RESEARCH ARTICLE

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Lovastatin Suppresses Morphine-Induced Inflammation and Cell Death in Pheochromocytoma-Like Neural Cells (PC12)

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Abstract

Background: Morphine has been shown to induce programmed cell death through the opioid μ (mu) receptor. It is shown statin has an anti-inflammatory, antioxidant and neuroprotective activities. In the present study, the effects of nanomollar concentration of lovastatin on cell death following the effect of morphine were investigated. **Methods:** PC12 cells were cultured in DMEM culture medium. The cell viability was measured by the MTT assay and LDH assay. The amount of nitric oxide produced was measured using Griess technique method. Concentrations of IL-1β, IL-6, IFNγ, and TNFα were measured by the ELISA method. **Results:** Lovastatin, in a dose-dependent manner, increased cell viability and suppressed cytotoxicity and cell death. It also decreased levels of IL-1β, IL-6, IFNγ, and TNFα as inflammatory factors compared with morphine-treated cells. **Conclusion:** lovastatin plays a supporting role against the destructive and cell death effects of morphine by reducing the inflammatory factors.

Keywords: Lovastatin- morphine- inflammation- cell death- PC12

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Introduction

Opioids by binding to their receptors and use for relieving acute and chronic pain [1, 2]. Long-term use of opioid compounds includes side effects such as intolerance, dependence, and ultimately addiction to these drugs [3-5]. It leads to an increase the inflammatory cytokines, NO, [6]. Other detrimental effects of drug abuse of this drugs include oxidative stress, apoptosis, and neuronal inhibition [7, 8]. Morphine is one of the 50 alkaloids in opium and one of the strongest analgesic opioid compounds.The sedative effect as well as the dangers associated with high doses are known for it [9]. Morphine has been shown to induce programmed cell death through opioid blockers [10, 11]. Morphine has also been reported to increase the expression of inflammatory agents, causing damage to nerve cells in the brain and, consequently, clinical disorders [12]. Morphine is known to induce cell death in neurons by increasing the mechanism of apoptosis in hippocampal cells [13, 14].

Statins include 6 types: atheostatin, fluvastatin, lovastatin, prostatin, rovastatin and simvastatin that lower blood cholesterol [15]. The liver is the major site of primary metabolism and the activity of statins and major organ in the synthesis of primary cholesterol [16]. In addition to inhibiting the cholesterol synthesis, statins decrease plasma cholesterol levels by increasing the LDL receptor [17]. Studies have shown that statins also have anti-inflammatory, antioxidant and neuroprotective activities [18]. Therefore, these drugs may be used in disorders such as cancer, diabetes, Alzheimer's, osteoporosis, depression and several other diseases. Previous studies have shown that statins reduce cell death in neurons [19, 20]. they can decrease the IL-6 and TNFα levels in MS patients [21]. Previous studies have been shown that The statins lead to suppress the apoptosis and reduce the nitric oxide levels by reducing the expression of nitric oxide synthase (iNOS) [22, 23]. In another study, it has been shown that the atorvastatin can reduce caspases-3 and ultimately reducing brain damage [24]. Lovastatin, as a member of the statin drug, is routinely prescribed to treat hypercholesterolemiaand by regulating the anti-apoptotic mechanism, reduces hippocampal cell death [25-27].

The present study investigates the the protective effect of lovastatin on Morphine induced cell death in PC12 cells by controlling the secretion of proinflammatory cytokines.

Materials and Methods

Cell culture

Culture medium DMEM (Gibco) including, PC12 cells, 10% fetal calf serum (FCS, Gibco), 100IU / ml penicillin (sigma) and 100 μg / ml streptomycin (sigma) in T-25 cm^2 culture flask, incubated at standard conditions (37 ºC in 5% CO_2 medium). The day after cell culture, we used PBS (pH 7.4 and 37°C) for washing. Next seven treatments were selected for morphine including; Negative control group: including culture medium, positive control

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group: Includes cell culture medium containing 1 mM morphine. Treatment 1: cell culture medium containing 1mM morphine and 10 nM lovastatin, Treatment 2: cell culture medium containing 1mM morphine and 50 nM lovastatin, Treatment 3: cell culture medium containing 1mM morphine and 100 nM lovastatin, Treatment 4: cell culture medium containing 1mM morphine and 200 nM lovastatin, Treatment 5: cell culture medium containing 1mM morphine and 500 nM lovastatin, Treatment 6: cell culture medium containing 1mM morphine and 1 µMlovastatin, and Treatment 7: cell culture medium containing 1mM morphine and 10 µMlovastatin. In the following, the cells were incubated at 37°C with 5 % CO_2.

