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Full Length Article

A comparison of mitochondrial toxicity of mephedrone on three separate parts of brain including hippocampus, cortex and cerebellum

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ABSTRACT

Mephedrone (4-methylmethcathinone) is a new and popular drug of abuse and also widely available on the internet and still legal in some parts of the world. The central nervous system is the target of mephedrone and recent evidence suggested that mephedrone could affect mitochondria in brain tissue. However, the underlying mechanisms of mephedrone toxicity in brain mitochondria have not yet been well understood. In this study, mitochondria from three separate parts of rat brain hippocampus, cortex, and cerebellum were obtained using differential centrifugation and were incubated with different concentrations of mephedrone (3, 6 and 12 μM). Then, the mitochondrial parameters toxicity were determined. The results showed that mephedrone (3, 6 and 12 μM) induced impairment in the activity of the mitochondrial complex II and IV. Also, mephedrone (3, 6 and 12 μM) increased mitochondrial reactive oxygen species (ROS) level, collapsed mitochondria membrane potential (MMP), induced swelling in the mitochondria and damaged the mitochondrial outer membrane (MOM) in the mitochondria obtained from hippocampus, cortex, and cerebellum, which in all cases is associated with the cytochrome c release. Furthermore, increased disturbance in oxidative phosphorylation was also shown by the decrease in ATP level in mephedrone-treated mitochondria indicating mitochondrial dysfunction in separate parts of the brain. This study suggests that mephedrone via increasing oxidative stress and impairment of the mitochondrial respiratory chain in the hippocampus, cortex, and cerebellum may play a key role in the neurotoxicity.

1. Introduction

In recent years, the use of designer 4-methylmethcathinoneas stimulants has been increased. One of these compounds is mephedrone, which has been abused through various routes (such as, oral and insufflation) (den Hollander et al., 2013; Hadlock et al., 2011; Lopez-Arnau et al., 2015; Robinson et al., 2012; Shortall et al., 2013). The structure and effects of mephedrone are similar to amphetamines (Hadlock et al., 2011). In some countries, mephedrone has easily been available through various legal routes, and often abused by young adults and adolescents. It has been shown that the desired effects of mephedrone occur in less than 1 h (from 15 to 45 min) after nasal insufflation and oral ingestion, respectively. Furthermore, mephedrone has the ability to cross the blood-brain barrier (BBB) and easily distribute into the brain (Martinez-Clemente et al., 2013). Peak brain tissue concentration was 0.004 μg/mg 2 min after a 1 mg/kg i.v. dose (Green et al., 2014). There are contradictions regarding the effects of prolonged use of mephedrone and as a public health concern (Hadlock et al., 2011; Lopez-Arnau et al., 2012, 2015; Martinez-Clemente et al., 2014; Shortall et al., 2013). It is often used to induced euphoria, enhance mood, and other stimulating effects. There are reports of side effect and death due to the use of mephedrone (Robinson et al., 2012; Shortall et al., 2013).

In the central nervous system (CNS), it has been documented that

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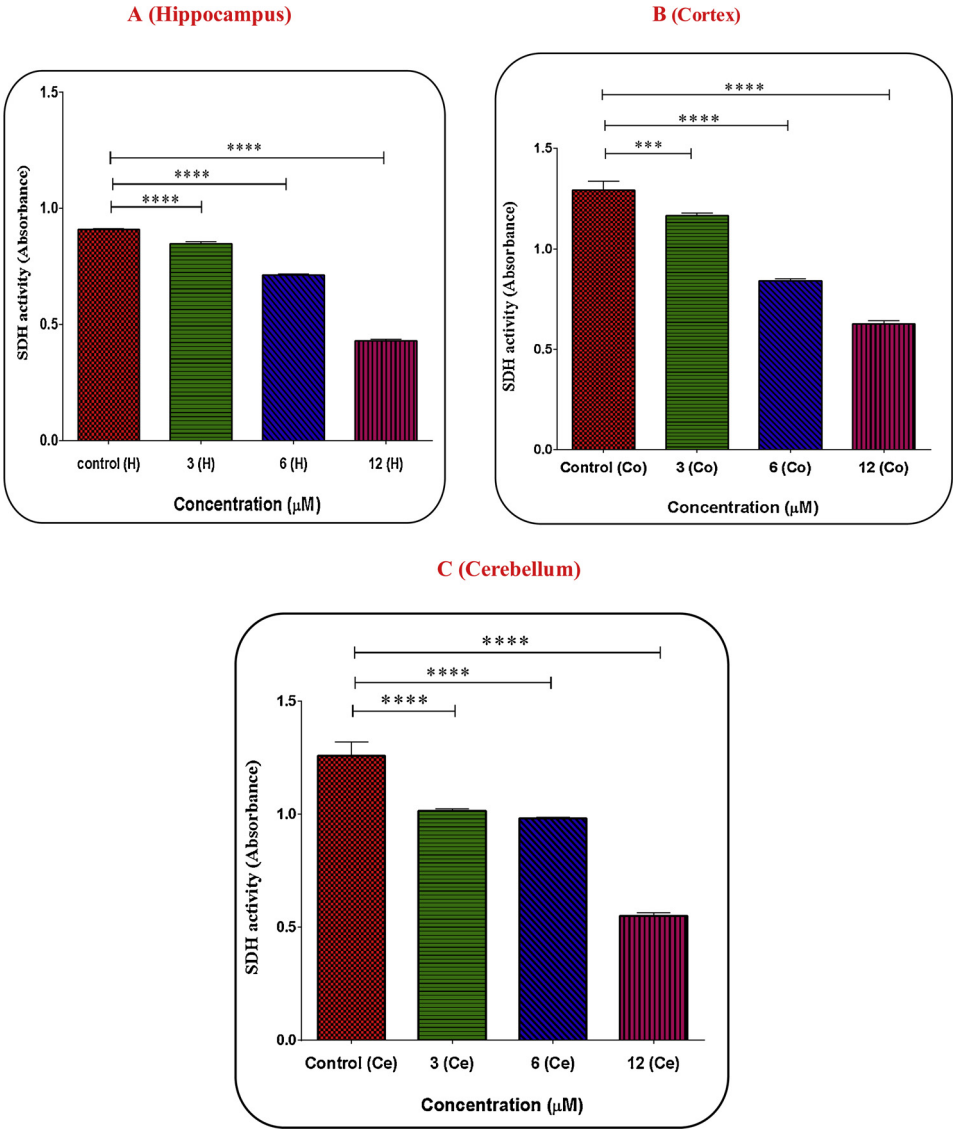

Fig. 1. SDH activity assay. The effect of different concentrations of mephedrone (3, 6 and 12 μM) on the SDH activity in mitochondria isolated from the (**A**) hippocampus (H), (B) cortex (Co) and (C) cerebellum (Ce). Data are presented as mean ± SD (n = 3 for each group). The one-way ANOVA test was carried out. *** and **** show a significant difference in comparison with the corresponding control group ($P < 0.001$ and $P < 0.0001$, respectively).

metabolic rate is high due to high oxygen consumption. Neurons require high levels of ATP to maintain normal function. The mitochondrial function and the oxygen supply are two important factors that generate the ATP of neurons through oxidative metabolism (Chaturvedi and Flint Beal, 2013; Kann and Kovács, 2007). Behavioral, cognitive and motor disorders are important characteristics of neurodegenerative diseases, in which brain function is not normal (Adam-Vizi, 2005). Evidence suggests that the phenomenon of oxidative stress plays a crucial role in the pathogenesis of neurodegenerative diseases (Green et al., 2014; Kann and Kovács, 2007). The mitochondrial complex I and complex III are one of the most important sources of reactive oxygen species (ROS) generation. Use of isolated mitochondria have significantly contributed to the understanding of this issue (Kann and Kovács, 2007).

