well-documented in adolescents, limited research exists on its effects during pregnancy, particularly on cerebellar development in offspring.

Objectives: This study examines the effect of mephedrone exposure during gestation on the morphometrical parameters of the cerebellum in Balb/C mouse offspring.

Methods: In this experimental study, 10 one-day-old male mouse pups were divided into a control and an experimental group, whose mothers received daily subcutaneous mephedrone injections (50 mg/kg) from days 5 to 18 of pregnancy. Cerebellar tissue from the pups was processed and stained with cresyl violet, and cerebellar layers and cell characteristics were assessed using histological methods.

Results: A significant reduction was observed in the thickness of the outer granular, molecular, Purkinje, and inner granular layers compared to the control group ($P < 0.05$). However, no significant differences were observed in white matter (WM) thickness, cell counts, or Purkinje cell morphology, including their area, perimeter, or diameters.

Conclusions: Exposure to mephedrone during pregnancy resulted in altered cerebellar development in mouse pups, specifically reducing the thickness of cerebellar layers.

Keywords: Mephedrone, Mice, Pregnancy, Cerebellum

1. Background

Addiction is a dependence on a stimulus that, despite its negative effects, provides psychological pleasure, increasing the desire for repeated use (1). Addiction, especially drug addiction, is a major global issue with profound physical and psychological effects. It is regarded as a "social disease" that undermines the cultural, economic, and social stability of nations. Addictive substances can be classified into three categories based on their effects: Depressants, hallucinogens, and stimulants. They can also be divided

into two types based on their origin: Natural and synthetic (2).

Although traditional addiction treatments and counseling methods are well-established, the rise of synthetic stimulants introduces new challenges. These substances, easily produced without the need for cultivation or international trade, are widespread because their chemical precursors are readily accessible in various industries. The widespread availability of these substances makes supply control challenging, directly affecting demand reduction efforts. Moreover, the effects of over 700 stimulants on the human brain

and body remain largely unexplored, complicating effective treatments.

Cathinone is an active compound found in the leaves of the khat plant, which has amphetamine-like properties and is commonly chewed in some African countries. Structurally, cathinone is similar to amphetamine, with the key difference being the presence of a carbonyl group in the cathinone molecule (Figure 1) (3).

A wide range of cathinones are currently misused as stimulants, particularly among young people. Some of these substances have limited medical applications. Methcathinone was originally used as an antidepressant but was withdrawn from clinical use due to instances of misuse (4). Diethylpropion is another cathinone analog currently used as an appetite suppressant. Some studies have reported neurotoxic effects of this drug in laboratory animals (5).

In vitro studies on human and animal cells have demonstrated that cathinones significantly elevate levels of dopamine, norepinephrine, and serotonin. Due to their structural similarities with other stimulants, cathinones share functions such as stimulating the release of monoamine neurotransmitters and inhibiting their reuptake in the synaptic cleft (6, 7). In addition, in vitro studies have shown that all cathinones, particularly mephedrone and MDPV, have the ability to cross the blood-brain barrier (8).

Among cathinone derivatives, mephedrone, methylone, and MDPV have garnered significant research attention due to their widespread use and associated health concerns. Mephedrone, in particular, has been shown to induce the release of dopamine in the striatum of rodents, both when administered alone or in combination with methamphetamines. Mephedrone was first synthesized in 1929 (9) and became popular as a recreational substance in some countries starting in 2003 (10). In 2008, it was introduced as a new psychoactive substance by the European Monitoring Center for Drugs and Drug Addiction (11).

A study in England revealed that 20 percent of ecstasy pills contain mephedrone, and the presence of this substance in the stimulant market is rapidly increasing. Mephedrone has been present in Iran since 2011 and is known among young people by slang names such as "Mef", "Salt", "Meow Meow", "Bath Salts", and "Ivory Wave". It is frequently used in combination with other substances, including bath salts, which often contain both mephedrone and MDPV (12).

Mephedrone is typically a white powder but can also be found in tablet or capsule form. It's mainly inhaled or

taken orally, with doses ranging from 0.5 to 1 gram per session (13). Effects begin within 15 to 45 minutes and last 2 to 5 hours (14). Overuse can cause euphoria, hallucinations, nausea, anxiety, elevated blood pressure, confusion, tremors, and other symptoms. Compared to ecstasy, mephedrone is associated with a stronger urge for repeated use, with inhalation linked to higher dependence compared to cocaine (13, 15). It inhibits norepinephrine reuptake and exhibits sympathomimetic effects (7, 16, 17).