Cell viability (%) measurement (MTT Assessment)

To quantify cell viability, MTT assay was performed as shown below: at first 15×103 cells were cultured to a 96-well cell culture plates with different treatments media for 24 hours. Next , we added 5 mg/mL of MTT solution to each well by incubation for 3 hours. The supernatant was drained from each well again and 100 µL of dimethyl sulfoxide (Sigma) It was added at 37 °C for 30 minutes. For measurement of the optical density of each well, we used a ELISA reader (EL800; USA) at 570 and 630 nm. This assessment was repeated 3 times.

Cell cytotoxicity measurement

Next, we measured cell cytotoxicity by LDH Cytoxicity Detection Kit (Roche, Germany). Further, 1×104 cells/mL densities for 12h were plated in 24 well culture plates, The cells were then cultured in different treatment media for 24 hours. After 24h, LDH activity's colorimetery was measured by calculation of samples absorbance at 490 or 492 nm using an ELISA Reader. This assessment was repeated 3 times.

Nitric oxide test

In this study, we used the Griess reaction for measurement of NO production amount in different treatments, after 48h [28]. This assessment was repeated 3 times.

Production of proinflammatory cytokines

After 24 hours, the rat inflammatory cytokine kit,) V-Plex rat kit(, was used to measure proinflammatory cytokines IL-1β, IL-6, INFγ and TNFα in different therapeutic environments. This assessment was repeated 3 times.

Statistical analysis of results

Data were expressed as Mean ± SEM. All calculations were performed by SPSS (version 21; SPSS Inc.). The statistical analyzes were performed using ANOVA and T-tests. In all cases, the P value of less than 0.05 was considered statistically significant.

Results

Cell viability%

After exposure to different concentrations of lovastatin and high concentrations of morphine for 24 hours, cell viability was measured using the MTT method. As shown in (Figure 1), Cell viability was shown to be about 99% in controls treatment. The results showed that cell viability in treatment 1-7 gradually increased compared with morphine treatment and decreased compared with control treatment ($p < 0.05$).Treatments 1 and 5 had the lowest and highest Cell viability, respectively ($p < 0.05$).

Cell cytotoxicity (%)

LDH assay was used for cell cytotoxicity measurement 24 h after exposure of different concentrations of lovastatin and high-concentration of morphine. Complete cytotoxicity was observed, with full loss of cell viability in morphine treatment. In control treatment the percentage of cell cytotoxicity was 2% . The results showed that the

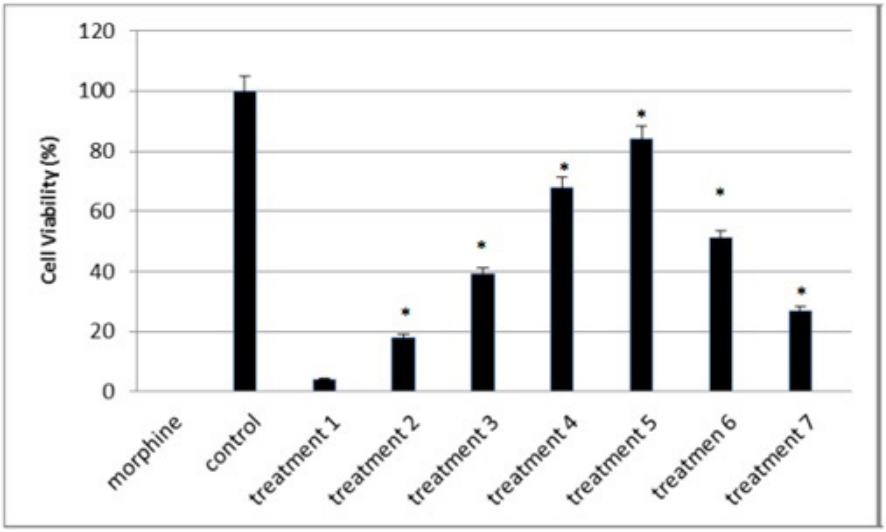
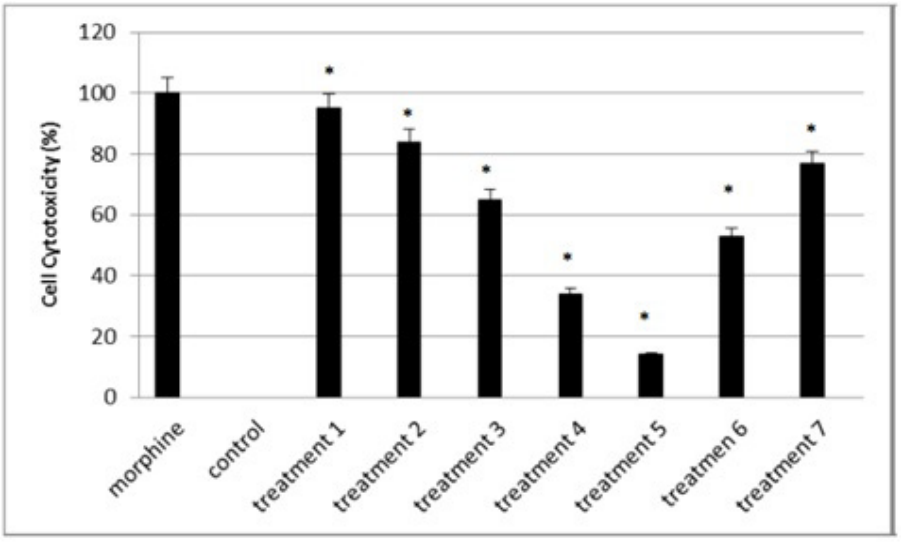