Survival and neuronal function are affected by mitochondrial function. Furthermore, factors such as ROS and changes in ATP/ADP ratio can induce a change in the mitochondrial membrane potential (MMP) (Adam-Vizi, 2005). Therefore, the defect of proper working of brain mitochondria could lead to severe energy deficiency, and also increased production of ROS in neuron and eventually neuronal demise (Chaturvedi and Flint Beal, 2013). The aim of the current study was to explore the impact of impairment in mitochondrial electron transfer chain in the different parts of brain including hippocampus, cortex and cerebellum with different cognitive memory and commanding functions following mephedrone exposure.

2. Materials and methods

2.1. Materials

Mephedrone and other chemicals were purchased from company Merck (Darmstadt, Germany) with the best analytical grade available.

2.2. Animals

Male Wistar rats (N = 18, including 12 rats for control group (n = 3) and different mephedrone concentrations (3 μM (n = 3), 6 μM (n = 3) and 12 μM (n = 3)), and 6 rats for groups received pretreatment with BHT or Cs.A (i.e. mephedrone (6 μM) + BHT (n = 3) and mephedrone (6 μM) + Cs A (n = 3)), weighing 250–300 g were housed under standard conditions (temperature 25 ± 2 °C, humidity 50–10%, 12 h light–dark cycle and free access to food and water). The

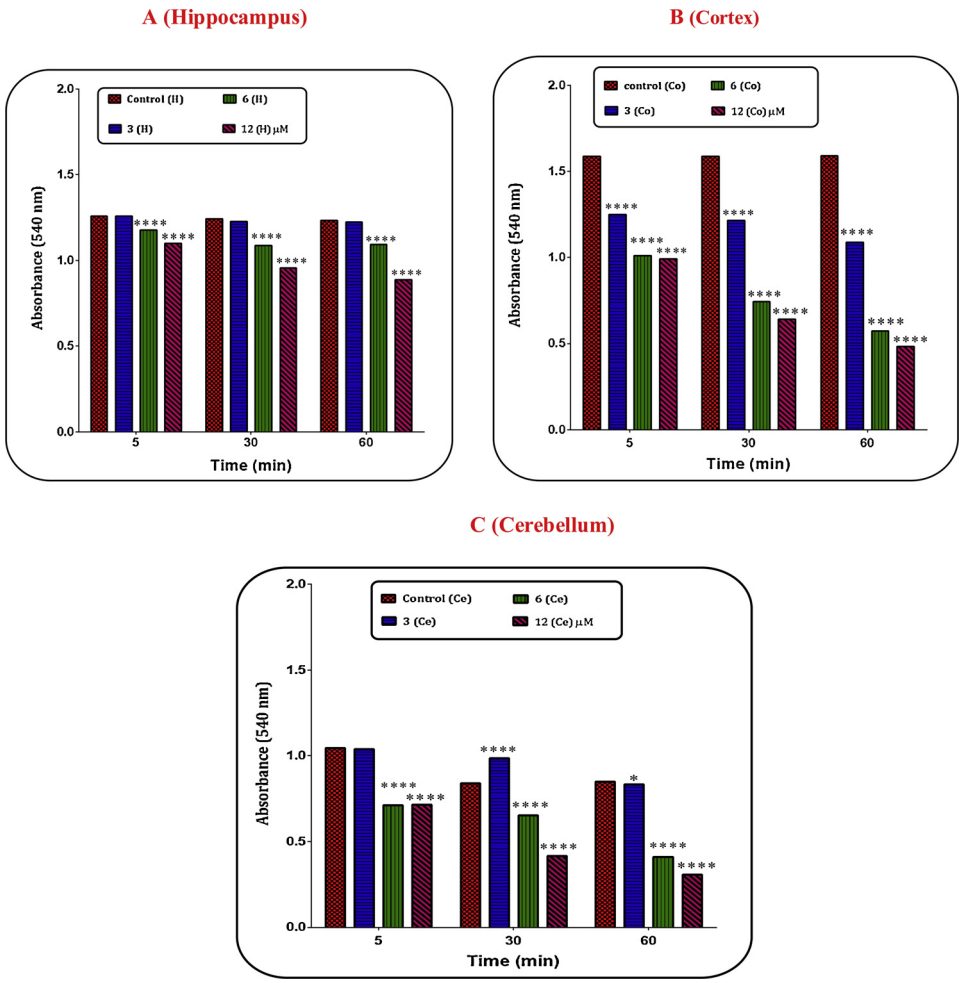

Fig. 2. Mitochondrial swelling assay. The effect of different concentrations of mephedrone (3, 6 and 12 µM) on the mitochondrial swelling in mitochondria isolated from the (**A**) hippocampus (H), (B) cortex (Co) and (C) cerebellum (Ce). Data are presented as mean ± SD (n = 3 for each group). The two-way ANOVA test was carried out.* and **** show a significant difference in comparison with the corresponding control group ($P < 0.05$ and $P < 0.0001$, respectively).

experimental protocols were approved by the Animal Ethics Committee of Shahid Beheshti University of Medical Sciences. All efforts were made to minimize the number and the suffering of rats used. After the rats were decapitated, brain tissues were quickly dissected out and rapidly rinsed using an isotonic saline buffer. These samples were used for the isolation of mitochondria as described below.

2.3. Preparation of mitochondria

After sacrifice, the brain was quickly removed and immersed in an ice-cold isolation buffer. In the next step, hippocampus, cortex, and cerebellum were isolated. Then, tissues (hippocampus, cortex, and cerebellum) was cut in small pieces for subsequent manual homogenization. The homogenates from hippocampus, cortex, and cerebellum were centrifuged at 1500 rpm, 2100 and 1000 rpm (respectively) for 10 min. In the next step, the obtained supernatants from hippocampus, cortex, and cerebellum were centrifuged at 9800 rpm, 11,100 and 12,000 rpm (respectively) for 10 min. Then, the supernatant was discarded. Finally, the mitochondrial pellet from hippocampus, cortex, and cerebellum were maintained into an ice-bath until the experiments. Normalizing and keeping the uniformity of the experimental conditions of the samples was accomplished based on the mitochondrial protein concentration from a Bradford test (BSA as a standard), adjusted to 0.5 mg protein per mL in all the experiments (Bradford, 1976; Ghazi-Khansari et al., 2006; Lopez-Arnau et al., 2015; Venediktova et al., 2017).

Mephedrone to prepare concentration of 3, 6 and 12 µM was dissolved in a small amount of DMSO 0.1%. Then, the desired concentrations reached the final volume with a buffer (the buffer associated with each experiment). In addition, all buffers are made in deionized water. In other words, the basis of all buffers is deionized water. To evaluate the toxicity, mephedrone is added to the mitochondrial suspension at concentrations of 3, 6 and 12 µM. The mitochondrial suspension is obtained by dispersing the mitochondria in the buffer. In this way, the mephedrone dilution (contain DMSO 0.1%) is carried out several times. The control group was exposed to 0.1% DMSO concentration, which has reached the final volume with a buffer. Then, added to the mitochondrial suspension. All stages of the test group are applied to the control group. In the control group, instead of concentrations of mephedrone buffer is used.

