Original article

Toxicity effects of methamphetamine on embryonic stem cell-derived neuron

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Background: Methamphetamine (MA) is the most popular recreational drug. According to potential neurotoxicity of this agent, it can cause deleterious effects on neural differentiation of embryo, if MA is used during the child bearing period. In recent decades, undifferentiated pluripotent embryo-derived stem cell lines, resembling early embryonic stages, have been used to analyze the toxic effects of components *in vitro*. Thus, this study aims at assessing toxic effects of MA on embryonic stem cell (ESC)-derived neuronal cells during differentiation in a pharmacological model. **Materials ans Methods**: ESC line Royan was used throughout this study. The effect of MA on neural differentiation was assessed during two periods, group 1: MA (10, 100, 200,500, 750, 1000 µM concentrations) was added during EB formation, group 2: MA (10, 50, 70, 100, 200, 500 µM concentrations) was added after the generation of neural precursors. Then cells were evaluated for neuronal markers by immunocytochemistry and RT-PCR. One way ANOVA followed by the post hoc test was used to analyze data. **Results:** The declining in outgrowth of dendrites was observed in neural morphology in a dose dependent manner. The ID50 (Inhibition of neuronal differentiation) of groups 1 and 2 were 130 and 400 µM, respectively. By using RT-PCR, in comparison with MAP2, no significant change was observed in Nestin expression. **Conclusions:** Our data on neuronal toxicity were consistent with *in vivo* and *in vitro* studies. We concluded that ESCs can be used as an efficient model to assess the toxicity of drugs.

Key words: Methamphetamine, Embryonic Stem Cell, Neural Differentiation, Toxicity.

INTRODUCTION

Methamphetamine (MA) is the most popular psychostimulant drug known as "Ice".[1-2] After cannabis, the widespread use of MA has faced a sharp upward trend among the adults over 12 years of age and women of child bearing ages during recent years [3] (United Nations Office on Drugs and Crime, 2004). MA is a cationic lipophilic molecule readily crossing the placenta. [4] Furthermore, it has been demonstrated that the following administration to the mother not only influences the fetal tissue and amniotic fluid by these compounds, but also involves neural differentiation and causes long-term motor defects in the progeny. [3, 5-6] Therefore, this idea has been progressed that the fetus may be at risk when MA users are not aware of being pregnant. [7] However there have been no enduring studies on the neurodevelopment consequences of prenatal MA exposure in humans.[3]

There is an essential need to incorporate embryotoxicity tests, in the embryonic period, into standard screening models for appraising the effects of pharmacological agents. Nowadays undifferentiated pluripotent embryo-derived stem cell lines, having both proliferation and differentiation capabilities, have been used to investigate embryotoxicity effects of chemical agents *in vitro*. It is believed that proliferation and differentiation capabilities represent early embryonic stages. In addition, this model has been validated by the differentiation of ES cells via formation of aggregate or embryoid body (EB) for detection of neurotoxic potential of compounds *in vitro*.[8,9] Many studies have described the effects of this drug *in vivo*[10] and *in vitro*[11, 12], but this study is the first report to use embryonic stem cell (ESC)-derived neuronal cells as a model for evaluating MA toxicity *in vitro* .Thus, this study aims at assessing the toxic effects of MA on these cells during differentiation in pharmacological model and determining whether it is a suitable model for evaluating MA toxicity.

MATERIALS AND METHODS

Culture and differentiation of ESCs

The C57BL/6 mouse strain derived ESC line Royan B1 was used in this study.[13] ESCs were kept in an

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undifferentiated, pluripotent state and differentiated into neural cells as described before. [12]

Toxicity studies on neural cell derivatives from mESCs

The effects of MA (Sigma–Aldrich, M8750-25G) on neural differentiation, after determining the dose response curve, was assessed during two periods, group 1: MA (10, 100, 200,500, 750, 1000 μM concentrations and control group) was added during EB formation until day 10, group 2: MA (10, 50, 70, 100, 200, 500 μM concentrations and control group) was added from day 6, after RA (retinoic acid) induction or the generation of neural precursors until day 10. Inhibition of neuronal differentiation (ID50) was determined by 50% reduction in EBs differentiating to neurons relative to control. At least, 75-100 EBs were assessed for each concentration of MA.

