Original Article

MDMA-Induced BV2 Microglial Cell Activation in Vitro

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Abstract

Background: 3,4-Methylenedioxymethamphetamine (MDMA) is a psychostimulant drug that induces neurotoxicity. Even though several psychostimulant substances activate microglia, little is known about MDMA's effects on these cells, and evidence of MDMA-induced microglial activation is equivocal.

Materials and Methods: This study employed a murine microglial cell line, BV2, to examine the effects of MDMA on the microglia morphological changes and the survival of microglia in vitro. MDMA was incorporated into the media at the time of plating, and cell number and mitochondrial dehydrogenase activity (MTT) levels were determined in vitro. The level of pro-inflammatory cytokine TNF-α was also determined.

Results: Treatment of BV2 cells with MDMA resulted in morphological changes, reduced cell viability after 24h incubation with the inhibitory concentration (IC_{50}) value of 243.6 µg/mL, and increased TNF-α level in a dose-dependent manner.

Conclusion: These findings proposed that MDMA could induce BV2 microglial cell activation in vitro and suggested that it has an essential role in developing MDMA use disorder.

Keywords: 3, 4-methylenedioxymethamphetamine, MDMA, Microglial activation, Neurotoxicity

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Introduction

Neurological insult is the most common form of MDMA neurotoxicity, which causes substantial damage to the brain by causing both apoptotic and necrotic cell death in the brain. Histopathological studies demonstrated neuronal damage inflicted by MDMA and immunohistochemically, visualized under microscopes (1, 2). It was indicated by the morphological changes, a decrease of intact neuronal cells, or several apoptotic markers. Besides that, biochemical studies have reported an immediate release of neurotransmitters upon MDMA administration, leading to excitotoxicity. In the long-term, MDMA causes the reduction of serotonin transporter and the depletion of dopamine and serotonin (3, 4). MDMA also induces a hyperthermic response that modulates the long-term neuronal damage caused by the drug. All effects are the mechanism outputs of MDMA actions starting from its administration into the body until the pharmacological changes taking place.

Besides the overproduction of neurotransmitter

releases, exposure to MDMA also causes the microglial cells in the brain to be activated in response to the danger-associated signals from the damaged neurons (5). As the immune cells, microglial act as sentinels, detecting the first signs of invasion by MDMA or tissue damage (6). Although microglia are not solely responsible for inflammatory and immune-mediated responses in the brain, they are well-positioned to respond quickly to environmental changes (7). The activation of microglia leads to specific differentiation of microglial phenotypes. Once activated, the cells will proliferate and migrate to the injury site, followed by changes in the morphology and the inflammatory secretion profiles (8). They undergo classical/pro-inflammatory (M1) or alternative/anti-inflammatory (M2) activated phenotypes. M1 phenotypes are more likely to be detrimental to the brain by inducing neuronal toxicity through secretion of pro-inflammatory cytokine and production of reactive oxygen species (ROS) (9). However, there is no direct evidence of the effects of MDMA on BV2 cells in vitro. In fact, in vitro models make it possible to examine molecular mechanisms in a straightforward and repeatable manner (10). Here we report MDMA-induced microglial activation by examining the morphological changes, cell viability, and TNF-α level at the different doses of MDMA exposure.

Methods

Study Design: This study was an in vitro experimental laboratory conducted at the Cell Culture Laboratory, Faculty of Medicine, Sultan Zainal Abidin University (UniSZA). This study aimed to investigate the effects of different doses of MDMA on BV2 cell viability, their morphological changes, and TNF-α cytokine level. BV2 cells were treated with varying concentrations of MDMA, and the percentage of cell viability was determined by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Morphological changes in the cells were observed under a microscope. Meanwhile, the TNF-α level for the selected doses of MDMA was determined using the Enzyme-Linked Immunosorbent Assay (ELISA) kit. The statistical analysis was carried out using Graph Pad Prism 6.

The BV2 cell line was purchased from Elabscience Biotechnology Co., Ltd (Elabscience), USA. Dulbecco's Modified Eagle Medium/F12 (DMEM/F12), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin EDTA, and penicillin/streptomycin were obtained from Gibco (Invitrogen, USA). Dimethyl sulfoxides (DMSO) were purchased from Merck (Germany). D,1-MDMA HCl were purchased from Labchem Sdn. Bhd., Malaysia. MDMA was dissolved in deionized water to produce a 10mg/mL stock solution. Trypan blue and MTT powder were purchased from Sigma-Aldrich (USA).

