EXPERIMENTAL STUDY

Effects of low-dose morphine suppress methamphetamine-induced cell death by inhibiting the ROS generation and caspase-3 activity

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ABSTRACT

OBJECTIVE: Methamphetamine in low doses can increase vigilance and power and at high doses has destructive effects that cause toxicity and death of various cell lines and affect the central nervous system. Morphine has also protective properties, which were observed in low concentrations, for nerve cells and also seem to have the ability to reduce cell death in neural cell lines.

MATERIALS AND METHODS: In this study, we used PC12 and U87 cell lines, which grew in DMEM culture media. Assays used in this study are listed below: MTT test for cell viability detection, LDH test for cytotoxicity measurement, caspase activity colorimetric assay kit (Bio-techne) for caspase 3 activity diagnosis, Rhodamine 123 for Detection of mitochondrial membrane potential. TUNNEL test for DNA fragmentation, fura-2 for Measurement of (Ca2+) ic and (Ca2+) m. fluorescence microscope for measurement of antioxidant enzyme activities.

RESULTS: morphine increased cell viability and the rhodamine-123 absorbance. It reduced cell cytotoxicity, caspase 3 activity, ic & m Ca2+ concentration, (.OH) generation, and DNA fragmentation in all concentrations of 1 pM t0 100 nM (p < 0.05) by optimal concentration of 1 pM.

CONCLUSION: morphine as a pain mediator can reduce the methamphetamine-induced cell death, may be due to its anti-inflammatory properties (Fig. 7, Ref. 52). Text in PDF www.elis.sk.

KEY WORDS: methamphetamine, morphine, apoptosis, cell death.

Introduction

Over the past decades, methamphetamine use has grown as an opiate in the world, slowly becoming a major global concern and involving 15 to 16 million people since 2007 (1, 2). Methamphetamine is known by the names of glass, crystal, ice, speed, and meth and it can be smoked, snorted, injected, swallowed, or inserted rectally (3, 4). Methamphetamine is inexpensive and easy to use, which is why it is rapidly expanding in the world. Methamphetamine secretes nerve mediators such as: dopamine, serotonin and norepinephrine, which leads to a sense of satisfaction, increased consciousness and increased energy in the users. Because of the half-life of 12 hours, these effects remain for many hours, more than cocaine, but long-term use leads to destructive effects on the central nervous system. Respiratory failure, myocardial problems, cardiomyopathy and increased risk of hepatitis and HIV viruses were observed in long-term amphetamine users (5–10).

Apoptotic genes expression analysis showed that methamphetamine can alter these gene expressions in the way of apoptosis. Furthermore, studies revealed that this drug by networking between mitochondrial, endoplasmic reticulum, and receptor-mediated apoptosis, can disrupt the striatal enkephalinergic neurons (11, 12). Methamphetamine involves in the JNK/SAPK-c-Jun Pathway, Mitochondrial Cell Death Pathway, Endoplasmic Reticulum (ER)-dependent Death Pathway and FasL/Fas Death Pathway (13–18).

Morphine as a member of the narcotic analgesics family affects the central nervous system and is used to treat pain. Morphine half-life is 1.5–7 hours and its products are available in oral form (tablets and capsules), injectable (intravenous, subcutaneous and muscular) and of course, it can also be inhaled (19–21). There is evidence that morphine in low dose can protect Oxidant-Induced Injuries and cell death in neuronal cell lines of human models by inhibition of glycogen synthase kinase-3β (GSK-3β). In this regard, many pathways are involved such as: phosphatidylinositol 3-kinase (PI3K), the target of rapamycin (TOR), JAK/STAT and the NO/cGMP/PKG pathway (19, 22–24).

On the other hand, morphine was introduced as an antioxidant due to the reduction of the ROS production (25). The inhibitory effects of morphine, in low dosages, at the disruptive effects of
methamphetamine on nerve cell lines have not yet been tested. Therefore, in this study, we intended to calculate the protective power of morphine on the U87, a human primary glioblastoma cell line (26) and also in the PC12 cell line, derived from a pheochromocytoma in adrenal medulla of the rat treated (27) with methamphetamine simultaneously. U87 is a common cell line that is used in many central nervous system studies. Therefore, we continued our studies on this cell line. On the other hand, methamphetamine causes cell death (28). Due to the opposite effects of methamphetamine and morphine on signaling pathways leading to apoptosis and ROS production, we suggested that by exposing different concentrations of morphine to a constant concentration of methamphetamine in the U87 and PC12 cell lines, we can see the protective effect of morphine on methamphetamine-induced cell death. By measurement of the cell cytotoxicity, cell viability, and apoptotic sings such as: intracellular and extracellular Ca\textsuperscript{2+} concentrations, we aimed to investigate the correctness of this issue.