Relative reverses transcription-polymerase chain reaction (RT-PCR) analysis

In order to evaluate the expression of the neural marker in cultured cells, total RNA was extracted by using the RNeasy Mini kit (Qiagen, Spain). For removing genomic DNA contamination, RNA samples were digested with DNase I (Fermentas; EN0521). Standard RT was performed using 2 μg total RNA, oligo (dT) and the RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas, K1622) according to the manufacturer's instructions. The cDNA samples were situated to polymerase chain reaction (PCR) amplification using mouse specific primers. Amplification conditions have been described in previous studies.[12] PCRs were carried out in triplicate. PCR products were separated on 2% Agarose (Fermentas) and stained with ethidium bromide, visualized and photographed on a UV transluminator (Uvidoc, UK). β-tubulin transcript was used for internal normalization. Finally, the intensity of neural-specific genes band to the density of β-tubulin was quantified with the TotalLab software. The primer sequences of Nestin, MAP-2 and β-tubulin have been previously reported.[12]

Immunofluorescence staining

Plated EBs were rinsed with PBS after 4 days, fixed with 4% paraformaldehyde (Sigma–Aldrich, P6148) at room temperature and permeabilized with 0.2% Triton X100 in PBS. Then cells were blocked with 10% goat serum/PBS tween-20 for 30 minutes at 37 ^{0}C, were incubated overnight at 4 ^{0}C in humidity chamber with anti-microtubule associated protein 2 (MAP2, Sigma–Aldrich, M1406) as mature neuron marker, and for β-tubulin III (Sigma– Aldrich, T8660) as immature neuron markers. At the end of the incubation time, cells were rinsed with PBS and incubated with fluorescence isothiocyanate (FITC)-conjugated anti-IgG (Sigma, F9006, and 1:100) for 60 minutes at 37 ^{0}C. After rinsing with PBS, nuclear staining was performed with PI (0.2μg/ml, Sigma Aldrich, P4170) and cells were analyzed under a fluorescent microscope (Olympus, Japan).

Statistical analysis

The data were stated as mean ± SD (standard deviation). One way ANOVA followed by the post hoc test were used to analyse data. Drug treatment and RT-PCR analysis were replicated at least three times. The difference between groups was considered as statistically reliable if the

$p < 0.05$. Data were expressed as mean ± SEM (standard error of mean).

RESULTS

ESCs derived neural cells with expressing of MAP2 and β-tubulin III markers by immunocytochemistry (Figure 1A and B) were treated for different concentrations of MA. ESCs were treated with MA from the time of EB formation (day 0) to the end of post-plating (day 10) in group 1, or only during the 4 days of post-plating in group 2 (Figure 1C). The declining in outgrowth was observed in neural morphology in a dose dependent manner (Figure 1D). The number of neurons emerging out of EBs gradually decreased with increasing concentrations of MDMA and at the concentration higher than 500μM of MA, the EBs did not plate and had a dark appearance. Inhibition of neuronal differentiation was determined to be the 50% reduction of EBs with neural extensions as compared to the control (ID50). The ID50 of groups 1 and 2 (as shown in Figure 1E) were 130 and 400 μM, respectively. Moreover, results of semi-quantitative RT-PCR at four days post-plating showed, that in spite of there being no significant change in Nestin expression, MAP2 was significantly reduced in concentrations greater than 200 and 500 μM in groups 1 and 2, respectively (Figure1 G and I).