Cell Culture and Treatment with Different Concentrations of MDMA: BV2 microglial cells were cultured in tissue culture flasks with Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were maintained in a humidified atmosphere of 5% CO2 at 37°C and cultured at an initial density of cells/flask. Then, the cells were plated into 96-well plates and treated with MDMA at concentrations ranging from 0 - 800 µg/mL.

Cell Viability Study by Using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) Assay: Cell viability was determined by using the MTT assay. BV2 cells were seeded in a 96-well plate at a density of $1x10^5$ cells/100 µl and treated with varying concentrations of MDMA (800, 600, 400, 200, 100, and 0 µg/ml) for 24 h. Then, 20 µl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) was added to the cells in the dark and incubated for four h, covered with aluminum foil. After incubation, 100 µl DMSO was added to each well to dissolve the formazan crystals formed, and absorbance was read at a wavelength of 490 nm as measurement wavelength and 570 nm as reference wavelength using the Tecan ELISA microplate reader. The potency of cell growth inhibition for the test agents will be expressed as the half-maximal (50%) inhibitory concentration, IC_{50}. The amount of color produced is directly proportional to the number of viable cells. Cell viability rate was calculated as the percentage of MTT absorption as follows: % survival = (mean experimental absorbance/mean control absorbance) × 100.

Visualization of the Morphology of BV2 Microglial Cells: After 24 h treatment with different doses of MDMA, the cell morphology was examined under a phase-contrast microscope. The images were captured at 20X magnification.

Quantitative Measurement of TNF-α: The concentrations of TNF-α in culture supernatants were measured by a Mouse TNF-α ELISA Kit (ab208348) according to the manufacturer's instructions. All reagents were provided in the kit by the manufacturer. Briefly, 50 µL of all samples and standards were added into a 96-well plate in duplicate. Then, 50 µL of Antibody Cocktail that was prepared early according to the manufacturer's instruction was added to each well. The plate was sealed and incubated for 1 hour at room temperature on a plate shaker set to 400 rpm. After that, all wells were washed with 350 µL 1X Wash Buffer three times. The plate was tapped gently against a clean paper towel until the liquid was removed entirely. 100 µL of TMB Development Solution was added to each well and incubated in the dark for 10 minutes on a plate shaker. Also, 100 µL of Stop Solution was added to each well and shaken for 1 minute to mix. The absorbance at 450 nm was determined using a microplate reader. The concentrations of TNF-α in culture supernatants were then calculated using their respective standards.

Statistical Analysis: Statistical analysis was undertaken in the GraphPad Prism computer program (GraphPad Software Inc., San Diego, CA, USA). Cell viability data and the half-maximal (50%) inhibitory concentration were analyzed using non-linear regression analysis. Comparison of the percentage of cell viability and TNF-α level between different dose groups was determined using a one-way analysis of variance (ANOVA) followed by Tukey's posthoc test. The results are the mean of at least three independent experiments. Data are expressed as mean ± SEM, and differences were statistically significant at $p \leq 0.05$.

Results

Effects of Different Doses of MDMA on the Cells' Viability: BV2 microglial cells were incubated with MDMA for 24 hours (0-800 µg/mL). The corresponding cell viability graphs are shown for MDMA (Figure 1).

The cytotoxicity of MDMA in BV2 microglial cells was evaluated based on its effect on cell proliferation by using the MTT assay. The cytotoxicity of MDMA was dose-dependent; the maximum cell death showed at a concentration of 600 ± 0.02 µg/mL. The inhibitory concentration (IC_{50}) of MDMA on BV2 microglial cells was 243.6 µg/mL. Figure 2 shows the multiple comparison test of different doses of MDMA on the percentage (%) of cell viability. The MDMA exposure at the doses of 200, 300, 400, 500, 600, and 800 µg/mL significantly reduced the % of viable cells as compared to the untreated cells (Control group) after 24H incubation ($p \leq 0.05$).

The effects of MDMA on the Morphology of BV2 Microglial Cells: As shown in Figure 3, untreated BV2 cells maintained the resting state in a traditional medium. Most of them exhibited round cytoplasm with some bipolar projections. However, in the MDMA treated groups, the cells were more significant, and the extent of amoeboid morphology seemed to be increased with the cytoplasmic area appearing minimal, which was seen in the 300 µg/mL MDMA group. BV2 cells treated with increasing concentrations of MDMA for 24h underwent dramatic morphological changes characterized by vacuolization and hypertrophy. At the higher concentrations of MDMA exposure, the cells undergo degeneration and apoptosis.

