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Exposure to mephedrone during gestation increases the risk of stillbirth and induces hippocampal neurotoxicity in mice offspring

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ABSTRACT

In recent years, abuse of synthetic cathinones, in particular, mephedrone, has increased among young adults worldwide. The study aim is to investigate the effects of mephedrone exposure during the gestational period on mice offspring outcomes, focusing on hippocampal neurotoxicity. The pregnant mice received mephedrone (50 mg/kg, sc) on a regular schedule (once daily on all days, from day 5 to 18 of gestation) or repeated schedule (thrice daily on day 5, 6, 11, 12, 17, and 18 of gestation) to simulate regular or recreational use of mephedrone, respectively. Results showed that the percentage of weight gain in pregnant mice was significantly lower in both regular and repeated schedule mephedrone groups ($p < 0.01$). Also, mephedrone significantly reduced the number and weight of delivered pups and increased the rate of stillbirth ($p < 0.05$). Immunohistochemistry and TUNEL assay showed an inhibition of cell proliferation ($p < 0.05$) and an increase of apoptosis ($p < 0.05$) in the hippocampus of delivered pups of the repeated schedule mephedrone group. This apoptotic effect was associated with enhanced expression of the pro-apoptotic Bax gene ($p < 0.05$) and reduction of expression of the anti-apoptotic Bcl-2 gene ($p < 0.05$) as evaluated by real-time PCR. The Morris water maze showed an impairment of spatial learning ($p < 0.05$) and reference memory ($p < 0.01$) in offspring born from litters exposed to mephedrone (repeated schedule). In conclusion, the present study has shown that regular and repeated exposure to mephedrone during the gestational period increases the risk of low birth weight and stillbirth. Also, repeated use of mephedrone impairs learning and memory processes through hippocampal damage.

1. Introduction

Mephedrone (4-methylmethcathinone), also called meow meow, drone, meph, and bubbles, is a synthetic derivative of cathinone, the natural psychostimulant alkaloid present in the khat plant. The chemical structure of mephedrone is closely related to the phenylethylamine family of illegal drugs, including methamphetamine and 3, 4-methylenedioxymethamphetamine (MDMA) (Schifano et al., 2011). In recent years, abuse of synthetic cathinones, in particular, mephedrone, has increased among adolescents and young adults in several countries (Measham et al., 2010; Hockenhull et al., 2016). Recreational use of mephedrone is associated with stimulant effects such as agitation, tachycardia, hypertension, euphoria, reduced appetite, insomnia, and even seizure (Wood et al., 2010a; Wood et al., 2010b; Winstock et al., 2011). The effects of mephedrone on the nervous system seem to be mediated mainly by stimulating the release of monoamine neurotransmitters (dopamine, serotonin, norepinephrine) and preventing their reuptake (Baumann et al., 2012; Hadlock et al., 2011; Luethi et al., 2017). In addition, repeated exposure to mephedrone has been shown to induce oxidative stress in the brain, inhibit hippocampal

Abbreviations: DAB, 3,3′-diaminobenzidine; DG, dentate gyrus; HRP, horseradish peroxidase; MDMA, 3, 4-methylenedioxymethamphetamine

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neurogenesis, and impair memory in adolescent rodents (López-Arnau et al., 2015; Ciudad-Roberts et al., 2016).

Drug abuse in pregnancy has increased over the last decades and has become an emerging public health problem (Keegan et al., 2010; Wendell, 2013). Recent studies have reported that street drugs and narcotics have a devastating outcome for the mother and the fetus. For example, it has been reported that maternal exposure to amphetamine, which has a similar structure to mephedrone, may increase the risk of preterm birth, low birth weight, small head circumference, learning disabilities, congenital anomalies, placental abruption, and postpartum hemorrhage (Lindsay and Burnett, 2013). Regarding mephedrone, no experimental study has yet evaluated its neurotoxic effects in the gestational period. Recent pharmacokinetic studies have revealed that mephedrone can cross the placenta and enter the fetal brain (Strange et al., 2017). Preliminary data of a study has proposed an association between mephedrone usage and premature birth (Jones et al., 2011). The aim of the present study is to investigate the effects of mephedrone exposure in the gestational period on neonatal outcomes, focusing on hippocampal neurotoxicity in mice offspring.

2. Materials and methods

2.1. Reagents and chemicals

Mephedrone hydrochloride was synthesized in our organic chemistry laboratory, following the method described by Lopez-Arnau et al. (Lopez-Arnau et al., 2012). The synthesized compound was a white powder with melting point of 240–242 °C. The structure of mephedrone was confirmed by carbon and proton nuclear magnetic resonance spectroscopy (Supplementary material). An in situ cell death detection (TUNEL) kit was purchased from Roche (USA). Rabbit monoclonal antibody against mouse Ki-67 protein (ab16667) and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (ab6721, a secondary antibody) were obtained from Abcam (USA). Hybrid-RTM microRNA extraction kit and cDNA synthesis kit (Superscript First-Standard Synthesis System) were purchased from GeneAll Biotechnology (South Korea). Proteinase K was bought from Thermo Scientific (USA). Normal goat serum and 3,3′-diaminobenzidine (DAB) were purchased from Sigma (USA).

2.2. Animals and study groups

Adult male and female Balb/C mice weighing 25–30 g were obtained from Laboratory Animals Research Center, Mashhad University of Medical Sciences, Mashhad, Iran. The mice were kept under controlled conditions of temperature (20 ± 2 °C) and light (12 h light/dark cycle) and had free access to food and water. All animal experiments complied with the National Institutes of Health guide for the care and use of laboratory animals, and was approved by the ethics committee at the Mashhad University of Medical Sciences.

Female mice were caged with fertile male mice at a ratio of 1:1 and the day in which vaginal plug was observed was considered as day 1 of gestation. Twenty-eight pregnant female mice were randomly allocated into four groups ($n = 7$): (1) the “regular schedule mephedrone group” was treated with 50 mg/kg of mephedrone (dissolved in saline, subcutaneous injection) once daily on all days from day 5 to 18 of gestation; (2) the “regular schedule control group” received saline as a control injection; (3) the “repeated schedule mephedrone group” was treated with 50 mg/kg of mephedrone thrice daily (subcutaneous, 2-h interval) on day 5, 6, 11, 12, 17, and 18 of gestation; (4) the “repeated schedule control group” received saline as a control injection. Regular and repeated treatment patterns were used to simulate regular and recreational (on the weekend) usage of mephedrone, respectively. The 2-h interval between injection was chosen because the elimination half-life of mephedrone is about 2 h and this mimics the modeled mephedrone boosting in humans (López-Arnau et al., 2015; Papaseit et al., 2016). The usual amount of mephedrone use over an evening/night has been estimated to be about 100–200 mg every hour or two hours (Measham et al., 2010). Considering dose translation based on the body surface area, 50 mg/kg in mice is approximately equivalent to 4 mg/kg in humans (i.e., 200 mg for young women with 50 kg weight) (Reagan-Shaw et al., 2008).