DISCUSION

There is not enough information about methamphetamine effects on neurogenic niches, especially in studying the consequences of gestational exposure to MA. [3,14] It is necessary to have a substitute model for studying these compounds and their effects on various stages of embryo development. On the other hand, ESCs have been commonly presented as a model for evaluating the toxicity of drugs *in vitro*. [8]

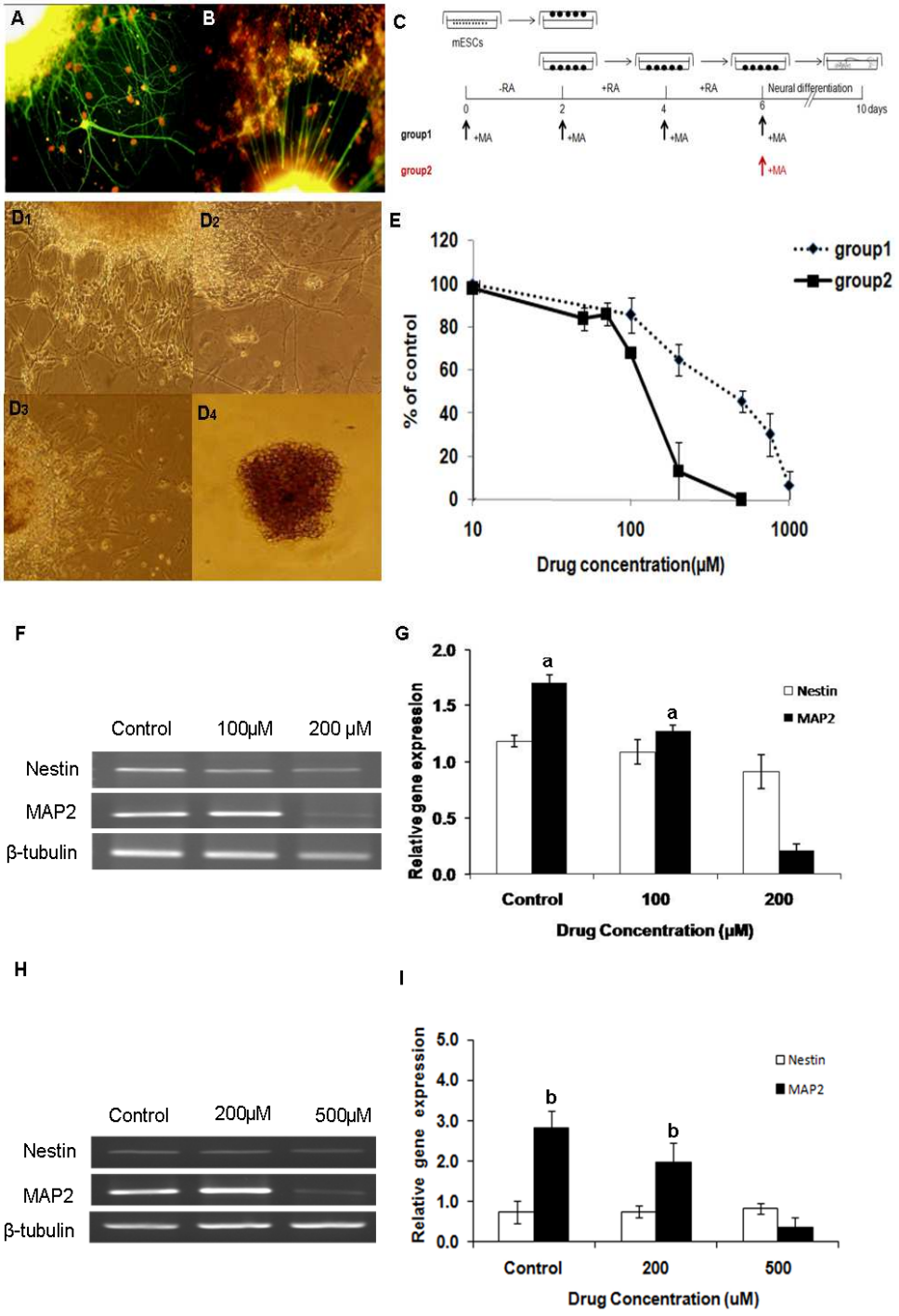

Figure 1. Immunostaining for neuronal cell, Neuron-like cell with dendritic processes, stained with anti-MAP2 and anti tubulin antibody respectively (A, B). Scale bar: 20μm. The nuclei were counterstained with PI. ESCs were treated with MA from the time of EB formation (day 0) to the end of post-plating (day 10) in group 1 or only during the 4 days of post-plating in group 2(C). Effect of different concentrations of MA on neural differentiation control were 10, 200, 500 μM respectively (D1, D2.D3, D4). Scale bar: 100μm. MA concentration–response curves during neuronal differentiation between group1 and group2 (E). RT-PCR and semi-quantitative analyses of MA on the relative gene expression of Nestin and MAP2 in group 1 (F and G)and group 2 (H and I). a: $p < 0.05$, control and 100 μM vs. 200 μM, b: $p < 0.05$, control and 200 μM vs. 500 μM

In our experiment we used ESCs-derived neuron as an in vitro model for evaluation of MA-induced neurotoxicity. According to the present study, ID50 is lower during neural precursor formation (group I, 130 μM) than during neural differentiation (group II, 400 μM), suggesting that the early phase of neural development which coincides with neural tube formation is more sensitive than the later phase when neural precursor cells differentiate into mature neurons.

Bento et al. presented the effect of MA on stem/progenitor cells obtained from early postnatal

mice and showed MA inhibited neuronal differentiation (1 and 10 μM) at the concentrations lower than decreased proliferation (100, 250, and 500 μM). This information leads to this point that differentiation is more sensitive than proliferation in subventricular zone stem cells.[15] The discrepancy ID50 between our results and the results of Bento's study could be explained with the different sources of used stem cells in the two experiments.