In Iran, approximately 10 percent of addicts are women. A study by Khajedalooee and Dadgar Moghadam in 2013 found that visible congenital abnormalities at birth were significantly more prevalent in infants whose mothers were addicted during pregnancy, compared to those born to non-addicted mothers (18). Research shows that substance abuse during pregnancy, including heroin, methadone, codeine, and morphine, poses risks to both the mother and child. The use of these substances is associated with complications such as fetal growth restriction, premature birth, stillbirth, and microcephaly (19-21).

The use of psychoactive drugs has not been as widespread as traditional drugs like cocaine, resulting in less information about their side effects during pregnancy. However, it has been shown that these substances decrease the consumer's appetite, leading to poor fetal growth. Prolonged use of ecstasy can cause long-term learning difficulties in infants and memory impairments later in life (22).

The cerebellum, located in the posterior cranial fossa and forming the roof of the fourth ventricle, is connected to the brainstem by three pairs of peduncles: Superior, middle, and inferior. Its primary function is to coordinate reflexes and voluntary muscle movements, regulating muscle tone, maintaining posture, and ensuring that movements are precise and efficient (23). The metencephalon, a part of the developing brain, forms the cerebellum. Initially, the upper lateral plate enlarges, and the posterior sections bend to create rhomboid edges, which eventually converge to form the cerebellar plate, consisting of the vermis and hemispheres. The cerebellar cortex after birth includes differentiated Purkinje cells, the molecular layer (ML) at the surface, and the internal granular layer (IGL) beneath the Purkinje cells (24). The ventricular layer, which gives rise to cerebellar nuclei and Purkinje cells, becomes the ependymal layer once Purkinje cells are positioned beneath the external granular layer (EGL) (25). As the cerebellum matures, its cortex folds into folia, and the EGL undergoes peak proliferation shortly after birth. Granule and Purkinje cells attain their final

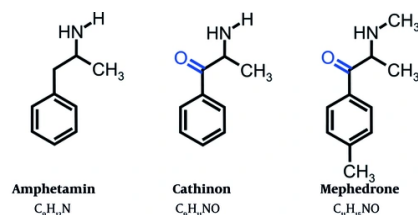


Figure 1. Comparison of the molecular structures of cathinone, mephedrone, and amphetamine

size postnatally, marking the completion of cerebellar development (26).

2. Objectives

This study was conducted to determine the impact of mephedrone exposure during pregnancy on neonatal outcomes, focusing particularly on the morphometric indices of the cerebellum in newborns.

3. Methods

This experimental study was conducted on brain samples of 10 one-day-old male mouse pups, divided into experimental and control groups. The experimental group consisted of pups whose mothers received daily subcutaneous mephedrone injections (50 mg/kg) from days 5 to 18 of pregnancy, while the control group received saline injections under the same conditions. The samples were obtained from our previous study (27).

3.1. Tissue Preparation

The brains were sectioned using a rotary microtome, which allowed for precise slicing at a thickness of 6 microns. Each brain was sliced coronally, yielding 200 - 300 sections per brain. Meticulous handling was required to prevent damage to the delicate cerebellar tissue. Selected slices were floated on warm water (45°C) to relax any folds, then transferred onto slides and dried on a hot plate at 50°C to melt the surrounding paraffin. The sections were stained using cresyl violet, which highlights neuronal structures, particularly the cell nuclei (28).

Cresyl violet staining was used because it is known to highlight neuronal structures. We chose these characteristics because changes in cell nucleus size and shape can be indicative of developmental alterations or cellular stress. In stained sections: (A) neuronal cell bodies (Perikaryon) appeared purple; (B) Purkinje cells,

large cells with purple nuclei and clear cytoplasm, were organized in a single layer beneath the ML; (C) granular cells, smaller with purple nuclei and cytoplasm, were also identifiable.

After staining, coverslips were applied using Entellan adhesive, and slides were left to dry completely. Stained sections were photographed using an Olympus BX53 optical microscope at different magnifications: (A) 400x for measuring cerebellar layer thickness by drawing the longest perpendicular line across the layers; (B) 20x for white matter (WM) thickness; (C) 100x for cell counting, particularly in the densest cerebellar regions.

A grid with squares of 400 μm^2 was used for cell counting, and cells were counted based on morphometric characteristics. Data on cell counts and layer thicknesses were recorded in Excel for statistical analysis.

3.2. Statistical Analysis

The data were analyzed using SPSS software (version 20). Normality was assessed with the Kolmogorov-Smirnov and Shapiro-Wilk tests. For normally distributed independent quantitative data, an independent *t*-test was applied. For non-normally distributed data, such as the area of Purkinje cells, the Mann-Whitney U test was used. Results were expressed as mean $\pm$ standard deviation (SD), with a significance level of $\alpha = 0.05$.