2.3. Animal follow-up and tissue sampling

Each litter was caged singly until parturition, and its body weight, water intake, duration of pregnancy, the number of delivered pups, and the percentage of stillbirths were recorded. One day after delivery, the body weight of all pups from each litter was measured, and the average value for each litter was used in the statistical analysis. For immunohistochemistry and TUNEL assay, only one male pup per litter was randomly sampled to be sacrificed for removal of the brain. The brain was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned coronally with a thickness of 5-μm, and then the sections were placed on poly-L-lysine-coated glass slides.

For real-time PCR, one male pup per litter was randomly chosen and the hippocampus of the right brain hemisphere was removed, rapidly frozen in liquid nitrogen, and stored at −80 °C. The remaining pups were housed until the 60th postnatal day when the learning and memory of one male pup from each litter were evaluated by the Morris water maze test. Because there are sex differences in hippocampal neurogenesis, learning, and memory (Duarte-Guterman et al., 2015), the test was carried out using only male pups to avoid the hormonal effect of the estrus cycle on learning and memory performance.

2.4. Immunohistochemistry

The brain sections were deparaffinized with xylene, rehydrated in graded ethanol, and heated in PBS/EDTA solution for antigen retrieval. The tissue sections were immersed in a methanol/H_2O_2 solution (1:100) for 15 min in the dark to block endogenous peroxidase. Then, after washing with PBS containing 0.025% Triton X-100, the sections were incubated for 1 h with 10% normal goat serum. Next, the sections were covered with an antibody against Ki-67 (diluted 1:100 with 1% BSA), as a cell proliferating marker. After overnight incubation at 4 °C, the sections were washed with the PBS/Triton X-100 solution and treated with the HRP-labeled secondary antibody (diluted 1:70 with 1% BSA) for 60 min at room temperature. Then, the sections were washed with PBS, treated for 15 min with DAB solution (0.03% *w/v* DAB and 0.2% *v/v* H_2O_2 in PBS), washed again with running water, and were counterstained with Harris-Hematoxylin solution. Finally, the sections were dehydrated in ascending grade ethanol, cleared with xylene, and mounted onto coverslips.

2.5. TUNEL assay

The hippocampal sections were deparaffinized, rehydrated, and immersed in methanol/H_2O_2 solution to block endogenous peroxidase. Then, the tissue sections were incubated with proteinase K solution for 20 min at 21–37 °C. After washing with PBS, they were incubated overnight with TUNEL reaction mixture at 4 °C. Then, the sections were washed with PBS and treated with peroxidase (POD) for 2 h at room temperature. The sections were rewashed with PBS, incubated for 15 min with DAB solution in the dark, and counterstained with Harris-Hematoxylin. Finally, the sections were dehydrated in ascending grade ethanol, cleared with xylene, and mounted onto coverslips.

2.6. Stereological analysis

The number of Ki-67 and TUNEL positive cells in the CA1, CA2, CA3, and dentate gyrus (DG) regions of the hippocampus was counted using the optical dissector technique as described in detail previously

Table 1
Sequences of primers for interest and reference genes. The sequences of genes were retrieved from the NCBI database and primer sets were designed and analyzed in a basic local alignment search tool to avoid secondary structure and homology with other sections of the genome. MT: Melting temperature.

Gene	Sequence (5′→3′)	MT	Product size (bp)
β-Actin	F: CACTGTCGAGTCGCGTCC	60.7	102
	R: CGCAGCGATATCGTCATCCA	60.5	
Bax	F: TTTGCTACAGGGTTTCATCCAG	60.3	148
	R: CATATTGCTGTCCAGTTCATCTC	61.1	
Bcl-2	F: GCCTTCTTTGAGTTCGGTG	57.3	162
	R: ATATAGTTCCACAAAGGCATCC	58.4	

(Haghir et al., 2017). Briefly, the tissue sections were photographed at a magnification of 400× using a light microscope (Olympus BX51, Japan) equipped with a DP12 digital camera. The images were analyzed by ImageJ software. The rectangular grids were superimposed on the images and the stained cells were counted per unit area. The mean number of Ki-67 and TUNEL positive cells per unit area (N_A) in each hippocampal regions was calculated by the following formula:

$$N_A = \sum Q \div (a/f \times \sum p)$$

where "ΣQ" is the sum of counted cells in each section frame, "a/f" is the area associated with each frame, and "Σp" is the sum of frame-associated points hitting reference space.

2.7. Real-time PCR

Total RNA was isolated from the hippocampus using the Hybrid-R™ microRNA extraction kit according to the manufacturer's instructions. Reverse transcription reaction was performed using the Superscript First-Standard Synthesis System. Quantitative PCR was done on the Step One Real-time PCR system using SYBR Green I reaction mix containing 1X Real Q master mix and 5 µM primers for Bax or Bcl-2 (Table 1). Thermal cycling condition was as follows: 95 °C for 5 min (initial denaturation) followed by 40 cycles of 95 °C for 10 s (denaturation), 60 °C for 10 s (annealing), and 72 °C for 30 s (extension). The fluorescent signal was captured at the end of the extension step. To confirm the specificity of each reaction, melting curve analysis was carried out from 55 to 95 °C. The β-actin was used as the normalizer gene and each sample was tested in triplicate. Finally, fold changes in gene expression were determined using the delta-delta Ct method.

2.8. Morris water maze

Spatial learning and memory of mice (60-day-old) born from treated litters were determined using the Morris water maze method. A circular pool with a diameter of 136 cm and a high of 60 cm was filled with water (23–25 °C) to a depth of 28.5 cm. The pool was divided into four quadrants, which were labeled as north (N), south (S), east (E), and west (W). An escape platform (10 cm diameter) was placed in the center of the northeast quadrant of the pool and submerged to a depth of 0.5 cm from the water surface. The pool was located in an isolated room and a number of fixed visual cues were placed on the walls around the pool. Each mouse underwent four trials on each of the five consecutive days. In each trial, the animal was placed in the pool, facing the pool wall, and allowed to swim to find the platform. If a mouse did not find the platform within 60 s, it was guided to the platform by the examiner and allowed to stay there for 20 s. After completion of the fourth trial, the mouse was removed, dried, and placed in its holding box. The latency time to find the platform and the length of the swimming path was recorded by a video tracking system. On the sixth day (probe day), to assess reference memory, the platform was removed, and the mice were allowed to swim for 60 s. The time spent in the target quadrant was recorded for each animal and compared between groups (Hosseini et al., 2011).