Materials and methods

Cell culture

DMEM culture media (Gibco) was used for PC12 and U87 cell growth. 10 % fetal bovine serum (FBS, Gibco), 1 % non-essential amino acid (NEAA, Sigma), 2 mM L-glutamine (Sigma), 100 IU/ ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) were used as supplement in T-25 cm\textsuperscript{2} tissue culture flasks. The cultures were incubated at 37 °C in 5 % CO\textsubscript{2} medium and it was repeated once in two days. In the time of 70 to 80 % confluence, the cell cultures were trypsinated using trypsin-EDTA 0.25 % (Sigma) and were subcultured at a density of 1×10\textsuperscript{4} cells/well in 24-well culture plates.

Cell treatment

The PC12 and U87 cells were washed with PBS in pH 7.4, the day after plating the cells. There were seven treatments for PC12 cells and seven treatments for U87 cells by the same concentration of methamphetamine and morphine, including; control: culture medium, Treatment 1: 1mM methamphetamine, Treatment 2: 1 mM methamphetamine/1 pM morphine, Treatment 3: 1 mM methamphetamine/10 pM morphine, Treatment 4: 1 mM methamphetamine/100 pM morphine, Treatment 5: 1 mM methamphetamine/1 nM morphine, Treatment 6: 1 mM methamphetamine/10 nM morphine, and Treatment 7: 1 mM methamphetamine/100 nM morphine. Then, the cells were placed in the incubator at 37 °C with 5 % CO\textsubscript{2}. The cells were cultured in DMEM culture medium containing 0.2 % BSA.

Cell viability (%) measurement (MTT assay)

In this study, the cell viability was quantified by MTT assay. In this regard, 15×10\textsuperscript{4} cells were loaded into a 96-well plate and 200 μL of DMEM media, which contains 0.2 % BSA was added. After 24h incubation, 200 μL of each treatment media was added to the wells. The cells were separately incubated with different treatment media for 24 hours.

Cell cytotoxicity measurement

Cell cytotoxicity was quantified in this study by LDH Cytotoxicity Detection Kit (Roche, Germany).

Caspase-3 assay

PC12 and U87 cells were cultured in the different treatment media condition. The caspase activity colorimetric assay kit (Bio-techne) was used for the measurement of treated cells lysates caspase-3 activity according to the manufacturer’s protocol and using a plate reader.

Detection of mitochondrial membrane potential (MMP)

MMP was measured using the cell permeable cationic fluorescence probe rhodamine 123, for quantitative analysis. In summary; PC12 and U87 cells 3×10\textsuperscript{4} cells/well were cultured and treated in different treatment media. Next, the cells were washed with PBS and incubated by 1 μM rhodamine 123 for 30 min at 37 °C in the dark. Then, an ELISA Reader was used for measurement of cells absorbance at 488 excitation and 525 nm emission. The reference wavelength was more than 630nm. All the experiments were replicated independently at least three times. Within each experiment, we replicated each condition four times.

Fig. 1. The effects of different treatments on the cell viability of the U87 and PC12 cells. A: For U87. B: for PC12 cell cultures. All data represented by the mean ± S.E.M (p < 0.05).
Quantification of apoptosis incidence

Fixation for all cells in this study was performed by 4 % w/v paraformaldehyde in PBS with pH = 7.4 for 10 min at room temperature. For identification of the apoptotic cells by TUNEL (Terminal Uridine deoxynucleotidyl transferase dUTP Nick End Labeling) staining, we used an in situ cell death detection kit (Roche), based on manufacturer’s protocol.

**Measurement of (Ca²⁺) ic and (Ca²⁺) m**

Measurement of intracellular (Ca²⁺) ic and mitochondrial (Ca²⁺) m calcium concentration was carried out on the base of previous studies (29).

(Ca²⁺) ic values were calculated using the equation described by Grynkiewicz (30). Relative (Ca²⁺) m was measured with the fluorescent probe Rhod 2-AM following methods described previously (31).

**Measurement of antioxidant enzyme activities**

Antioxidant enzyme activities and protein damage assay were carried out on the base of previous studies (32). Briefly, in order to visualize intracellular ROS, cells were incubated with treatment media for 24 h, and then washed three times with Krebs–Ringer–Hepes (KRH) buffer, and cells were incubated for 1 h at 37 °C. Fluorescence (Ex. 490 nm and Em. 525 nm) was visualized using a fluorescence microscope.