The effects of MDMA on the TNF-α Level: Based on the cytotoxicity assay and morphological observations, we assumed that the doses of MDMA above the IC_{50} (243.6 µg/mL) would activate the BV2 cells. As indicated by the cells' morphology, 300 µg/mL of MDMA caused some changes in the morphology without producing a substantial cell deficit. Hence, we conducted the subsequent experiment by incubating the cells with 300, 500, and 700 µg/mL MDMA at different time frames, i.e., 0H, 3H, 6H, 12H, and 24H, to see whether there were any effects on TNF-α protein released. Our results demonstrated a treatment effect of 700 µg/mL MDMA at 12H incubation and 500 µg/mL MDMA at 24H incubation on the TNF-α level (Figure

4). MDMA on BV2 microglial cells, and even little is

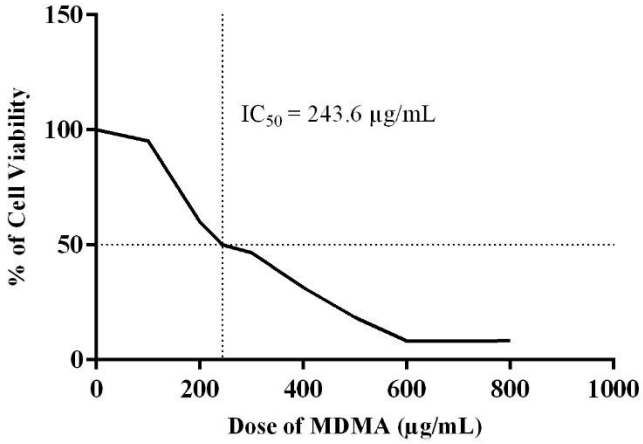

Figure 1. The IC_{50} value of MDMA in BV2 cells that were exposed to MDMA at the range of concentration 0-800 µg/mL and incubated for 24 hours. The IC_{50} value of MDMA was 243.6 µg/mL.

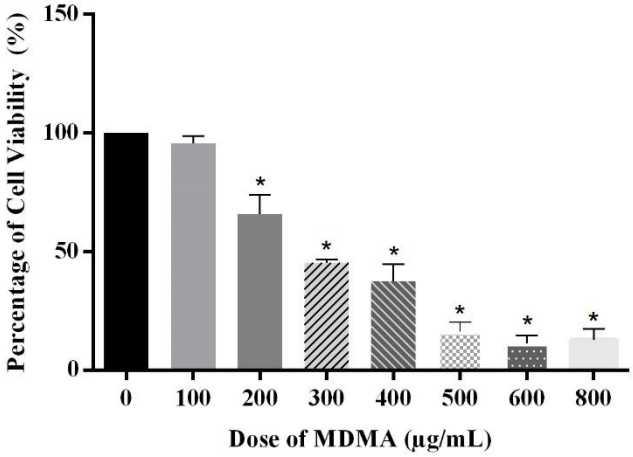

Figure 2. The effects of different doses of MDMA on the percentage (%) of cell viability (% of control) at 24 hours of incubation. Statistical analysis was carried out using one-way ANOVA followed by Tukey's Multiple Comparisons Test. * $P \leq 0.05$ vs Control Group. Data represent the mean ± SEM of three independent experiments.

Discussion

MDMA is administered systemically and may exert pharmacological effects on the function of the organs, mainly the brain. Many investigations consider how these effects originate within neurons, making it essential to explore the neuronal effects of MDMA due to the potential to contribute to neurotoxicity significantly. Moreover, elucidating these neuronal mediators of toxicity could reveal novel targets for treating the complex neurotoxicity produced by MDMA. There is far less known about the effects of known about its impact on the functions of the microglial cells. The purpose of this investigation was to assess the effects of MDMA on the viability of BV2 microglial cells and its activation through the changes in the cell's morphology and TNF-α production.

The present study found that the % of cell viability reduced with increased MDMA doses. It shows that MDMA is toxic to the BV2 microglial cells in a dose-dependent manner. MDMA seems toxic to the microglial cells at a dose of more than 300 µg/mL, in which 400 µg/mL to 800 µg/mL cause cell degeneration and apoptosis (Figure 2). However, it was demonstrated that 300 µg/mL of MDMA could