2.4. Mitochondrial succinate dehydrogenase (complex II) activity

The activity of SDH was assayed using of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) test. Briefly, 100 µl mitochondrial suspensions (0.5 mg protein/ml) was incubated with different concentrations of mephedrone (3, 6 and 12 µM) at 37 °C for 60 min. In the next step, MTT (0.4%) was added to the medium and incubated at 37°C for 30 min. Finally, The product formazan crystals was dissolved in100 µl DMSO, and then absorbance at 570 nm was measured with an ELISA reader (Tecan, Rainbow Thermo, Austria) (Zhao et al., 2010).

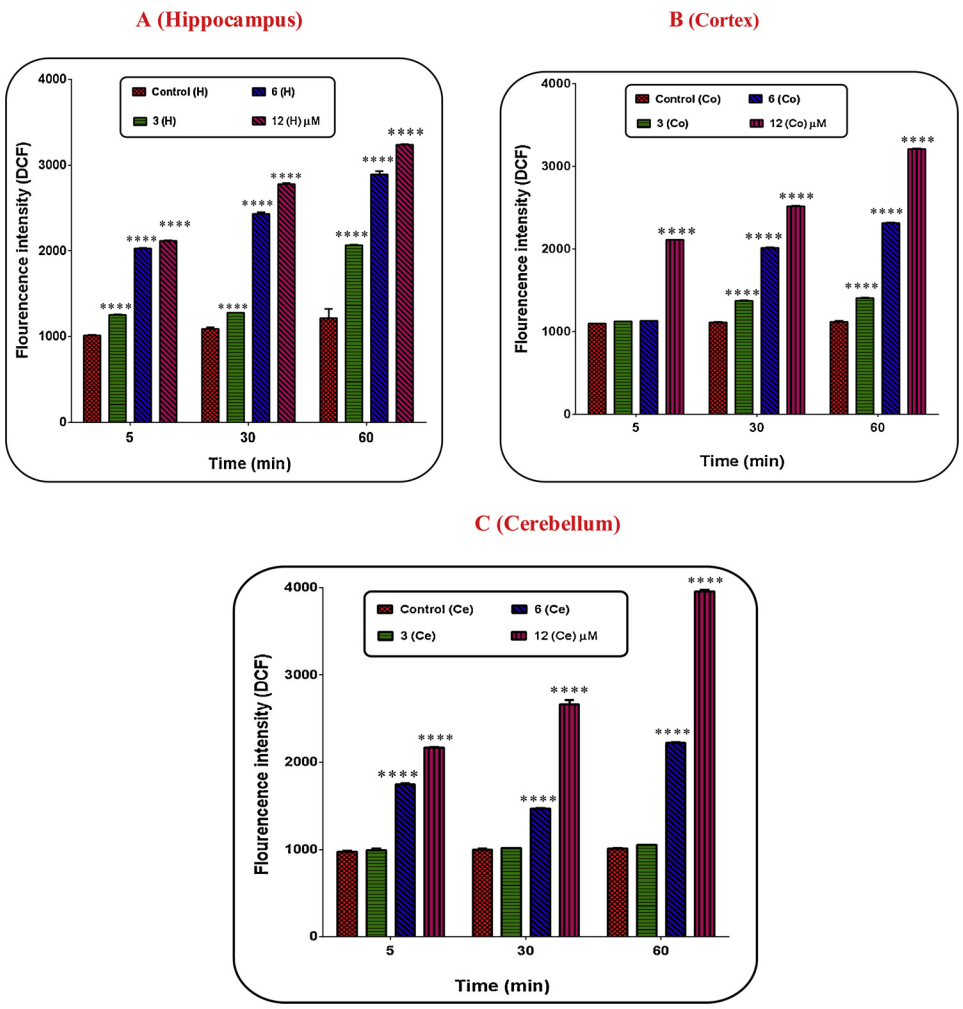

Fig. 3. Evaluation of mitochondrial ROS formation. The effect of different concentrations of mephedrone (3, 6 and 12 µM) on the ROS formation in mitochondria isolated from the (**A**) hippocampus (H), (B) cortex (Co) and (C) cerebellum (Ce). Data are presented as mean ± SD (n = 3 for each group). The two-way ANOVA test was carried out. **** shows a significant difference in comparison with the corresponding control group ($P < 0.0001$).

2.5. Mitochondrial swelling

Briefly, isolated mitochondria (from Hippocampus, Cortex, and Cerebellum) the all groups were suspended in swelling buffer and incubated at 37 °C with different concentrations of mephedrone (3, 6 and 12 µM). The absorbance was measured at 5, 30 and 60 min using an ELISA reader (Tecan, Rainbow Thermo, and Austria) at 540 nm A decrease in the absorbance indicates anincrease in mitochondrial swelling (Zhao et al., 2010).

2.6. Measurement of ROS level

Mitochondrial ROS formation induced by mephedrone in isolated mitochondria obtained from hippocampus, cortex, and cerebellum (normalized to 100 mg of mitochondrial protein) was measured using 2,7-dichlorofluorescein diacetate (DCFH-DA) (final concentration of 10 µM) as a reagent in modified respiratory buffer, including KCl (130 mM), $MgCl_2$ (5 mM), NaH_2PO_4 (20 mM), ADP (1.7 mm), succinate (5 mM) and $FeCL_3$ (0.1 mm), at pH 7.4 (Ex: 488 nm and Em: 527 nm).

2.7. Mitochondria membrane potential (MMP) assay

The Rhodamine 123 (Rh 123) redistribution technique was used for MMP measurement. Then, the isolated mitochondria were suspended in 1 ml of MMP buffer, including 220 mM sucrose, 68 mM D-mannitol, 10 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 50 mM EGTA, 5 mM sodium succinate, 10 mM HEPES and 2 mM rotenone, then Rh 123 (10 µM) was added. Mitochondrial fluorescence was analyzed using a fluorimeter (Ex: 490 nm and Em: 535 nm) (Baracca et al., 2003).

2.8. Assay of ATP level

The ATP levels were measured using Luciferin/Luciferase Enzyme system (Tafreshi et al., 2007). Bioluminescence intensity was measured using Sirius tube luminometer (Berthold Detection System, Germany).

2.9. Cytochrome c oxidase activity

In this study, the mitochondrial cytochrome c oxidase activity and mitochondrial outer membrane (MOM) (from hippocampus, cortex, and cerebellum) were measured using a cytochrome-c oxidase assay kit (Sigma, St. Louis, MO). Experimental procedures were performed according to the manufacturer's protocol.

2.10. Cytochrome c release assay

The concentration of cytochrome *c* was determined by using the Quantikine Rat/Mouse Cytochrome *c* Immunoassay kit provided by R & D Systems, Inc. (Minneapolis, Minn.).