**Results**

**Cell culture**

**Cell Viability (%)**

Different concentrations of morphine and constant methamphetamine concentration was added to the PC12 and U87 cell cultures, so, after 24 h, MTT assay was used for the cell viability measurement. Control treatments showed 99 % of cell viability as the result in both cell lines. In treatment 1, 1 mM of methamphetamine caused all the cells to die, so 0 % of cell viability was clear in both treatments. Results showed that exposure of the cells to the 2–7 treatment media decreased the cell viability of these treatments compared to the control cells (p < 0.05). The percentage of cell viability were increased in 2–7 treatments compared to the treatment 1, respectively (p < 0.05). The lowest and highest cell viability was for treatment 1 (0 % for both cell lines) and treatment 2, respectively (Fig. 1) (p < 0.05).
Cell cytotoxicity (%)

PC12 and U87 cells were exposed to different concentrations of morphine and constant concentration of methamphetamine (1 mM), so, after 24 h, the cell cytotoxicity was measured by LDH assay. Control treatments, showed us 2% of cell cytotoxicity as the result in both cell lines. In treatment 1, 1 mM of methamphetamine caused all the cells to die, so the cytotoxicity 100% was clear in both treatments. The results showed that exposure of the cells to 2–7 treatment media increased the cell cytotoxicity of these treatments compared to the control cells (p < 0.05). The percentage of cell cytotoxicity was decreased in 2–7 treatments compared to treatments-1 in both cell lines (p < 0.05). In both cell lines, the lowest cell cytotoxicity was in treatment 2 and the highest cell cytotoxicity was in treatment 1, respectively (Fig. 2) (p < 0.05).

Cell death index

The cells were exposed to different concentrations of morphine and constant concentration of methamphetamine, so after 24 h, cell death was measured by TUNEL assay. Control treatments showed us 1% of cell death as the result for PC12 and U87 cells. In treatment 1, all of the cells died because of methamphetamine and the percentage of cell death was 100%. The results showed that exposure of the cells to 2–7 treatment media caused cell death increases compared to the control cells, respectively (p < 0.05). The percentage of cell death decreased in 2–7 treatments compared with treatments 1, but as concentration increased the protective potential of morphine decreased (p < 0.05). The results confirmed similarity in both cell lines (Fig. 3).

Caspase-3 assay

In most cases, apoptosis eventually mediates a common pathway through the result obtained in case of caspase-3 activation. Furthermore, results showed that Caspase3 activation after 24h in treatments 2–7 was increased compared to the control treatments (p < 0.05). The caspase3 activation in control treatments of PC12 and U87 cells was lower than in other treatments (treatments 1–7) (p < 0.05). Caspase-3 activation in treatments 2–7 were lower compared to the treatment 1, as morphine concentration increased its protective potential decreased in both cell lines (Fig. 4) (p < 0.05).
Mitochondrial membrane potential (Rhodamine-123 absorbance)

Through the apoptosis processes, in most cases caspase-3 activation leads to mitochondrial membrane potential ($\Delta \varphi_m$) change, that eventually mediate a common pathway of cell death, which is named apoptosis.

To checking this $\Delta \varphi_m$ change in the treated cells, both cell lines were exposed to different treatment media, after 24 h, $\Delta \varphi_m$ was measured by Rhodamine-123 staining and colorimetry assay. Furthermore, RH-123 absorption in all treatments after 24 h was decreased compared to the control treatment in PC12 and U87 cells ($p < 0.05$). The RH-123 absorption in control cells was higher than other treatments (treatments 1-7) ($p < 0.05$). RH-123 absorption in treatments 2-7 were higher compared to treatment 1, as morphine concentration increased its protective potential decreases ($p < 0.05$). The results were similar for both cell types (Fig. 5).

($Ca^{2+}$) ic and ($Ca^{2+}$) m

Exposure of PC12 and U87 cells to different media had specific and obvious effect on ($Ca^{2+}$) ic and ($Ca^{2+}$) m. In 2–7 treatments, the ($Ca^{2+}$) ic were increased in comparison with the control groups in both cell lines ($p < 0.05$) (Fig. 6). It seems that $Ca^{2+}$ might have accumulated in mitochondria, because morphine decreased concentration of ($Ca^{2+}$) ic in 2–7 treatments. So, we evaluated changes in ($Ca^{2+}$) m in cells loaded with the mitochondrial $Ca^{2+}$ indicator by microscope. After comparison of the treatment inhibitors with treatment 1, a significant decrease in ($Ca^{2+}$) m was observed both in the PC12 and the U87 cells (Fig. 6).