Figure 1. Dose-Dependent Effects of Different Treatments on PC12 Cell Viability. Treatment 1: 1mM morphine and 10 nM lovastatin, Treatment 2: 1mM morphine and 50 nM lovastatin, Treatment 3: 1mM morphine and 100 nM lovastatin, Treatment 4: 1mM morphine and 200 nM lovastatin, Treatment 5: 1mM morphine and 500 nM lovastatin, Treatment 6: 1mM morphine and 1 µMlovastatin, and Treatment 7: 1mM morphine and 10 µMlovastatin. All data represented as the mean ± SEM ($p < 0.05$).



Figue 2. Dose-Dependent Effects of Different Treatments on PC12 Cell Cytotoxicity: 1mM morphine. Treatment 1: 1mM morphine and 10 nM lovastatin, Treatment 2: 1mM morphine and 50 nM lovastatin, Treatment 3: 1mM morphine and 100 nM lovastatin, Treatment 4: 1mM morphine and 200 nM lovastatin, Treatment 5: 1mM morphine and 500 nM lovastatin, Treatment 6: 1mM morphine and 1 µMlovastatin, and Treatment 7: 1mM morphine and 10 µMlovastatin. All data represented as the mean ± SEM ($p < 0.05$

percentage of cell cytotoxicity in the treatments1-7 was significantly reduced compared with morphine treatment ($p <0.05$). Morphine and treatment 5 had the highest and lowest the percentage of cell cytotoxicity, respectively (Figure 2). ($p < 0.05$).

Nitric oxide concentration

NO assay was used for cell cytotoxicity measurement 24 h after exposure of different concentrations of lovastatin and 1mM morphine. Nitric oxide concentration was 193 µl/ml for treatment . The control treatment showed 45 µl/ml of Nitric oxide concentration as shown in Figure 3. It was indicated that the concentration of nitric oxide in the cells in treatments1-7 was significantly reduced compared with morphine treatment ($p <0.05$). Morphine treatment and treatment 5 had the highest and lowest levels of nitric oxide, respectively ($p < 0.05$).

Evaluating Cytokines with ELISA

IL-1β, IL-6, INFγ and TNFα levels were measured using ELISA to identify the role of morphine in inflammation and the protective effect of lovastatin using specific antibodies. morphine lead to increased the Cytokine IL-1β, IL-6, INFγ and TNFα levels compared with control group ($p<0.05$). The findings showed The level of cytokines in the treatments2-6 was significantly