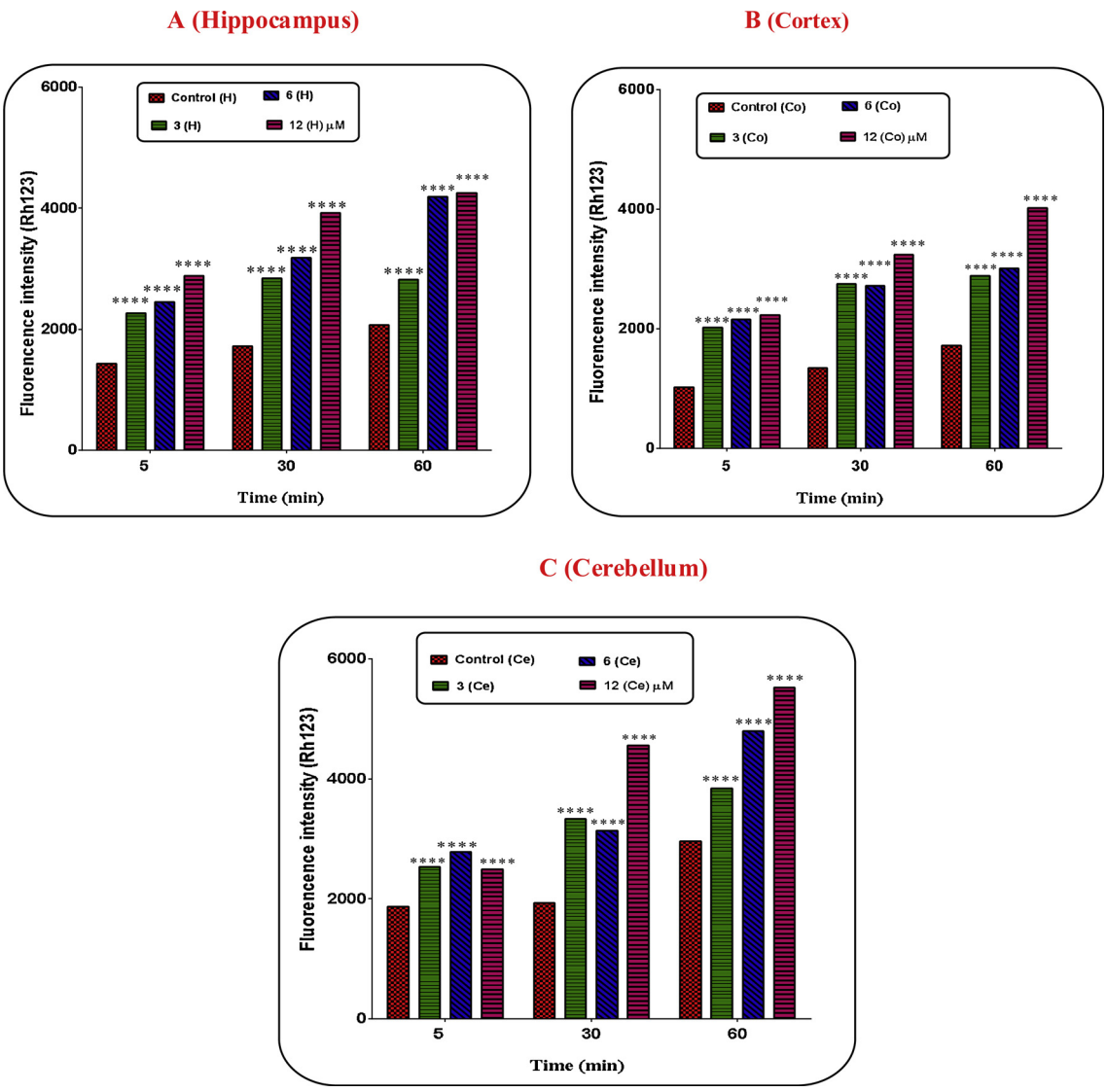

Fig. 4. The mitochondria membrane potential (MMP) assay. The effect of different concentrations of mephedrone (3, 6 and 12 µM) on the MMP in mitochondria isolated from the (**A**) hippocampus (H), (B) cortex (Co) and (C) cerebellum (Ce). Data are presented as mean ± SD (n = 3 for each group). The two-way ANOVA test was carried out. **** shows a significant difference in comparison with the corresponding control group ($P < 0.0001$).

2.11. Statistical analysis

Results are presented as means ± SD. All statistical analyses were performed using the GraphPad Prism software, version 5. Assays were performed in triplicate and the means of the triplicates were used for statistical analysis. The effects of group (control group and different concentrations of mephedrone (3, 6, and 12 µM)) on the SDH activity, mitochondrial ATP level, mitochondrial cytochrome c oxidase activity, and MOM integrity in each part of the brain (i.e. the hippocampus, cortex and cerebellum) were analyzed using one-way analysis of variance (ANOVA). Comparison of each group with the control group was done using the Dunnett's multiple comparisons test. The effects of group (control group and different concentrations of mephedrone (3, 6, and 12 µM)) and time (5, 30 and 60 min) on the mitochondrial swelling, mitochondrial ROS formation and MMP in the three parts of the brain (main effects and interaction of these effects) were analyzed using a 4 × 3 (group × time) two-way ANOVA. Multiple comparisons following two-way ANOVA was conducted using the Bonferroni's multiple comparisons test. The effects of group (control group, different concentrations of mephedrone (3, 6, and 12 µM), mephedrone (6 µM) + BHT and mephedrone (6 µM) + Cs A) on the cytochrome c release in the three parts of the brain were analyzed using one-way ANOVA followed by the Bonferroni's multiple comparisons test. Statistical significance was set at $P < 0.05$.

3. Result

3.1. Effects of mephedrone on succinate dehydrogenase (SDH) activity

SDH activity which is the measure of mitochondrial functionality was assessed using the MTT test after 1 h of incubation of isolated mitochondria (from hippocampus, cortex, and cerebellum) with different concentrations of mephedrone (3, 6 and 12 µM). There was a significant difference on the SDH activity among the experimental groups (hippocampus: $F_{(3, 8)} = 2500.00$, $P < 0.0001$; cortex: $F_{(3, 8)} = 448.20$, $P < 0.0001$; and cerebellum: $F_{(3, 8)} = 251.30$, $P < 0.0001$, one-way ANOVA). The results of Dunnett's multiple comparisons test showed a significant decrease in the SDH activity following addition of all applied concentrations of mephedrone on the mitochondria isolated from different parts of brain compared to control group (hippocampus [3 µM: $q = 10.28$, $P < 0.0001$; 6 µM: $q = 32.38$, $P < 0.0001$; 12 µM: $q = 79.57$, $P < 0.0001$]; cortex [3 µM: $q = 6.32$,