Measurement of antioxidant enzyme activities

Results revealed that the exposure of PC12 and U87 cells to different treatment media had a clear effect on ROS (.OH) generation. The (.OH) generation in treatments 2–7 was increased compared to the control cells ($p < 0.05$) and it was decreased in treatments 2–7 compared to the treatment 1 ($p < 0.05$). It was clear that overload of intracellular and mitochondrial $Ca^{2+}$ caused enhanced accumulation and cytochrome c release in ROS pathway in treatment 1 cells, and this event was reverse in 2–7 treatments in compared to the treatment 1. These observations occurred in both cell types (Fig. 7).

Discussion

The abuse of methamphetamine over the last few decades has become a major and growing global dilemma, like cocaine abuse.
cell shrinkage, while cell death due to cellular swelling and membrane fracture is called necrosis. Methamphetamine induces apoptosis in dopaminergic and serotonergic neurons of mesencephalic and cortex cell culture. Also, in the PC12 cell line, derived from a pheochromocytoma in adrenal medulla of the rat, methamphetamine causes non-apoptotic cell death (48, 49).

Extra experiments showed that morphine can reduce tert-butyl hydroperoxide destructive effects on H9c2 cells (rat cardiomyoblast) and have positive effects on cell viability. Morphine can increase total antioxidant capacity of H9c2 cells, can reduce the ROS production, protein carbonylation, and lipid peroxidation (19).

In this study, we tried to investigate the protective effects of morphine on cell cytotoxicity, low viability and apoptotic behaviors of PC12 and U87 cell lines culture treated by methamphetamine. It has been shown previously that methamphetamine increases the cell cytotoxicity and apoptosis and reduces cell viability of neuronal cell culture (50). The PC12 as a rat model and U87 as a human nerve cell line have been widely used to study the molecular mechanisms of neuronal cell death (51, 52). In this way, we have affected the different concentrations of morphine from 1 pM to 100 μM at a constant concentration of methamphetamine in pc12 cells to the achievement of best methamphetamine/morphine ratio to reduce the harmful effects of methamphetamine by concomitant use of methamphetamine and morphine. In both cell lines, morphine reduced cell cytotoxicity and increased cell viability in a dose-dependent manner, so that its optimum concentration was 1 pM. This indicates that morphine has the ability to reduce inflammation and apoptosis, especially at low concentrations. On the other hand, our results showed that morphine could reduce (.OH) production. In this experiment, the optimum concentration was also 1 pM. In low concentrations, morphine can reduce (.OH) production, which reduces the production of inflammatory cytokines such as: IL-1, IL-6, IL-10 and TNFα, thus reducing inflammation, and therefore reducing apoptosis. Morphine at low concentrations increases the cAPM’s concentration. This increase in concentration leads to the activation of the Erk 1 & 4 and the Erk 2 & 3 paths. These pathways are associated with the reduction of apoptosis. As the result, morphine reduces inflammation and increased differentiation, leading to a reduction in methamphetamine-induced cell death in PC12 and U87 cell lines in a dose-dependent manner. Our study showed that morphine can reduce intracellular and mitochondrial Ca2+. It prevents mitochondrial membrane destruction as the result, so the cytochrome c will not enter the cytosol as an apoptotic signal and will not activate the mitochondrial apoptotic pathway. Further, we used rhodamine 123 for monitoring of mitochondrial inner membrane electron potential as a marker of mitochondrial function. Afterwards, TUNNEL test was performed for DNA fragmentation detection. Our results, like previous results, showed that morphine in a dose-dependent manner, by 1 pM optimal concentration, prevents disruption of the mitochondrial inner membrane and DNA fragmentation. The study of caspase 3 activity was consistent with previous information and argued that caspase 3 activity as an effector caspase in the pathway of apoptosis decreases in lower concentrations of morphine. As the result of this study, we can say that morphine has a maximum effect on the reduction of methamphetamine-induced cell death in the opti-

![Fig. 7. The antioxidants and reduce agents of endogenous reactive oxygen species (ROS) production in treated cells of U87 and PC12. A: .OH generation in U87 cells. B: .OH Generation in PC12 cells. All data represented by the mean ± S.E.M (p < 0.05).](image-url)
nal concentration of 1 pM, which indicates the anti-inflammatory properties of morphine in the neural cells and the differentiation role of this substance in very low doses. On the other hand, as morphine concentration increases, its anti-inflammatory and anti-apoptotic effects decrease, so that in higher concentrations it can reduce cAMP and increase inflammation and apoptosis.

Conclusion

Finally, we can say that low levels of morphine have anti-inflammatory, anti-apoptotic and neuroprotective properties. It can be therefore used to treat inflammatory and neurogenic diseases. It also reduces the effects of methamphetamine abuse. However, at high concentrations, the opposite and destructive effects of morphine are apparent.

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Received February 16, 2019.
Accepted March 8, 2019.