2.9. Statistical analysis

The changes in maternal body weight, maternal water intake, and offspring learning during different days were analyzed by the repeated measures two-way analysis of variance (ANOVA), with the schedule of administration (regular or repeated schedule injection) and treatment (saline or mephedrone) as between-subjects factors and day as the within-subjects factor. Other data were analyzed using the two-way ANOVA followed by the unpaired Student's *t*-test. The results were presented as mean ± SEM and probability level < 0.05 was considered significant.

3. Results

3.1. Effects of mephedrone on litters and delivered pups

The results of the repeated measures ANOVA demonstrated that day, as within-subjects factor, affected weight gain ($F_{(2, 120)} = 431.050$, $p < 0.0001$) and water intake ($F_{(3, 144)} = 122.373$, $p < 0.0001$) of pregnant mice, indicating that the body weight and water intake steadily increased during pregnancy (Fig. 1). Also, a significant effect of treatment was found on both weight gain ($F_{(1, 24)} = 37.657$, $p < 0.0001$) and water intake ($F_{(1, 24)} = 8.635$, $p = 0.007$). In the regular administration schedule, mephedrone significantly reduced the maternal body weight gain ($F_{(1, 12)} = 27.320$, $p = 0.0002$) during pregnancy. Similarly, repeated administration of mephedrone reduced the maternal body weight gain ($F_{(1, 12)} = 14.549$, $p = 0.002$), and

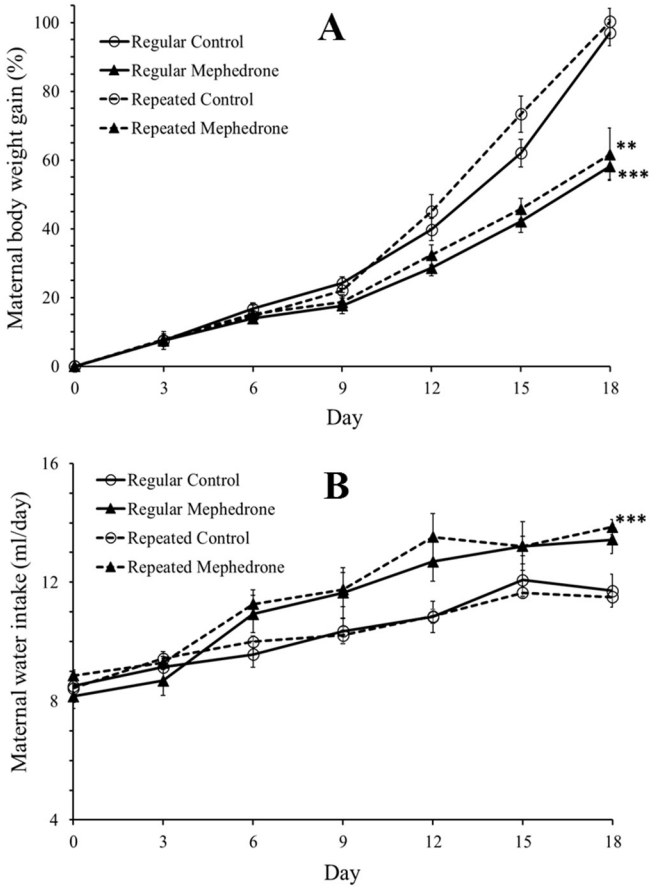

Fig. 1. Effects of mephedrone exposure during pregnancy on the maternal body weight (A) and water intake (B). Values are expressed as mean ± SEM ($n = 7$). $^{**}p < 0.01$ versus its corresponding control group; $^{***}p < 0.001$ versus its corresponding control group.

Table 2
Effects of maternal exposure to mephedrone on the duration of pregnancy and characteristics of delivered pups. Values are expressed as mean ± SEM ($n = 7$).

Parameter	Group			
	Regular Control	Regular Mephedrone	Repeated Control	Repeated Mephedrone
Pregnancy duration (day)	19.4 ± 0.4	19.6 ± 0.3	19.3 ± 0.4	19.4 ± 0.2
Pup number	11.1 ± 0.26	7.1 ± 1.12*	12 ± 0.44	7.4 ± 1.25**
Pup weight (g)	1.67 ± 0.05	1.41 ± 0.02**	1.59 ± 0.02	1.46 ± 0.04**
Stillbirth (%)	1.43 ± 12.6	35.1 ± 14.62*	2.4 ± 1.55	46.2 ± 12.6*

* $p < 0.05$ versus its corresponding control group.
** $p < 0.01$ versus its corresponding control group.

increased water intake ($F_{(1, 12)} = 24.394$, $p = 0.0003$). There was no interaction between schedule and treatment regarding weight gain (schedule * treatment $F_{(1, 24)} = 0.007$, $p = 0.935$) and water intake (schedule * treatment $F_{(1, 24)} = 0.319$, $p = 0.577$).

Table 2 shows the effects of mephedrone on the duration of pregnancy and characteristics of delivered pups. Two-way ANOVA showed no significant effect for treatment, schedule of administration, or treatment-by-schedule interaction for the duration of pregnancy. However, a significant effect of treatment on the pup number ($F_{(1, 24)} = 23.841$, $p < 0.0001$) and pup weight ($F_{(1, 24)} = 32.153$, $p < 0.0001$) was found. Both regular and repeated administration of mephedrone significantly decreased the number (regular: $p = 0.011$; repeated: $p = 0.005$) and the body weight (regular: $p = 0.002$; repeated: $p = 0.009$) of delivered pups compared to their corresponding controls. In addition, treatment factor had a significant effect on the percentage of stillbirths ($F_{(1, 24)} = 15.928$, $p = 0.001$). Administration of mephedrone with both the regular and repeated schedule significantly increased the percentage of stillbirths compared to their corresponding controls ($p = 0.041$ and $p = 0.013$ for regular and repeated, respectively). The factor of schedule and treatment-by-schedule interaction had no significant effects on the pup number, pup weight, and stillbirth.

3.2. Effect of maternal exposure to mephedrone on hippocampal cell proliferation of delivered pups

Immunohistochemistry staining of the endogenous cell proliferation marker Ki-67 in the hippocampus of delivered pups is shown in Fig. 2. Two-way ANOVA has showed a significant effect of treatment on the number of proliferating cells in the CA1 ($F_{(1, 24)} = 11.386$, $p = 0.003$), CA2 ($F_{(1, 24)} = 4.841$, $p = 0.038$), CA3 ($F_{(1, 24)} = 7.764$, $p = 0.010$), and DG ($F_{(1, 24)} = 9.734$, $p = 0.005$) regions of the hippocampus. Repeated mephedrone administration decreased the number of proliferating cells in all CA1 ($p = 0.001$), CA2 ($p = 0.025$), CA3 ($p = 0.015$), and DG ($p = 0.035$) regions. However, regular mephedrone administration had no significant effect on the number of proliferating cells in any regions of the hippocampus. Also, the effect of the factor of schedule and interaction of treatment-by-schedule was not significant.