In these experiments, it was indicated that the toxicity increment *in vitro* had direct correlation with time in mESCs derived neural cells. We have previously reported that MDMA (a synthetic methamphetamine derivative) is a moderate teratogen and such a dose and time dependent toxicity is observed for both group 1 and group 2[12], Furthermore, similar effects have been described with MA in N27 dopaminergic neuronal cell,[16] microglial [17] and mesencephalic cells *in vitro*.[18]

In addition, ID50 in both *in vivo*[19,20] and *in vitro*[1, 21] studies were very close to our results in some experiments, for instance, in neocortical neuron after 96 hours of 500 μM MA produced prominent immunoreactivity and nuclear fragmentation in approximately 50% in treated groups.[2] Meanwhile, MA significantly decreased MAP2 expression in concentrations greater than 200 and 500 μM in groups 1 and 2, respectively but the expression of Nestin was not influenced by MA at the dose levels experimented. Similar to our results, Putzke et al.[22] reported diminishing MAP2 expression in the cortex of adult male Wistar rats following administration of MA. *In vitro* and *in vivo* studies have shown that there is a correlation between reduced expression of MAP2 [23](as an important cytoskeletal protein) and neuronal disorders, such as schizophrenia, depression, and impulsive disorders. MA can also lead to abnormal behaviour in a wide range of species. Thus, by evaluation of MAP2 expression it seems that it can be used as a proper marker to pursue the molecular plasticity influenced by exposure to these compounds. [23]

Regarding ESC abilities such as tissue-specific properties especially on the formation of neuroectoderm and mesoderm, it could act as a system to evaluate inhibiting or inducing effects on the differentiation processes of early embryonic stages. [15,24]

In conclusion, according to the results of this study, It is proposed that embryonic stem cells are a suitable model for the assessment of drug toxicity, especially when assessment of drug in vivo is difficult and a large number of animals are required to be sacrificed to achieve a similar conclusion.[23]

Finally, the application of novel technologies to ES cells in the future will influence toxicology research considerably.

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