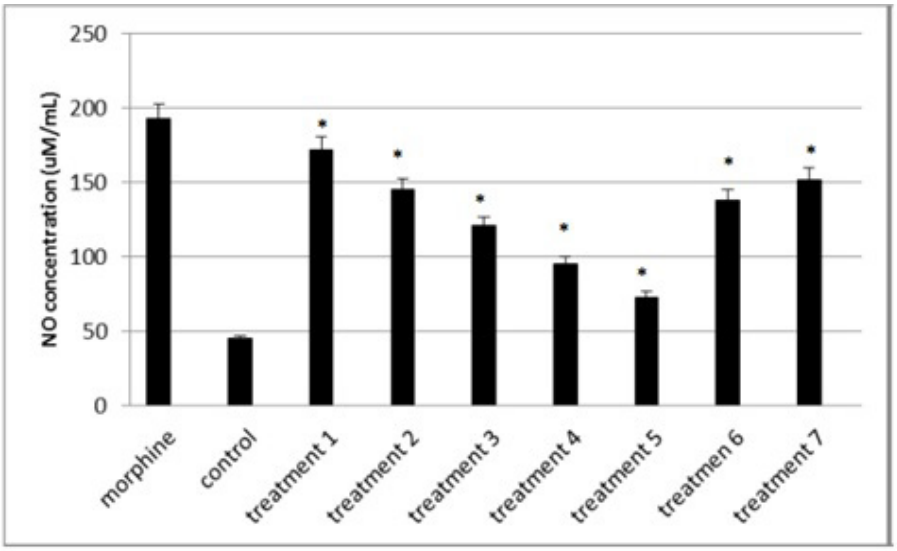


Figure 3. Dose-Dependent Effects of Different Treatmentson Nitric Oxide Concentration. Control: culture medium, Positive control of morphine: 1mM morphine .Treatment 1: 1mM morphine and 10 nM lovastatin, Treatment 2: 1mM morphine and 50 nM lovastatin, Treatment 3: 1mM morphine and 100 nM lovastatin, Treatment 4: 1mM morphine and 200 nM lovastatin, Treatment 5: 1mM morphine and 500 nM lovastatin, Treatment 6: 1mM morphine and 1 µMlovastatin, and Treatment 7: 1mM morphine and 10 µMlovastatin. All data represented as the mean ± SEM ($p < 0.05$)

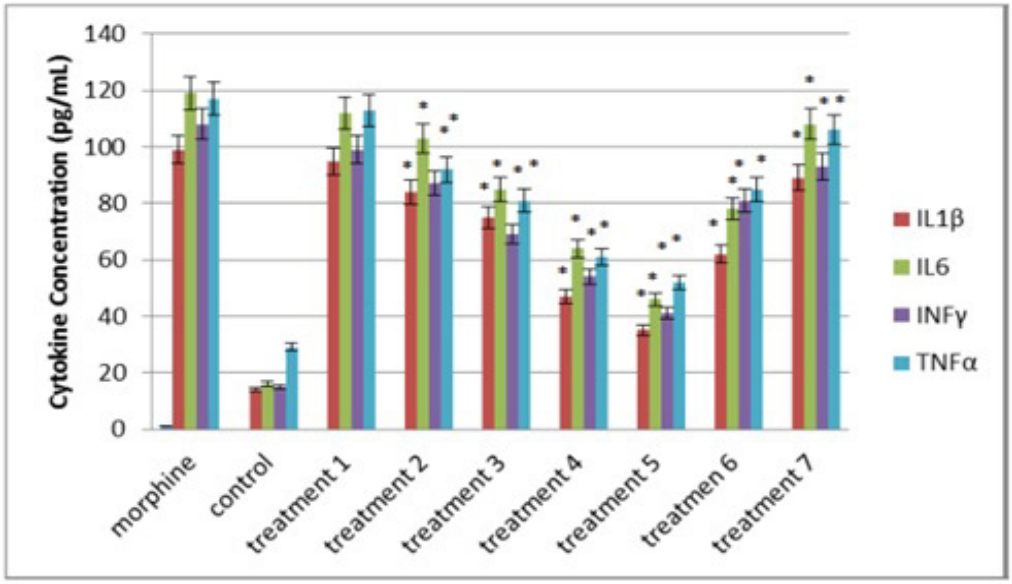


Figure 4. Dose-Dependent Effects of Different Treatments on the Level of Cytokines. Control: culture medium, Positive control of morphine: 1mM morphine .Treatment 1: 1mM morphine and 10 nM lovastatin, Treatment 2: 1mM morphine and 50 nM lovastatin, Treatment 3: 1mM morphine and 100 nM lovastatin, Treatment 4: 1mM morphine and 200 nM lovastatin, Treatment 5: 1mM morphine and 500 nM lovastatin, Treatment 6: 1mM morphine and 1 µMlovastatin, and Treatment 7: 1mM morphine and 10 µMlovastatin. All data represented as the mean ± SEM ($p < 0.05$)

reduced compared with the morphine treatment ($p < 0.05$) (Figure 4).