3.3. Effect of maternal exposure to mephedrone on hippocampal cell apoptosis of delivered pups

TUNEL-positive cells were observed in the all regions of the hippocampus in delivered pups of the repeated mephedrone group (Fig. 3A). Statistical analysis showed a significant effect of treatment factor on the number of apoptotic cells in CA1 ($F_{(1, 24)} = 8.732$, $p = 0.007$), CA2 ($F_{(1, 24)} = 17.454$, $p = 0.0003$), CA3 ($F_{(1, 24)} = 8.458$, $p = 0.008$), and DG ($F_{(1, 24)} = 21.636$, $p = 0.0001$) regions. A significant increase in the number of apoptotic cells was seen in the CA1 ($p = 0.049$), CA2 ($p = 0.005$), CA3 ($p = 0.003$), and DG ($p = 0.0002$) regions in the repeated mephedrone group compared to its corresponding control (Fig. 3B). However, regular mephedrone administration had no significant effect on the number of apoptotic cells in any regions of the hippocampus. Effect of the factor of schedule and interaction of treatment-by-schedule on apoptosis was not significant.

Real time-PCR data showed that there was a significant interaction between treatment and schedule on the expression of Bax gene (schedule * treatment $F_{(1, 20)} = 4.796$, $p = 0.041$). The expression of this pro-apoptotic gene was significantly higher in the hippocampus of pups delivered from the repeated mephedrone group in comparison with the control group ($p = 0.021$, Fig. 4). On the other hand, the expression of the anti-apoptotic Bcl-2 gene was significantly lower in the hippocampus of pups delivered from the repeated mephedrone group ($p = 0.022$). No differences were found in the expressions of Bax and Bcl-2 genes between the pups of the regular mephedrone group and regular control group.

3.4. Effect of maternal exposure to mephedrone on learning and memory of offspring

Analysis of data obtained from the Morris water maze showed that training session (day), as within-subjects factor, affected latency time ($F_{(4, 96)} = 41.138$, $p < 0.0001$) and swimming distance ($F_{(4, 96)} = 36.039$, $p < 0.0001$) to reach the hidden platform. This indicated that all groups of offspring improved their ability to locate the platform over the five days of training (Fig. 5). Also, a significant effect of treatment was found on the both latency time ($F_{(1, 24)} = 8.227$, $p = 0.008$) and swimming distance ($F_{(1, 24)} = 9.292$, $p = 0.006$). In regular administration schedule, mephedrone had no significant effect on the latency time ($F_{(1, 12)} = 1.481$, $p = 0.247$) and swimming distance ($F_{(1, 12)} = 3.348$, $p = 0.092$). On the other hand, in the repeated administration schedule, mephedrone significantly increased the latency time ($F_{(1, 12)} = 7.854$, $p = 0.016$) and swimming distance ($F_{(1, 12)} = 6.924$, $p = 0.022$) variables. No significant effect was found for schedule as between-subject effect, and for treatment-by-schedule interaction regarding the latency time (schedule: $F_{(1, 24)} = 0.0002$, $p = 0.987$; schedule*treatment $F_{(1, 24)} = 1.413$, $p = 0.246$) and swimming distance (schedule: $F_{(1, 24)} = 0.499$, $p = 0.487$; schedule * treatment $F_{(1, 24)} = 0.018$, $p = 0.895$).

Analysis of data obtained from the subsequent probe trial showed a significant effect of treatment on the percentage of time spent in the target quadrant ($F_{(1, 24)} = 15.903$, $p = 0.001$). The time spent in this quadrant was significantly lower in both regular ($p = 044$) and repeated ($p = 0.004$) mephedrone groups compared to the controls. The effect of the administration schedule ($F_{(1, 24)} = 0.002$, $p = 0.961$) and interaction of treatment-by-schedule ($F_{(1, 24)} = 0.001$, $p = 0.976$) on the percentage of time spent in the target quadrant was found to be statistically non-significant (Fig. 6).

4. Discussion

Information on the effects of mephedrone use in pregnancy on fetal and neonatal outcomes is limited. Since a number of previous studies