4. Results

4.1. Cerebellar Layer Thickness

The thickness of the outer granular layer was significantly lower in the experimental group ($26.19 \pm 4.19 \mu m$) compared to the control group ($31.92 \pm 2.08 \mu m$; $P < 0.05$). Similarly, the ML thickness was significantly reduced in the experimental group ($6.94 \pm 0.6 \mu m$)

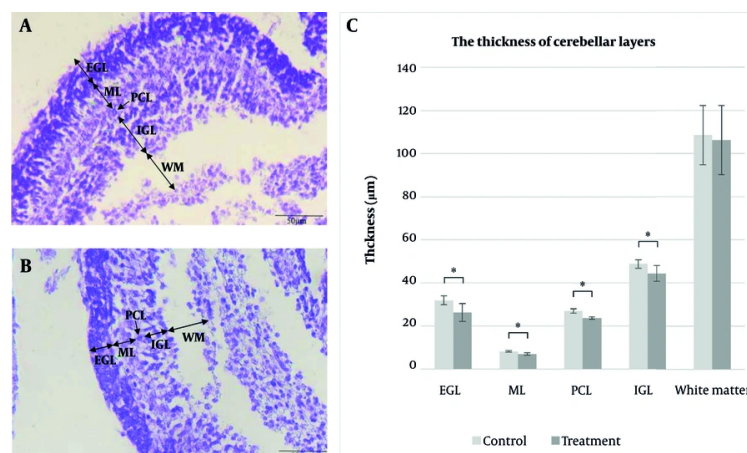


Figure 2. A, the thickness of the white matter (WM) and gray matter layers in the cerebellum of pup male mice of control; B, and experimental groups (staining: Cresyl violet, magnification 400x); C, the thickness of the external granular layer (EGL), molecular layer (ML), Purkinje cell layer (PCL), and internal granular layer (IGL) was significantly reduced after exposure to mephedrone, but the WM thickness was not affected significantly (values represent the means $\pm$ SEM, $n = 5$). * $P < 0.05$ versus its corresponding control group.

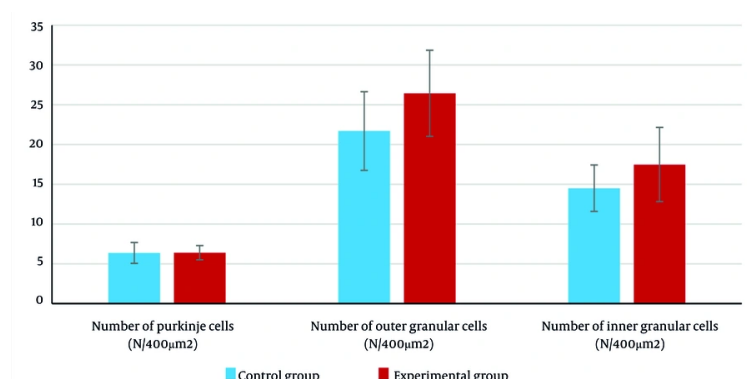


Figure 3. Number of the cells in the cortex of the cerebellum in experimental and control groups

versus the control group ($8.20 \pm 0.34 \mu\text{m}$; $P < 0.05$, Figure 2).

The thickness of the Purkinje layer was significantly reduced in the experimental group ($23.64 \pm 0.59 \mu\text{m}$) compared to the control group ($26.92 \pm 1.00 \mu\text{m}$; $P < 0.05$). The inner granular layer thickness also reduced significantly in the experimental group ($44.35 \pm 3.65 \mu\text{m}$) compared to the control group ($48.72 \pm 2.06 \mu\text{m}$; $P < 0.05$). Although the WM thickness was reduced in the experimental group ($106.22 \pm 16.06 \mu\text{m}$) compared to the control group ($108.57 \pm 13.77 \mu\text{m}$), the difference was not significant.

4.2. Cell Counts

The number of Purkinje cells increased slightly in the experimental group (6.40 ± 0.9) compared to the control group (6.37 ± 1.31), but the difference was not significant. The count of outer granular cells increased in the experimental group (26.44 ± 5.42) relative to the control group (21.70 ± 4.94), but this change was also not statistically significant ($P > 0.05$). Inner granular cell numbers were higher in the experimental group (17.48 ± 4.67) compared to the control group (14.51 ± 2.92), but