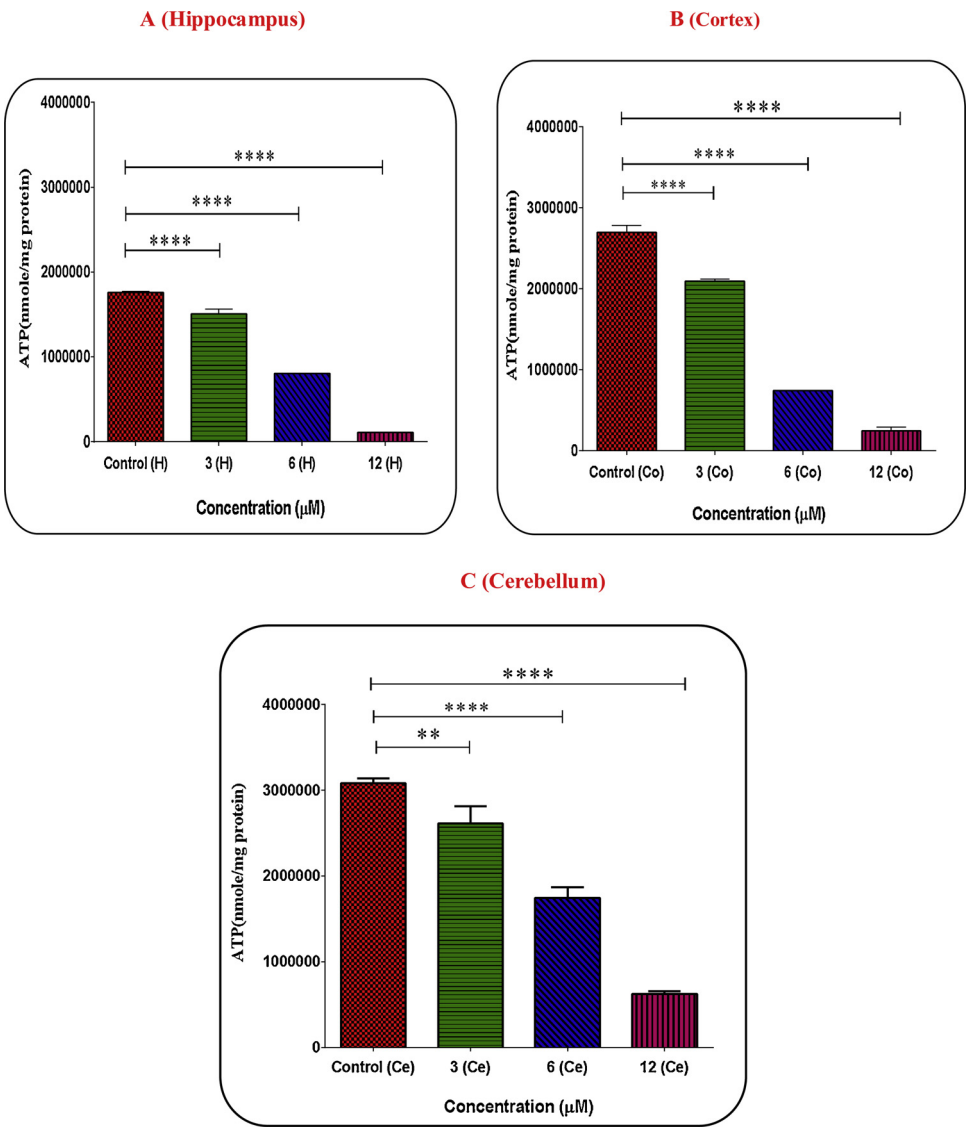

Fig. 5. Evaluation of ATP levels. The effect of different concentrations of mephedrone (3, 6 and 12 μM) on the ATP levels in mitochondria isolated from the (**A**) hippocampus (H), (B) cortex (Co) and (C) cerebellum (Ce). Data are presented as mean ± SD (n = 3 for each group). The one-way ANOVA test was carried out.** and **** show a significant difference in comparison with the corresponding control group ($P < 0.01$ and $P < 0.0001$, respectively).

$P < 0.0001$; 6 μM: $q = 22.31$, $P < 0.0001$; 12 μM: $q = 32.86$, $P < 0.0001$]; and cerebellum [3 μM: $q = 9.34$, $P < 0.0001$; 6 μM: $q = 10.56$, $P < 0.0001$; 12 μM: $q = 26.97$, $P < 0.0001$]) (Fig. 1A–C).

3.2. Effects of mephedrone on mitochondrial swelling

In this study, we assayed the mitochondrial swelling as an indicator of mitochondrial membrane permeability transition (MPT). A significant main effect of time (hippocampus: $F_{(2,\ 16)} = 314.70$, $P < 0.0001$; cortex: $F_{(2,\ 16)} = 6151.00$, $P < 0.0001$; and cerebellum: $F_{(2,\ 16)} = 6615.00$, $P < 0.0001$) and group (hippocampus: $F_{(3,\ 8)} = 473.00$, $P < 0.0001$; cortex: $F_{(3,\ 8)} = 15439.00$, $P < 0.0001$; and cerebellum: $F_{(3,\ 8)} = 38158.00$, $P < 0.0001$) as well as significant interaction effect of time by group (hippocampus: $F_{(6,\ 16)} = 69.62$, $P < 0.0001$; cortex: $F_{(6,\ 16)} = 1225.00$, $P < 0.0001$; and cerebellum: $F_{(6,\ 16)} = 490.20$, $P < 0.0001$) was found on mitochondrial swelling. The results of Bonferroni multiple comparisons showed that higher concentrations of mephedrone resulted in greater mitochondrial swelling in different parts of the brain over time (Fig. 2A–C).

3.3. Effects of mephedrone on ROS formation

The results showed that the level of ROS formation was significantly affected by time (hippocampus: $F_{(2,\ 16)} = 1224.00$, $P < 0.0001$; cortex: $F_{(2,\ 16)} = 35985.00$, $P < 0.0001$; and cerebellum: $F_{(2,\ 16)} = 6695.00$, $P < 0.0001$) and group (hippocampus: $F_{(3,\ 8)} = 5865.00$, $P < 0.0001$; cortex: $F_{(3,\ 8)} = 81504.00$, $P < 0.0001$; and cerebellum: $F_{(3,\ 8)} = 13862.00$, $P < 0.0001$) as well as the interaction effect of time by group (hippocampus: $F_{(6,\ 16)} = 113.40$, $P < 0.0001$; cortex: $F_{(6,\ 16)} = 8123.00$, $P < 0.0001$; and cerebellum: $F_{(6,\ 16)} = 3106.00$, $P < 0.0001$). According to the results of Bonferroni multiple comparisons, higher concentrations of mephedrone resulted in higher level of ROS formation in the mitochondria isolated from the hippocampus, cortex and cerebellum over time (Fig. 3A–C).

3.4. Effects of mephedrone on mitochondrial membrane potential (MMP)

In this study, we used Rh 123 as a cationic fluorescent dye for the measurement of MMP collapse. The results indicated a significant main effect of time (hippocampus: $F_{(2,\ 16)} = 931.70$, $P < 0.0001$; cortex: $F_{(2,\ 16)} = 55141.00$, $P < 0.0001$; and cerebellum: $F_{(2,\ 16)} = 16242.00$,