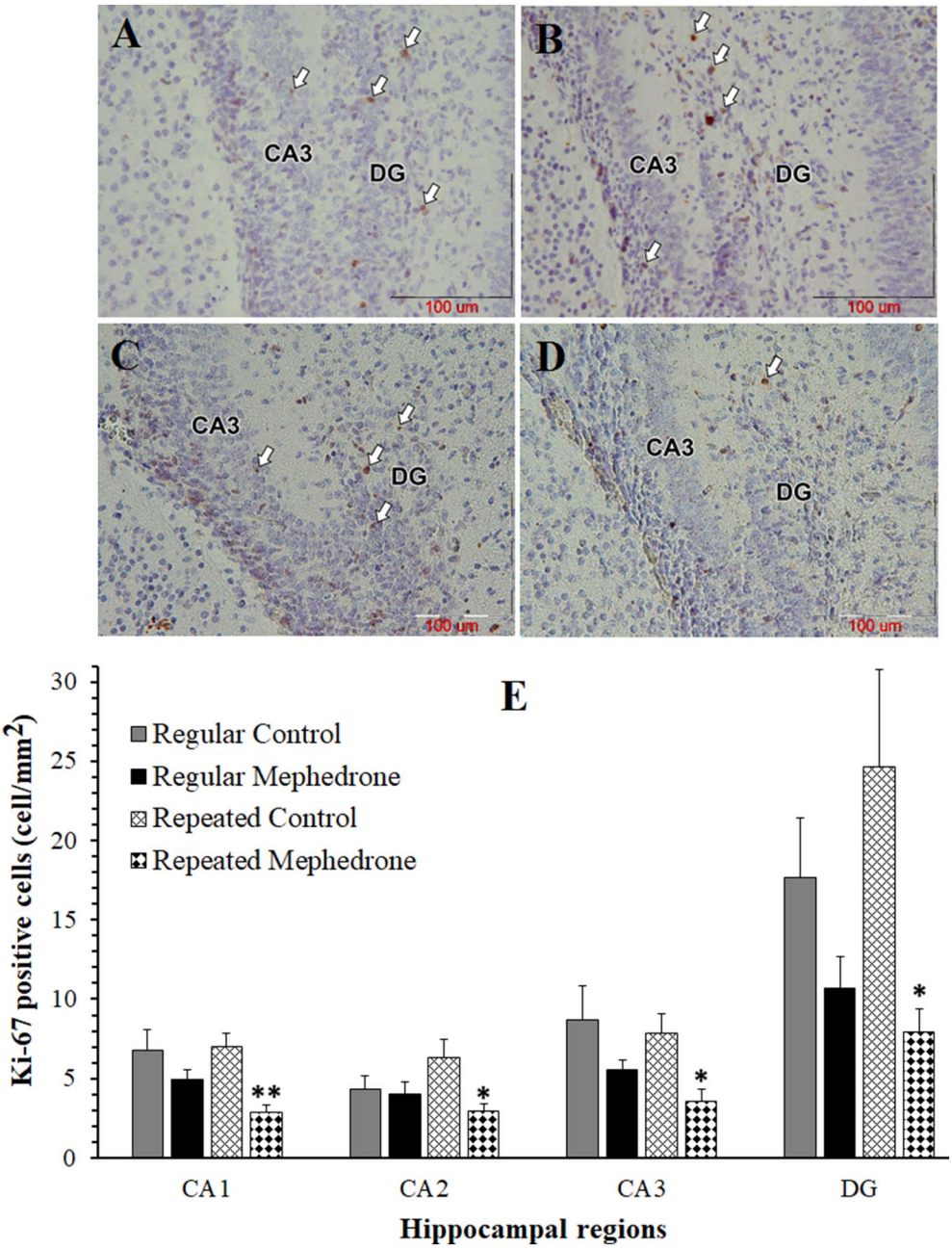

Fig. 2. Effect of maternal exposure to mephedrone on cell proliferation in the hippocampus of delivered pups. A–D: photomicrograph of immunohistochemistry for cell proliferation marker Ki-67 in the CA3 and dentate gyrus (DG) regions of the hippocampus (A: regular control group; B: regular mephedrone group; C: repeated control group; D: repeated mephedrone group). E: quantitative analysis of Ki-67-positive cells. Arrows point to representative Ki-67 positive cells. The cells were counterstained with hematoxylin. Values are expressed as mean ± SEM (n = 7). $^{*}p < 0.05$ versus its corresponding control group; $^{**}p < 0.01$ versus its corresponding control group.

have reported some potential neurotoxic effects for mephedrone in adolescent rodents (Motbey et al., 2013; López-Arnau et al., 2015; Ciudad-Roberts et al., 2016), this study was aims to evaluate the effects of mephedrone exposure in pregnancy on neonatal outcomes, focusing on hippocampal damage. Results of the current study have shown that this drug has deleterious effects on delivered pups including increased incidence of stillbirth, decreased birth weight, reduced hippocampal neurogenesis, and impaired learning and memory performances.

In the pregnant mice, both mephedrone groups showed enhanced water intake, which is in agreement with clinical evidence that users of the khat plant or its cathinone derivatives experience oral dryness and thirst (Yarom et al., 2010; Ashrafioun et al., 2016). Mephedrone also reduced the rate of weight gain in pregnant mice, which could be the result of decreased appetite or increased metabolic rate (Jones et al., 1992; Dargan et al., 2010; Winstock et al., 2011). One possible limitation of the present study is that we did not measure food consumption in the pregnant mice. However, previous clinical studies have shown decreased appetite in young and adult mephedrone users (Dargan et al., 2010; Winstock et al., 2011). Consistent with our results, it has been reported that adolescent rats who received mephedrone gained weight at a slower rate than control rats (Motbey et al., 2013). Also, repeated exposure to mephedrone was found to cause a transient decline in weight gain of adolescent rats (López-Arnau et al., 2015). In addition to pregnant mice, the neonates of both mephedrone treated groups had low birth weight, indicating that intrauterine growth was impaired while fetuses were exposed to this drug. It has been shown that infants of khat-chewer mothers (either habitually or occasionally) had a significantly lower birth weight, which supports the findings of