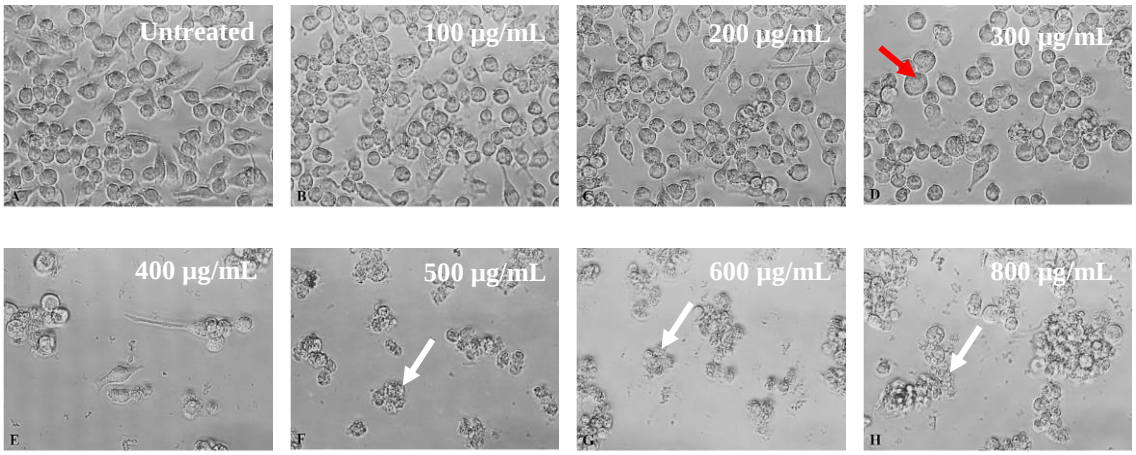

Figure 3. MDMA triggers the changes in BV2 cell morphology. Phase-contrast images showing the morphology of BV2 cells treated with different concentrations of MDMA for 24 h. Red arrows indicate the activated cell and white arrows indicate the degenerating cells.

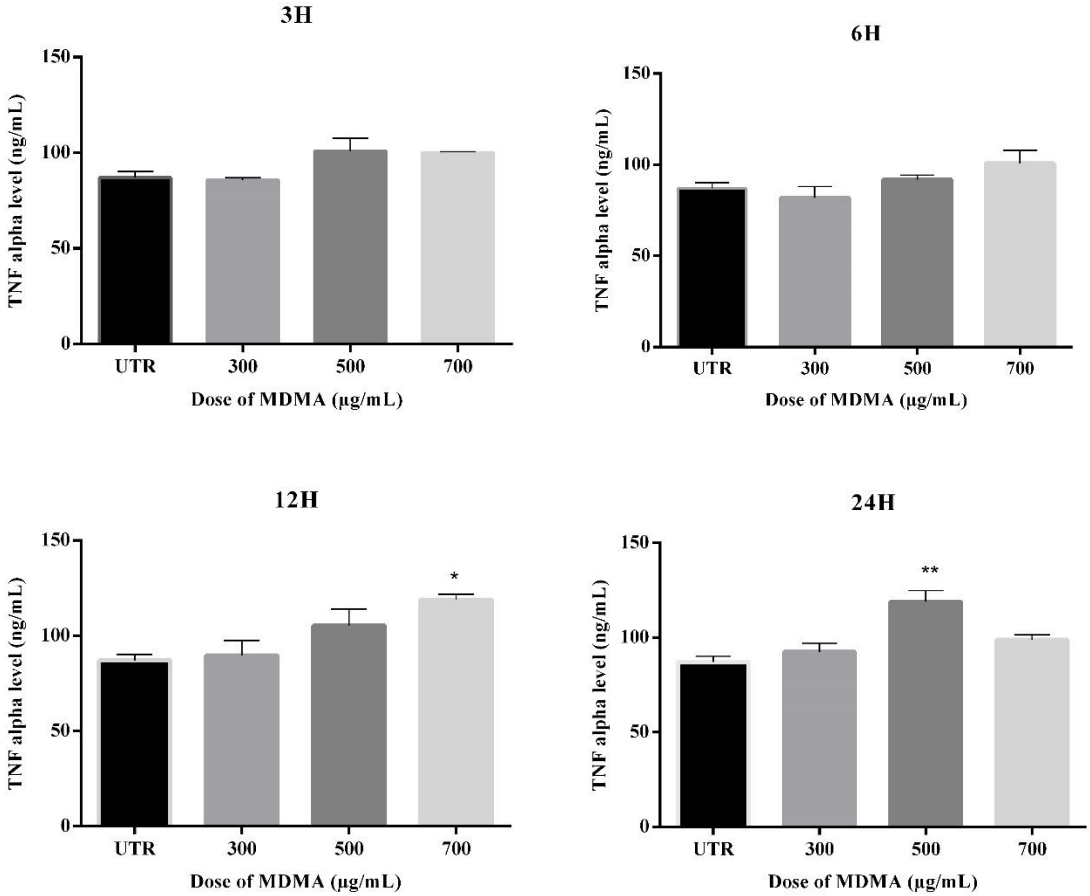

Figure 4. The effects of 300, 500, and 700 µg/mL MDMA on the TNF-α level at 3H-24H incubation. Statistical analysis was carried out using one-way ANOVA followed by Tukey's Multiple Comparisons Test. Data represent the mean ± SEM of three independent experiments.