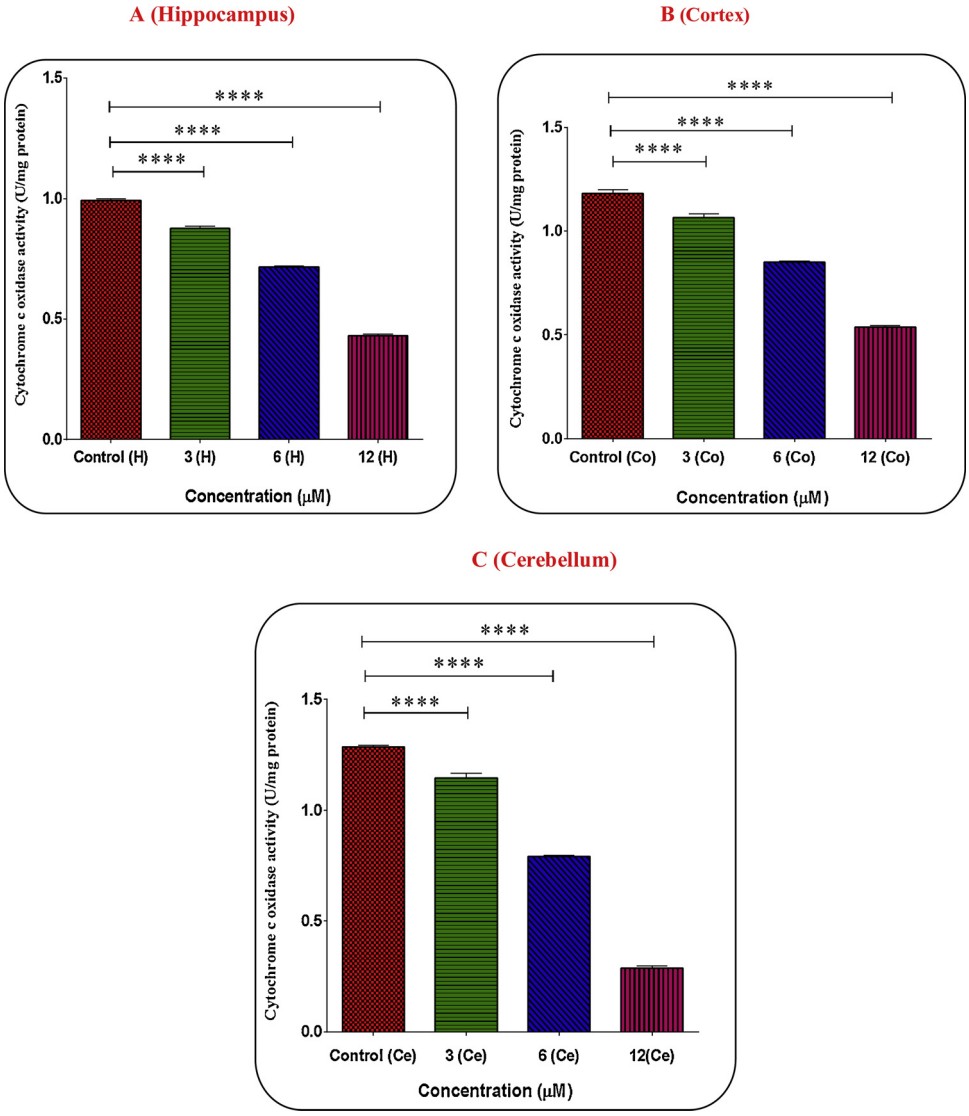

Fig. 6. Mitochondrial cytochrome-c oxidase (complex IV) activity assay. The effect of different concentrations of mephedrone (3, 6 and 12 μM) on the complex IV activityin mitochondria isolated from the (**A**) hippocampus (H), (B) cortex (Co) and (C) cerebellum (Ce). Data are presented as mean ± SD (n = 3 for each group). The one-way ANOVA test was carried out. **** shows a significant difference in comparison with the corresponding control group ($P < 0.0001$).

$P < 0.0001$) and group (hippocampus: $F_{(3, 8)} = 473.40$, $P < 0.0001$; cortex: $F_{(3, 8)} = 32958.00$, $P < 0.0001$; and cerebellum: $F_{(3, 8)} = 10523.00$, $P < 0.0001$) as well as significant interaction effect of time by group (hippocampus: $F_{(6, 16)} = 81.32$, $P < 0.0001$; cortex: $F_{(6, 16)} = 3339.00$, $P < 0.0001$; and cerebellum: $F_{(6, 16)} = 1379.00$, $P < 0.0001$) on MMP. The results of Bonferroni multiple comparisons showed that higher concentrations of mephedrone resulted in greater MMP collapse in different parts of the brain (i.e. hippocampus, cortex and cerebellum) over time (Fig. 4A–C).

3.5. Effects of Mephedrone on mitochondrial ATP level

Mitochondrial electron transfer chain (mETC) is required for ATP production. Therefore, we decided to measure the ATP levels in mitochondria isolated from hippocampus, cortex, and cerebellum following the addition of mephedrone (3, 6 and 12 μM). The results showed a statistically significant difference on the ATP levels among different groups in the hippocampus ($F_{(3, 8)} = 1828.00$, $P < 0.0001$, one-way ANOVA), cortex ($F_{(3, 8)} = 1475.00$, $P < 0.0001$, one-way ANOVA), and cerebellum ($F_{(3, 8)} = 236.00$, $P < 0.0001$, one-way ANOVA). As shown in Fig. 5A–C, compared to the control group, mephedrone in all applied concentrations (3, 6 and 12 μM) induced significant decrease on mitochondrial ATP levels in the mitochondria isolated from the hippocampus (3 μM: $q = 10.30$, $P < 0.0001$; 6 μM: $q = 38.77$, $P < 0.0001$; 12 μM: $q = 67.16$, $P < 0.0001$), cortex (3 μM: $q = 14.30$, $P < 0.0001$; 6 μM: $q = 46.47$, $P < 0.0001$; 12 μM: $q = 58.20$, $P < 0.0001$), and cerebellum (3 μM: $q = 4.70$, $P < 0.01$; 6 μM: $q = 13.46$, $P < 0.0001$; 12 μM: $q = 24.70$, $P < 0.0001$).

3.6. Effects of mephedrone on mitochondrial cytochrome-c oxidase (complex IV) activity

The complex IV activity as a key enzyme in the mitochondrial respiratory chain was determined in the mitochondria isolated from hippocampus, cortex, and cerebellum, and after the exposure to the different concentration of mephedrone (3, 6 and 12 μM). We found a significant difference between the experimental groups regarding the complex IV activity in the hippocampus ($F_{(3, 8)} = 3857.00$, $P < 0.0001$, one-way ANOVA), cortex ($F_{(3, 8)} = 1339.00$, $P < 0.0001$, one-way ANOVA), and cerebellum ($F_{(3, 8)} = 3663.00$, $P < 0.0001$, one-way ANOVA). The results showed that mephedrone at concentrations of 3, 6 and 12 μM significantly reduced the activity of complex IV