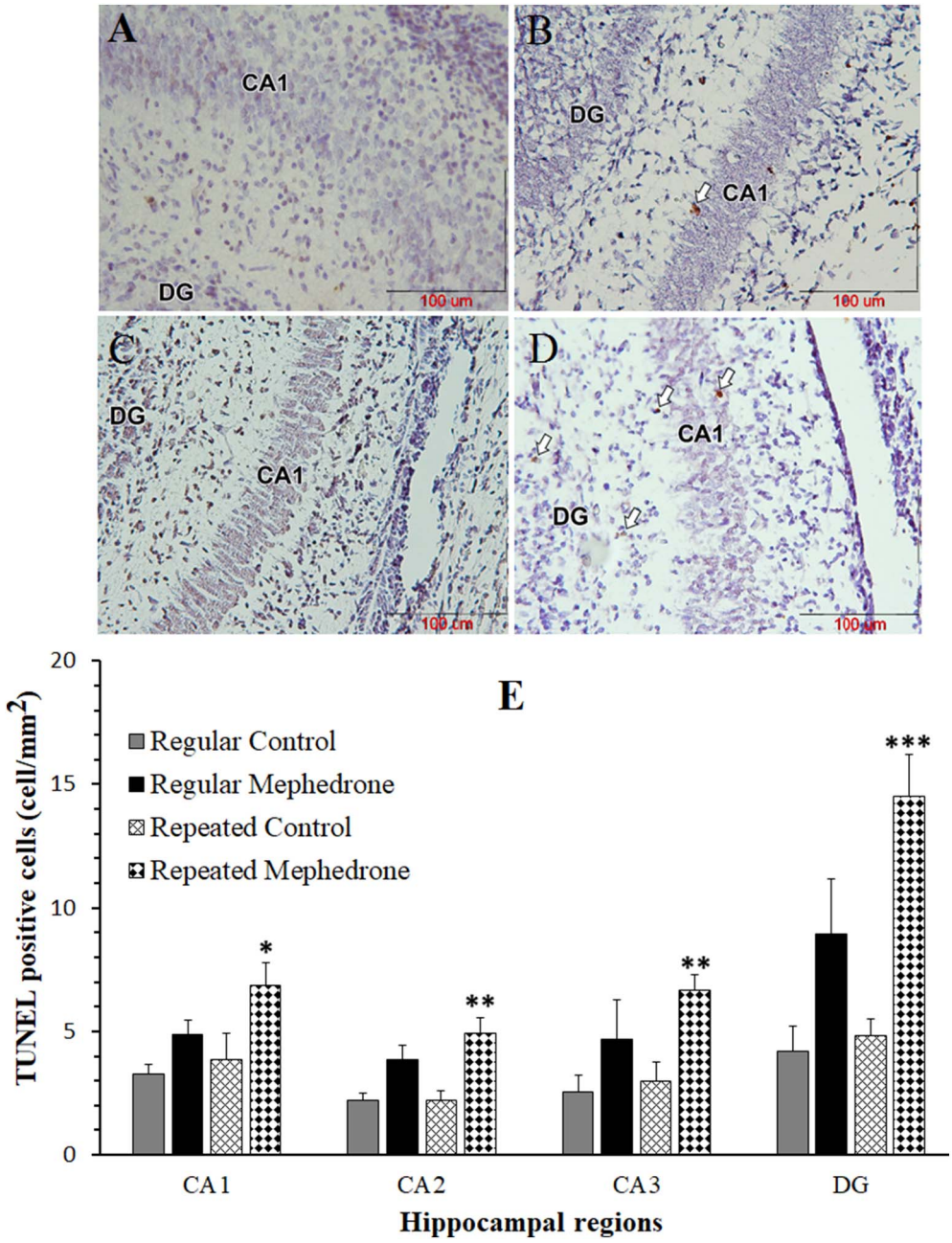

Fig. 3. Effect of maternal exposure to mephedrone on apoptosis in the hippocampus of delivered pups. A–D: Representative images of TUNEL assay in the CA1 and dentate gyrus (DG) regions of the hippocampus (A: regular control group; B: regular mephedrone group; C: repeated control group; D: repeated mephedrone group). E: quantitative analysis of TUNEL-positive cells. Arrows point to representative TUNEL-positive cells. The cells were counterstained with hematoxylin. Values are expressed as mean ± SEM (n = 7). $*p < 0.05$ versus its corresponding control group; $**p < 0.01$ versus its corresponding control group; $***p < 0.001$ versus its corresponding control group.

the present study (Ghani et al., 1987).

Previous studies have shown that khat, cathinone, and methamphetamine (which structurally is similar to mephedrone) are embryotoxic and have a teratogenic effect on the fetus (Yamamoto et al., 1992; Islam et al., 1994; McElhatton et al., 1999; Mwenda et al., 2003). In the present work, both regular and repeated exposure to mephedrone increased the rate of stillbirth. This highlights the importance of early screening of mephedrone abuse during pregnancy.

Immunohistochemistry and TUNEL assay revealed that repeated exposure to mephedrone in gestational stage inhibits cell proliferation and induces apoptosis in developing hippocampus of neonates. Inhibitory effect of mephedrone on viability and proliferation of neural cells has been shown previously in a number of in vitro and in vivo experiments (Martínez-Clemente et al., 2014; Siedlecka-Kroplewska et al., 2014; López-Arnau et al., 2015; Ciudad-Roberts et al., 2016). In cultured cortical neurons, mephedrone decreased the neuronal viability in a concentration-dependent manner (Martínez-Clemente et al., 2014). Also, a structural analog of mephedrone, 3-fluoromethcathinone, was shown to induce G0/G1-phase cell cycle arrest in HT22 mouse hippocampal cells (Siedlecka-Kroplewska et al., 2014). In adolescent male mice, administration of 25 mg/kg of mephedrone (four times a day, every 2 h) decreased the newly formed cells in the dentate gyrus of the hippocampus (Ciudad-Roberts et al., 2016). In another study, administration of 25 mg/kg of mephedrone in the weekend consumption pattern (thrice daily, for two consecutive days) led to astrogliosis and loss of hippocampal serotoninergic neuronal markers (Martínez-Clemente et al., 2014). The anti-proliferative and pro-apoptotic effects of mephedrone might be attributed to oxidative stress induced by this

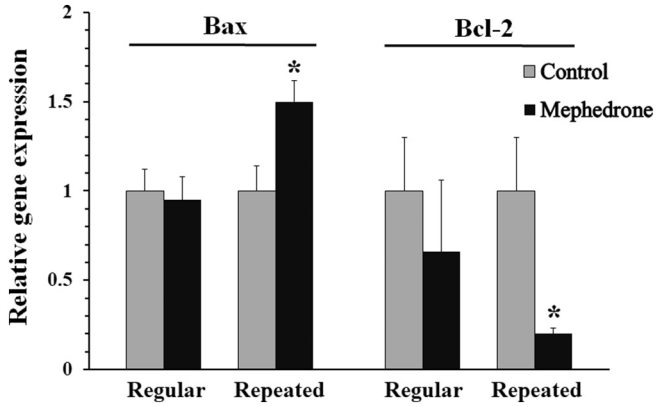

Fig. 4. Effect of maternal exposure to mephedrone on the expressions of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) genes in the hippocampus of delivered pups. Values are expressed as mean ± SEM ($n = 6$). *$p < 0.05$ versus its corresponding control level.

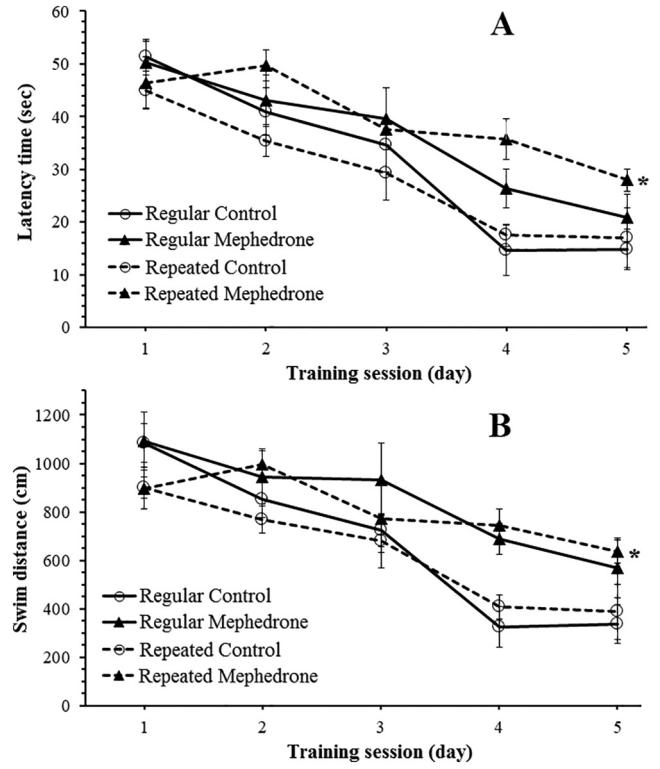

Fig. 5. Effect of maternal exposure to mephedrone on learning of offspring mice. A: The latency time to find the hidden platform during training days. B: The traveled distance to reach the platform during training days. Values are expressed as mean ± SEM (n = 7). *$p < 0.05$ versus its corresponding control group.

drug in the brain tissue. It has been shown that single and repeated exposure to mephedrone increased lipid peroxidation in the brain cortex and hippocampus of adolescent and adult rodents (Budzynska et al., 2015; López-Arnau et al., 2015; Ciudad-Roberts et al., 2016). Also, extensive studies have shown that oxidative stress plays an important role in the molecular toxicity of psychostimulant drugs (e.g. MDMA and methamphetamine) (Riezzo et al., 2013). Increased reactive oxygen species can activate mitochondria-dependent pathways of apoptosis, in which losing the balance between pro- and anti-apoptotic proteins ultimately results in the release of mitochondrial apoptogenic elements that induce apoptosis in caspase-dependent or -independent mechanisms (Sinha et al., 2013). The MDMA was shown to up-regulate pro-apoptotic protein Bax and down-regulate anti-apoptotic protein Bcl-2 in the rat hippocampus (Asl et al., 2012). Similarly, our results