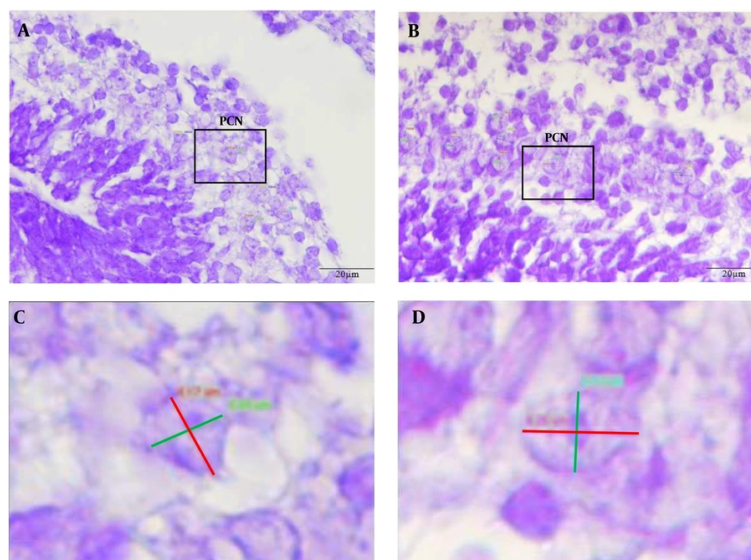


Figure 4. A, The Purkinje cell nucleus (PCN) characteristics in the cerebellum of pup male mice of control; and B, experimental groups. (staining: cresyl violet, magnification 1000x). The red line in C, and D, is large diameter and green line in C, and D, is small diameter in the higher magnification of the Purkinje cell nucleus (PCN) in A, control and B, experimental group.

this difference was not statistically significant (Figures 3 and 4).

4.3. Purkinje Cell Characteristics

The environment of Purkinje cells' nucleus (surrounding area) showed a slight reduction in the experimental group (24.67 ± 1.40) compared to the controls (24.74 ± 1.45), but the change was not significant. The area of Purkinje cells also reduced slightly in the experimental group (32.43 ± 4.04) in comparison with the control group (32.49 ± 3.72), but this difference was not significant (Figures 4 and 5).

The small diameter of Purkinje cells increased slightly in the experimental group (5.41 ± 0.4) compared to the control group (5.22 ± 0.39), but this difference was not significant. The large diameter of Purkinje cells decreased in the experimental group (7.11 ± 0.62) compared to the control group (7.33 ± 0.50), but this change was not significant (Figures 4 and 5).

5. Discussion

This study aimed to assess the effects of mephedrone exposure during pregnancy on neonatal outcomes, particularly examining the morphometric indices of the cerebellum in newborns. The results indicated that prenatal exposure to mephedrone led to alterations in

the cerebellar cortex of mice offspring. There is controversy regarding the impact of mephedrone exposure on tissues. The studies on the impact of mephedrone exposure during pregnancy on fetal and neonatal outcomes are limited. Several *in vivo* studies show no evidence of mephedrone-related toxicity (13, 29, 30). These findings may be influenced by varying metabolic responses and different methods of administering mephedrone (29-31).

A number of studies identify potential neurotoxic effects of mephedrone in adolescent rodents (32-34). The inhibitory effect of mephedrone on the survival and proliferation of neural cells is demonstrated in several *in vitro* and *in vivo* experiments (32, 33, 35, 36). In cultured cortical neurons, mephedrone reduces neuronal survival in a concentration-dependent manner (35). In adolescent male mice, administration of 25 mg/kg of mephedrone (four times a day, every 2 hours) leads to a decrease in the number of newly formed cells in the dentate gyrus of the hippocampus (33). According to one study, administering 25 mg/kg of mephedrone over two consecutive days during weekends results in astrogliosis and the loss of serotonergic markers in the hippocampus (35).

The effect of mephedrone can be attributed to several mechanisms. The anti-proliferative and pre-apoptotic effects of mephedrone may be due to oxidative stress in

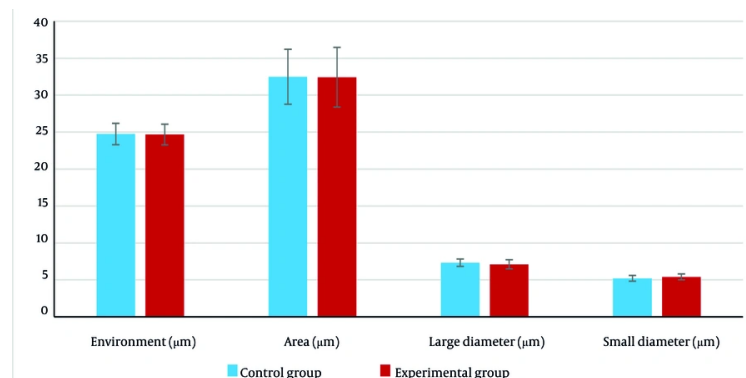


Figure 5. Purkinje cell nucleus (PCN) characteristics in experimental and control groups

brain tissue. Several studies demonstrated that both single and repeated exposure to mephedrone lead to increased lipid peroxidation in the cerebral cortex and hippocampus of adolescent and adult rodents (32, 33, 37).