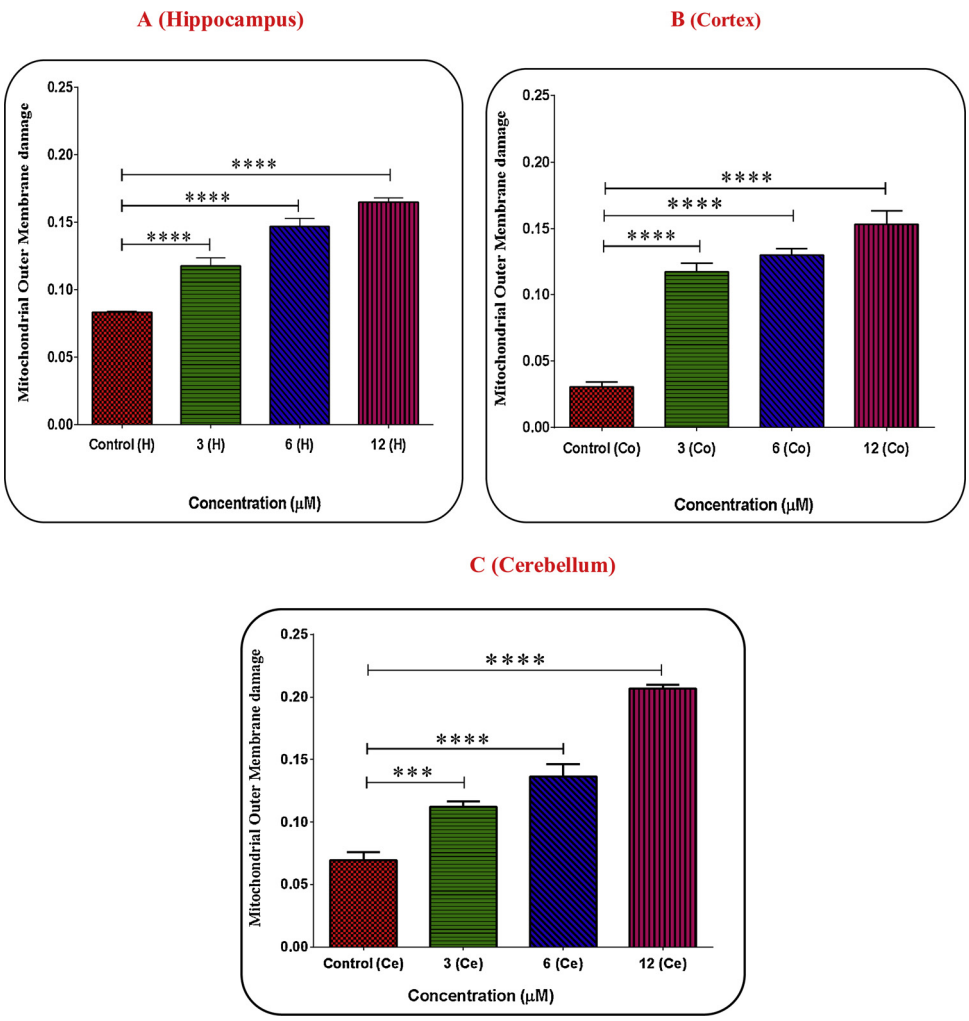

Fig. 7. Evolution of mitochondrial outer membrane (MOM) integrity. The effect of different concentrations of mephedrone (3, 6 and 12 μM) on the MOM integrity in mitochondria isolated from the (A) hippocampus (H), (B) cortex (Co) and (C) cerebellum (Ce). Data are presented as mean ± SD (n = 3 for each group). The one-way ANOVA test was carried out. *** and **** show a significant difference in comparison with the corresponding control group ($P < 0.001$ and $P < 0.001$, respectively).

in the mitochondria isolated from the hippocampus (3 μM: $q = 20.85$, $P < 0.0001$; 6 μM: $q = 49.87$, $P < 0.0001$; 12 μM: $q = 101.3$, $P < 0.0001$), cortex (3 μM: $q = 10.58$, $P < 0.0001$; 6 μM: $q = 30.27$, $P < 0.0001$; 12 μM: $q = 58.88$, $P < 0.0001$), and cerebellum (3 μM: $q = 13.57$, $P < 0.01$; 6 μM: $q = 47.66$, $P < 0.0001$; 12 μM: $q = 96.03$, $P < 0.0001$) compared with the control group (Fig. 6A–C).

3.7. Effects of mephedrone on mitochondrial outer membrane (MOM) integrity

In this study, the MOM integrity was measured in the presence/absence of detergent n-dodecyl β-D-maltoside. This ratio represents the percentage of MOM damage. The results of the current study revealed a significant difference on the MOM damage between the different groups in the three parts of the brain (hippocampus: $F_{(3, 8)} = 184.00$, $P < 0.0001$; cortex: $F_{(3, 8)} = 192.00$, $P < 0.0001$; and cerebellum: $F_{(3, 8)} = 228.00$, $P < 0.0001$, one-way ANOVA). As shown in Fig. 7A–C, exposure to different concentrations of mephedrone (3, 6 and 12 μM) induced a significant rise in the MOM damage in isolated hippocampus (3 μM: $q = 9.28$, $P < 0.0001$; 6 μM: $q = 17.05$, $P < 0.0001$; 12 μM: $q = 21.96$, $P < 0.0001$), cortex (3 μM: $q = 15.88$, $P < 0.0001$; 6 μM: $q = 18.20$, $P < 0.0001$; 12 μM: $q = 22.42$, $P < 0.0001$), and cerebellum (3 μM: $q = 7.92$, $P < 0.001$; 6 μM: $q = 12.44$, $P < 0.0001$; 12 μM: $q = 25.50$, $P < 0.0001$) compared to the control group in a concentration-dependent manner.

3.8. Effects of mephedrone on cytochrome c release

We found a statistically significant difference on the cytochrome c release between the experimental groups in different parts of the brain (hippocampus: $F_{(5, 12)} = 2165.00$, $P < 0.0001$; cortex: $F_{(5, 12)} = 1148.00$, $P < 0.0001$; and cerebellum: $F_{(5, 12)} = 2589.00$, $P < 0.0001$, one-way ANOVA). The results of Bonferroni multiple comparisons indicated that, as shown in Fig. 8A–C, there was a statistically significant difference in the release of cytochrome c between the mephedrone-treated mitochondria (3, 6 and 12 μM) and control mitochondria in different parts of the brain (i.e. the hippocampus, cortex and cerebellum). Considerably, the pretreatment of mitochondria with The MPT inhibitor such as Cs A or BHT (an antioxidant) inhibited cytochrome c release from mephedrone (6μM) treated mitochondria. These results demonstrate the role of oxidative stress and MPT pore opening in the release of cytochrome c.

4. Discussion

Mephedrone has been abused as a recreational drug in many countries. Recent reports indicate that mephedrone has similar effects to cocaine and amphetamines. Mephedrone has side effects (even at low