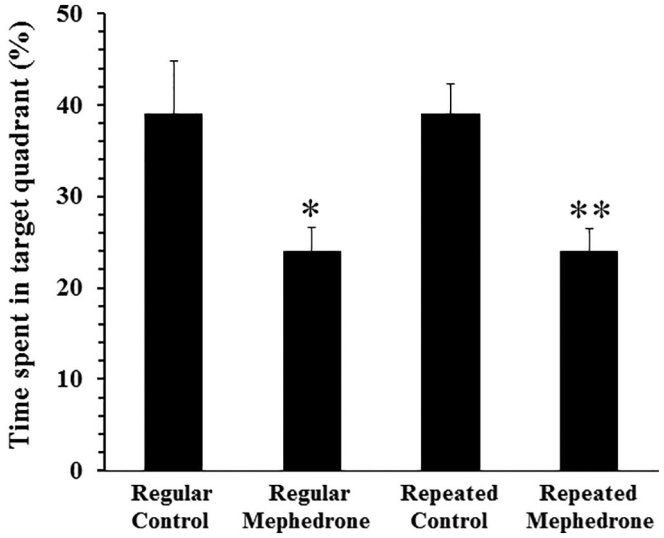

Fig. 6. Effect of maternal exposure to mephedrone on memory performance of offspring mice. The percentage of time spent in the target quadrant during the probe trial is shown for each group. Values are expressed as mean ± SEM (n = 7). *$p < 0.05$ versus its corresponding control group; **$p < 0.01$ versus its corresponding control group.

have shown that repeated mephedrone administration enhanced the expression of Bax and reduced the expression of Bcl-2 genes in the hippocampus of delivered pups. More studies on the caspase family and other members of the Bcl-2 family are required to determine the exact mechanism of the pro-apoptotic effect of mephedrone.

Given that the hippocampus plays a key role in the processes of learning and memory, we hypothesized that mephedrone impairs these processes. Results of the Morris water maze showed an impairment of spatial learning and reference memory in mice born from mephedrone-treated litters. This suggests that the negative effects of mephedrone abuse in pregnancy may remain for a long time after delivery. Such long-term impairment of learning and memory most probably results from antiproliferative and pro-apoptotic effects of mephedrone on the hippocampus. Although the present study is the first to show maternal exposure to mephedrone damages the hippocampus and impairs learning and memory of offspring mice, the results are in line with a number of previous studies in adolescent rodents (Motbey et al., 2012; López-Arnau et al., 2015; Ciudad-Roberts et al., 2016). Two recent studies have demonstrated that repeated mephedrone injection could impair reference memory one week after the cessation of drug exposure (López-Arnau et al., 2015; Ciudad-Roberts et al., 2016). Also, it has been shown that repeated doses of mephedrone weakened novel object recognition five weeks after drug exposure, indicating long-term memory impairment (Motbey et al., 2012). All these findings indicate that frequent use of mephedrone may impair memory retrieval.

In conclusion, the present study demonstrates that regular and repeated exposure to mephedrone during the gestational period increases the risks of low birth weight and stillbirth. Also, repeated use of mephedrone impairs learning and memory processes potentially through inducing hippocampal damage.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in online version.

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References

Ashrafioun, L., Bonadio, F.A., Baik, K.D., Bradbury, S.L., Carhart, V.L., Cross, N.A., Davis, A.K., Feuille, M., Harper, A.R., Lackey, J.H., 2016. Patterns of use, acute subjective experiences, and motivations for using synthetic cathinones ("bath salts") in recreational users. J. Psychoactive Drugs 48 (5), 336–343.

Asl, S.S., Farhadi, M.H., Moosavizadeh, K., Saraei, A.S.K., Soleimani, M., Jamei, S.B., Joghataei, M.T., Samzadeh-Kermani, A., Hashemi-Nasl, H., Mehdizadeh, M., 2012. Evaluation of Bcl-2 family gene expression in hippocampus of 3, 4-methylenedioxymethamphetamine treated rats. Cell J. 13 (4), 275.

Baumann, M.H., Ayestas, M.A. Jr, Partilla, J.S., Sink, J.R., Shulgin, A.T., Daley, P.F., Brandt, S.D., Rothman, R.B., Ruoho, A.E., Cozzi, N.V., 2012. The designer methcathinone analogs, mephedrone and methylone, are substrates for monoamine transporters in brain tissue. Neuropsychopharmacology 37 (5), 1192–1203.

Budzynska, B., Boguszewska-Czubara, A., Kruk-Slomka, M., Kurzepa, J., Biala, G., 2015. Mephedrone and nicotine: oxidative stress and behavioral interactions in animal models. Neurochem. Res. 40 (5), 1083–1093.

Ciudad-Roberts, A., Duart-Castells, L., Camarasa, J., Pubill, D., Escubedo, E., 2016. The combination of ethanol with mephedrone increases the signs of neurotoxicity and impairs neurogenesis and learning in adolescent CD-1 mice. Toxicol. Appl. Pharmacol. 293, 10–20.

Dargan, P., Albert, S., Wood, D., 2010. Mephedrone use and associated adverse effects in school and college/university students before the UK legislation change. QJM 103 (11), 875–879.

Duarte-Guterman, P., Yagi, S., Chow, C., Galea, L.A., 2015. Hippocampal learning, memory, and neurogenesis: effects of sex and estrogens across the lifespan in adults. Horm. Behav. 74, 37–52.

Ghani, N.A., Eriksson, M., Kristiansson, B., Qirbi, A., 1987. The influence of khat-chewing on birth-weight in full-term infants. Soc. Sci. Med. 24 (7), 625–627.

Hadlock, G.C., Webb, K.M., McFadden, L.M., Chu, P.W., Ellis, J.D., Allen, S.C., Andrenyak, D.M., Vieira-Brock, P.L., German, C.L., Conrad, K.M., 2011. 4-Methylmethcathinone (mephedrone): neuropharmacological effects of a designer stimulant of abuse. J. Pharmacol. Exp. Ther. 339 (2), 530–536.

Haghir, H., Hami, J., Lotfi, N., Peyvandi, M., Ghasemi, S., Hosseini, M., 2017. Expression of apoptosis-regulatory genes in the hippocampus of rat neonates born to mothers with diabetes. Metab. Brain Dis. 32 (2), 617–628.

Hockenhull, J., Murphy, K.G., Paterson, S., 2016. Mephedrone use is increasing in London. Lancet 387 (10029), 1719–1720.

Hosseini, M., Nemati Karimooy, H., Hadjzadeh, M., Safari, V., 2011. Inducible nitric oxide synthase inhibitor aminoguanidine, differently affects Morris water maze tasks of ovariectomized and naive female rats. Acta Physiol. Hung. 98 (4), 421–432.