A study highlighted the central role of oxidative stress in the molecular toxicity of psychostimulant drugs (e.g., MDMA and methamphetamine) (38). An increase in reactive oxygen species can activate mitochondria-dependent apoptotic pathways by disrupting the balance between pro-apoptotic and anti-apoptotic proteins, leading to the release of apoptogenic factors from mitochondria and the induction of apoptosis through caspase-dependent or independent mechanisms (39). One study indicated that exposure to mephedrone during pregnancy might disrupt learning and memory processes, likely by causing damage to the developing infants' hippocampus (27).

Molecularly, cathinones, including mephedrone, share structural similarities with other stimulants and function by stimulating the release of monoamine neurotransmitters (dopamine, norepinephrine, serotonin) and inhibiting their reuptake in the synaptic cleft. Specifically, mephedrone has been shown to induce the release of dopamine in the striatum of rodents. It also inhibits norepinephrine reuptake and exhibits sympathomimetic effects (29, 31).

Another study reported that mephedrone may contribute to neurotoxicity by increasing oxidative stress and impairing mitochondrial respiratory chain function in the hippocampus, cortex, and cerebellum (40). The cerebellum primarily regulates muscular functions, including balance and movement, while also

contributing to cognitive functions such as language processing and memory. The use of drugs such as alcohol, caffeine, and heroin has been shown to lead to structural changes in the higher brain cells, particularly in the cerebellum and the Purkinje cells (41). A study showed that administering morphine during gestation and lactation leads to Purkinje cell loss and decreases the size and thickness of the Purkinje cell layer (PCL) in the cerebellar cortex of 18- and 32-day-old infant mice (42). Several mechanisms can be considered regarding the effect of morphine on the central nervous system. The loss of Purkinje cells in morphine-treated animals may result from apoptosis or necrosis (43). Moreover, morphological changes in astrocytes caused by morphine can increase both Ca^{2+} levels and carbonyl oxidation production, which may subsequently promote apoptosis or necrosis in neurons (44). Several studies have shown that morphine, like heroin, can reduce the proliferation and differentiation of Purkinje cells while increasing cell death in the cerebellum (43, 45). The N-methyl-D-aspartate receptor-caspase pathway can induce neurotoxic effects of opioids (46). Indeed, cell death can occur as a result of mitochondrial damage (47).

We hypothesize that exposure to mephedrone during pregnancy may affect the morphometric parameters of the cerebellum in the offspring of Balb/C mice. Our results indicated a significant reduction in the gray matter thickness of newborns exposed to mephedrone, while the thickness of the WM remains unaffected. The findings align with previous studies on morphine's impact on cerebellar cortical layer thickness (48, 49). Based on the results, the number of molecular, Purkinje, and granule cells, as well as the area, perimeter, and

major and minor diameters of Purkinje cell nuclei in the cerebellum of newborns exposed to mephedrone, are not significantly impacted. The absence of changes in the number of cerebellar neurons may be due to alteration in gray matter thickness due to reduction in glial cells and damage to nerve fibers.

5.1. Conclusions

This experiment resulted in a significant reduction in the thickness of several cerebellar cortex layers, including the outer granular, molecular, Purkinje, and inner granular layers. However, no significant differences were observed in the thickness of WM, cell counts, or the morphological characteristics and the environment of Purkinje cells' nucleus. These findings suggest that mephedrone exposure primarily influenced the thickness of cerebellar layers rather than cellular density or Purkinje cell structure. Overall, this study demonstrated that exposure to mephedrone during pregnancy could induce alterations in the cerebellar cortex of mice offspring.

Footnotes

Authors' Contribution: M. J. G. and Gh. N. designed the experiments. S. K. performed experiments and collected data. S. K. and Gh. N. discussed the results and strategy. Gh. N. supervised, directed, and managed the study. S. K., M. J. G., and Gh. N. gave final approval of the version to be published.

Conflict of Interests Statement: The authors declare no conflict of interests.

Data Availability: The dataset presented in the study is available on request from the corresponding author during submission or after publication.

Ethical Approval: Ethical approval for this study was conducted only after due clearance and approval from the Ethics Committee of Golestan University of Medical Sciences (IR.GOUMS.AEC.1402.018).

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