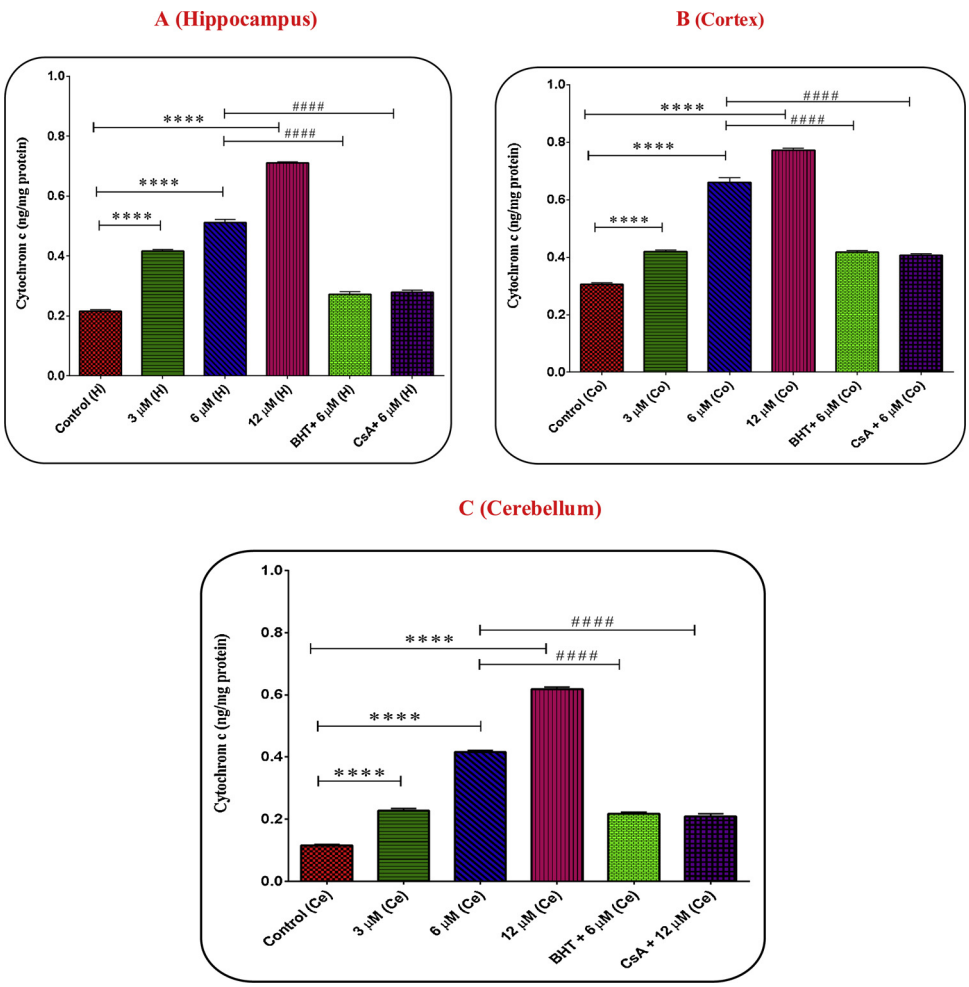

Fig. 8. Evolution of cytochrome c release. The effect of different concentrations of mephedrone (3, 6 and 12 µM) on cytochrome c release in mitochondria isolated from the (A) hippocampus (H), (B) cortex (Co) and (C) cerebellum (Ce). Data are presented as mean ± SD (n = 3 for each group). The one-way ANOVA test was carried out. **** shows a significant difference in comparison with the corresponding control group ($P < 0.0001$).#### Shows a significant difference in comparison with mephedrone (6 µM) treated mitochondria ($P < 0.0001$).

doses) on various organs such as the brain and heart (Karila et al., 2016; Motbey et al., 2012; Opacka-Juffry et al., 2014). Mephedrone is also able to cause changes in the mitochondrial respiration (Pantano et al., 2017). However, the mechanism of the effect of mephedrone on the CNS and the development of neurotoxicity is not well known. This study was designed to evaluate the effect of mephedrone on mitochondrial respiration and oxidative stress in different parts of brain including hippocampus, cortex, and cerebellum.

The mitochondria as a vital organelle play an important role in brain development and neuronal growth due to the effect on energy metabolism. Thus, dysfunction of mitochondria plays a role in the pathophysiology of various diseases, in which all the respiratory chain function has been impaired. Furthermore, dysfunction of the respiratory chain can cause neuronal degeneration and neurological disease (Wirtz and Schuelke, 2011). Each of the part of brain requires the proper functioning of the various parts of the mitochondrial respiratory chain, For example, the hippocampus and cerebellum require the proper function of mitochondrial complex I and mitochondrial ATP production (complex V) (Wirtz and Schuelke, 2011). The results showed that mephedrone reduced SDH activity (mitochondrial complex II) in isolated mitochondria from the hippocampus, cortex, and cerebellum. Our reports also showed that mephedrone significantly increases the level of ROS in the mitochondria isolated from the hippocampus, cortex, and cerebellum of treated rats. In recent years, the study of the role of ROS in the CNS has been considered. In the CNS, the generation of ROS is adjusted by the antioxidant defense system. However, an antioxidant defense is ineffective against the excessive release of ROS, and subsequently, oxidative stress occurs. Furthermore, oxidative stress and ROS (at a high level) are often associated with loss of cognitive functions and synaptic plasticity (Beckhauser et al., 2016; Massaad and Klann, 2011).The hippocampus and neo-cortex play a vital role in memory consolidation. But, both of them are very susceptible to oxidative damage. Commonly, various evidence exists that an increase of oxidative processes is the major cause of neurodegeneration (Budzynska et al., 2015). In memory function, researches show that ROS play as a double-edged sword (Massaad and Klann, 2011). Generally, the CNS is vulnerable to ROS and oxidative stress. In the brain compared to other organs, oxygen consumption and lipid content are higher and its antioxidant defense is weaker (Beckhauser et al., 2016). Furthermore, excessive ROS causes suppression of long-term potentiation (LTP) in the hippocampus and cerebellum (Beckhauser et al., 2016).

It is possible that raised ROS formation and oxidation of membrane protein thiol groups triggers MPT pores opening, a vital event in mitochondrial dysfunction, followed by induction of swelling in mitochondria, and decline of MMP (Pourahmad et al., 2010). In this research, we showed that mephedrone can cause increased swelling in isolated mitochondria from the hippocampus, cortex, and cerebellum. Also, results showed the decline of MMP (an indicator of MPT pore opening) after incubation of rat hippocampus, cortex, and cerebellum

mitochondria with different concentrations of mephedrone. In this study, mephedrone also reduced the activity of mitochondrial complex IV. We indicated that mephedrone was able to disrupt the mitochondrial respiratory chain.

It has already been reported that mitochondrial function plays an important role in the neuronal processes and oxygen consumption in CNS is much higher than other organs (Beckhauser et al., 2016; Cardoso et al., 2010). Mitochondrial oxidative phosphorylation (OXPHOS) provides ATP for normal neuronal function. The OXPHOS system is the main source of ROS generation (Beckhauser et al., 2016; Cardoso et al., 2010). The results showed that mephedrone decreases the ATP content of mitochondria isolated from the hippocampus, cortex, and cerebellum. Finally, we suggest that mephedrone can change the function of the hippocampus, cortex, and cerebellum, which all require high levels of ATP for their normal function.

It was shown that ROS in high content leads to apoptosis (Beckhauser et al., 2016). Ultimately, we showed the release of cytochrome c from hippocampus, cortex, and cerebellum mitochondria, which is related to the opening of large MPT pores in the outer membrane or inhibiting some segments of the respiratory chain in the inner membrane. The release of cytochrome c (Cyt c) as a key part of the electron transfer between complexes III and IV will impairs mitochondrial respiratory chain activity (Pourahmad and Hosseini, 2012). Previous works showed that Cyt c exit from mitochondria into cytosol plays an important role in the onset of apoptosis (Katoh et al., 2004; Ko et al., 2005).

The results of our study showed that mephedrone can disrupt mitochondrial respiratory chain in the isolated mitochondria from the hippocampus, cortex, and cerebellum. Subsequently, mephedrone induces oxidative stress, increases ROS generation and impairment of mitochondrial complexes II and IV. It also reduces the ATP content and increases the Cyt crelease in mitochondria isolated from the different parts of brain. We suggest that the abuse of mephedrone could induce neurotoxicity through mitochondrial respiratory disturbances and their consequences leading to the neuronal death and the onset of neurological disease in the brain.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

Jalal Pourahmad: Contributed by formulating the research question(s), designing the study, carrying out the experiments, analyzing the data, and writing the paper. **Enayatollah Seydi**: Contributed to this research by analyzing the data, and writing the paper. **Behnaz Atabaki:** Contributed to this research by carrying out the experiments and analyzing the data as her Doctor of Pharmacy thesis. The thesis was carried out under supervision of Prof. Jalal Pourahmad at Shahid Beheshti University of Medical Sciences, Tehran, Iran. **Parvaneh Naserzadeh** and **Ghorban Taghizadeh:** Contributed by analyzing the data and writing the paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2019.02.014.

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