activate the BV2 microglial cells, observed by its morphological changes via the less in the bipolar projections, and exhibited a large, rounded cytoplasm or amoeboid shape. According to Hoogland et al.

(2015), microglial cells are activated based on the changes in their morphology, a significant increase in the number and size of microglia compared to the control group, and a substantial increase in expression of a microglial marker. When all three criteria are negative, microglia are inactive. If one or more criteria are positive, microglia are activated. If results are contradictory (e.g., increased expression of the microglial marker but the morphology is negative), microglia are judged as moderately activated (11). In the early phase of acute neuroinflammatory response, the microglia also increase immediately due to the microglial activation program (12).

In this study, a dose of 300 µg/mL of MDMA incubated for 24h could activate microglial cells in vitro, as seen by the changes in their morphological features and cell viability. Evidence on the effects of MDMA on BV2 cells in-vitro is scarce and limited. The previous study has reported the results of Meth on microglial activation, a psychostimulant drug categorized under a similar group as MDMA. METH exposure has exerted immunomodulatory effects in the brain through the involvement of microglial cells by increasing the release of pro-inflammatory phenotypes and reducing anti-inflammatory phenotypes. In addition, previous studies have shown that microglial cell activation can be induced by LPS exposure at a dose of 1 µg/mL (13-15). Despite in-vitro, in vivo studies on the effects of MDMA on microglial activation were documented in several articles. For instance, MDMA produced a neuroinflammatory reaction in the rat brain by increasing the number of activated microglial cells and causing an increase in the specific parameters such as interleukin-1 beta (IL-1β), IL-1 receptor antagonist (IL-1ra), and the rise of CD11b and GFAP immunoreactivity (5,16-18). The doses of MDMA used to induce neurotoxicity in male rats are either a single dose of 12.5 mg/kg, through intraperitoneal injection (i.p), or 18-20 mg/kg subcutaneously (5,18,19). Therefore, the effects of MDMA on the microglial cell in vitro should be investigated to understand its mechanism, as it could be a preliminary finding for future studies on the potential of a therapeutic compound to treat MDMA neurotoxicity.

In terms of TNF-α, a critical activator of microglial activation (20), we found that BV2 cells could regulate toxicity and cell death at a dosage of 300 g/mL MDMA with no significant changes in TNF- levels as compared to untreated cells. Exposure to 700 µg/mL MDMA at 12h and 500 µg/mL at 24h significantly increased the TNF-α level. According to a study using primary rat and human neuronal cultures, interleukin-1b (IL-1b) and tumor necrosis factor-α (TNF-α) are the two pro-inflammatory cytokines that are commonly raised in neurodegenerative diseases that cause neuronal death and apoptosis in vitro (21). In a study on dopamine neuron degeneration and glial activation, MDMA increased TNF-α in caudate-putamen (CPU) of adult mice (22). Besides that, many studies conducted in vivo demonstrated the increase of cytokine production and other neurotoxic parameters after MDMA exposure, such as IL-1ra, IL-1RI, 5-HT, Cannabinoid CB2 Receptor, HRP-conjugated Isolectin B4 (ILB4), and glial fibrillary acidic protein (GFAP) response (17,18,23,24). Therefore, further studies should be conducted to evaluate genes or protein expression to understand the mechanism of MDMA neurotoxicity. The findings of this study can be the basis for future studies involving the mechanism of MDMA on neurotoxicity through microglial cells.

Conclusion

In summary, we discovered that MDMA promotes changes in the viability, morphology, and production of TNF-α in BV2 microglial cells in vitro. The changes in these parameters indicated the activation of the microglial cells after MDMA exposure. The BV2 microglial cell activation was noticed in their morphological changes after MDMA administration to the cells at ≥300 µg/mL. Meanwhile, the level of TNF-α protein released increased compared to the control group depending on the dose of MDMA exposure and incubation time. Further study should be done on the evaluation of other chemokines produced by BV2 microglial cells following MDMA exposure to understand the mechanism underlying the neurotoxic effects of MDMA.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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