Islam, M., Al-Shabanah, O., Al-Harbi, M., Al-Gharably, N., 1994. Evaluation of teratogenic potential of khat (*Catha edulis* Forsk.) in rats. Drug Chem. Toxicol. 17 (1), 51–68.

Jones, J.R., Caul, W.F., Hill, J.O., 1992. The effects of amphetamine on body weight and energy expenditure. Physiol. Behav. 51 (3), 607–611.

Jones, D., Yates, L., Stephens, S., Dunstan, H., Richardson, J., James, D., Thomas, S., 2011. In: Preliminary data on exposure to mephedrone in pregnancy. 2011. International Congress of the European Association of Poisons Centres and Clinical Toxicologists. Dubrovnik, Croatia, 213 abstract no. 63.

Keegan, J., Parva, M., Finnegan, M., Gerson, A., Belden, M., 2010. Addiction in pregnancy. J. Addict. Dis. 29 (2), 175–191.

Lindsay, M.K., Burnett, E., 2013. The use of narcotics and street drugs during pregnancy. Clin. Obstet. Gynecol. 56 (1), 133–141.

Lopez-Arnau, R., Martinez-Clemente, J., Pubill, D., Escubedo, E., Camarasa, J., 2012. Comparative neuropharmacology of three psychostimulant cathinone derivatives: butylone, mephedrone and methylone. Br. J. Pharmacol. 167 (2), 407–420.

López-Arnau, R., Martínez-Clemente, J., Rodrigo, T., Pubill, D., Camarasa, J., Escubedo, E., 2015. Neuronal changes and oxidative stress in adolescent rats after repeated exposure to mephedrone. Toxicol. Appl. Pharmacol. 286 (1), 27–35.

Luethi, D., Kolaczynska, K.E., Docci, L., Krähenbühl, S., Hoener, M.C., Liechti, M.E., 2017. Pharmacological profile of mephedrone analogs and related new psychoactive substances. Neuropharmacology (in press).

Martínez-Clemente, J., López-Arnau, R., Abad, S., Pubill, D., Escubedo, E., Camarasa, J., 2014. Dose and time-dependent selective neurotoxicity induced by mephedrone in mice. PLoS One 9 (6), e99002.

McElhatton, P., Bateman, D., Evans, C., Pughe, K., Thomas, S., 1999. Congenital anomalies after prenatal ecstasy exposure. Lancet 354 (9188), 1441–1442.

Measham, F., Moore, K., Newcombe, R., Zoë, 2010. Tweaking, bombing, dabbing and stockpiling: the emergence of mephedrone and the perversity of prohibition. Drug Alcohol Today 10 (1), 14–21.

Motbey, C.P., Karanges, E., Li, K.M., Wilkinson, S., Winstock, A.R., Ramsay, J., Hicks, C., Kendig, M.D., Wyatt, N., Callaghan, P.D., 2012. Mephedrone in adolescent rats: residual memory impairment and acute but not lasting 5-HT depletion. PLoS One 7 (9), e45473.

Motbey, C.P., Clemens, K.J., Apetz, N., Winstock, A.R., Ramsey, J., Li, K.M., Wyatt, N., Callaghan, P.D., Bowen, M.T., Cornish, J.L., 2013. High levels of intravenous mephedrone (4-methylmethcathinone) self-administration in rats: neural consequences and comparison with methamphetamine. J. Psychopharmacol. 27 (9), 823–836.

Mwenda, J., Arimi, M., Kyama, M., Langat, D., 2003. Effects of khat (Catha edulis) consumption on reproductive functions: a review. East Af. Med. J. 80 (6), 318–323.

Papaseit, E., Pérez-Mañá, C., Mateus, J.-A., Pujadas, M., Fonseca, F., Torrens, M., Olesti, E., de la Torre, R., Farré, M., 2016. Human pharmacology of mephedrone in comparison with MDMA. Neuropsychopharmacology 41 (11), 2704–2713.

Reagan-Shaw, S., Nihal, M., Ahmad, N., 2008. Dose translation from animal to human studies revisited. FASEB J. 22 (3), 659–661.

Riezzo, I., Fiore, C., De Carlo, D., Karch, S.B., Neri, M., Emanuela Turillazi, T., Fineschi, V., 2013. The role of oxidative stress in methamphetamine and MDMA-induced toxicity. Mini-Rev. Org. Chem. 10 (4), 349–359.

Schifano, F., Albanese, A., Fergus, S., Stair, J.L., Deluca, P., Corazza, O., Davey, Z., Corkery, J., Siemann, H., Scherbaum, N., 2011. Mephedrone (4-methylmethcathinone; 'meow meow'): chemical, pharmacological and clinical issues. Psychopharmacology 214 (3), 593–602.

Siedlecka-Kroplewska, K., Szczerba, A., Lipinska, A., Slebioda, T., Kmiec, Z., 2014. 3-Fluoromethcathinone, a structural analog of mephedrone, inhibits growth and induces cell cycle arrest in HT22 mouse hippocampal cells. J. Physiol. Pharmacol. 65 (2), 241–246.

Sinha, K., Das, J., Pal, P.B., Sil, P.C., 2013. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. Arch. Toxicol. 87 (7), 1157–1180.

Strange, L.G., Kochelek, K., Keasling, R., Brown, S.D., Pond, B.B., 2017. The pharmacokinetic profile of synthetic cathinones in a pregnancy model. Neurotoxicol. Teratol. 63, 9–13.

Wendell, A.D., 2013. Overview and epidemiology of substance abuse in pregnancy. Clin. Obstet. Gynecol. 56 (1), 91–96.

Winstock, A., Mitcheson, L., Ramsey, J., Davies, S., Puchnarewicz, M., Marsden, J., 2011. Mephedrone: use, subjective effects and health risks. Addiction 106 (11), 1991–1996.

Wood, D., Greene, S., Dargan, P., 2010a. Clinical pattern of toxicity associated with the novel synthetic cathinone mephedrone. Emerg. Med. J. 28 (4), 280–282.

Wood, D.M., Davies, S., Puchnarewicz, M., Button, J., Archer, R., Ovaska, H., Ramsey, J., Lee, T., Holt, D.W., Dargan, P.I., 2010b. Recreational use of mephedrone (4-methylmethcathinone, 4-MMC) with associated sympathomimetic toxicity. J. Med. Toxicol. 6 (3), 327–330.

Yamamoto, Y., Yamamoto, K., Fukui, Y., Kurishita, A., 1992. Teratogenic effects of methamphetamine in mice. Nihon Hoigaku Zasshi 46 (2), 126–131.

Yarom, N., Epstein, J., Levi, H., Porat, D., Kaufman, E., Gorsky, M., 2010. Oral manifestations of habitual khat chewing: a case-control study. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 109 (6), e60–e66.