Discussion

In this study, the dose-dependent effect of lovastatin on morphine-induced cellular damage in PC12 cells was measured. Brain cells have a limited ability to proliferate, so increasing inflammation in neurological disorders is very important, and a drug that can prevent apoptosis and inflammation has a positive therapeutic potential against these disorders. Some previous studies suggested morphine can inhibit cell growth by inhibiting cell cycle and induce apoptosis in MCF-7 cells. [29]. It has been shown that morphine tolerance stimulates the mechanism of neuroinflammation [30]. Another study shows that apoptosis in hippocampal cells increases before and after morphine treatment, leading to cell death [31]. Atorvastatin has also been shown to inhibit homocysteine-induced apoptosis in endothelial progenitor cells, which has been linked to its role in suppressing oxidative stress and down-regulating the p38mapk / caspase-3 signaling pathway [32]. Statins reduce cell death in neurons and have a protective effect [19, 33].

Our findings showed that morphine increases cell cytotoxicity, cell death in the PC12 cell line and the Lovastatin reduces the destructive effect of morphine by increasing cell survival, suppressed cell cytotoxicity in the PC12.

Studies show that chronic use of morphine increases cytokine expression Inflammation such as IL6 and TNF-α and increased activity of astrocytes and microglia [34, 35]. It has been shown that morphine tolerance stimulates the mechanism of neuroinflammation [30]. Administration of morphine induces the production of proinflammatory cytokines (TNF-α, IL-1β and IL-6 (in nerve tissues [12]. Preoperative statin therapy reduces pro-inflammatory IL-6 and regulates anti-inflammatory IL-10 after cardiac surgery with cardioplegia [36]. Simvastatin selectively reduces the expression of IL-1β and inhibits the activation of microglia cells and astrocytes after TBI, indicating the anti-inflammatory role of statins [21].

It has also been shown that administration of lovastatin in rats with experimental TBI significantly reduced IL-1β and TNF-α in areas of brain injury, suggesting that the protective mechanisms of lovastatin may reduce the inflammatory response [37]. Another studies demonstrated the anti-inflammatory role of lovastatin [38, 39]. In this study it was shown that morphine increases the level of inflammatory cytokines and lovastatin reduces the level of inflammatory factors in PC12 cells. morphine-induced progressive increased malondialdehyde (MDA) level and nitric oxide (NO) in brain [40]. Other studies have also shown that morphine consumption leads to neurodegradation by activating TRPM2 and overproduction of NO [41, 42].

Nitric oxide increases glutamate release from presynaptic neurons, and elevated glutamate levels cause toxicity to nerve cellsAtorvastatin mediates the analgesic tolerance of morphine at least in part through nitric oxide in animal pain models of hot plate and tail pain flick. [43]. Lovastatin has also been shown to reduce Nitric oxide surface By reducing the expression of nitric oxide synthase (iNOS) in stimulated lipopolysaccharide (LPS) -stimulated RAW264.7 macrophage cells [22]. In the most recent study, the combined effect of lovastatin with metformin or glyclazide was significantly better in serum levels of AST, ALP, TNF-α, and hepatic TBARS, GSH, GST, SOD, and NOx than either drug [44]. Thus, these data suggest that lovastatin reduces inflammation by

reducing NO levels. In our study it was also shown that morphine increases the level of nitric oxide in PC12 cell and lovastatin inhibits the destructive effects of morphine by decreasing the level of nitric oxide in PC12 cell.

In general, this study showed that lovastatin plays a protective role against the destructive effects of morphine on cell death and cytotoxicity by by increasing cell survival and reducing inflammatory factors and the process of apoptosis.

Author Contribution Statement

Hossein Zhaleh and Ali-Akbar Rahimi conceived the study. Hossein Zhaleh and Ali-Akbar Rahimi developed the theoretical framework and performed the experiments. Hossein Zhaleh aided in the analysis. Hossein Zhaleh supervised the project. All authors discussed the results and contributed to the final manuscript.

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Funding Statement

This study is approved by the scientific committee of the Science and Research Branch, Islamic Azad University, Tabriz and is part of Mr. Ali-Akbar Rahimi's thesis in collaboration with Dr. Hossein Zhaleh at the Department of Biology, Faculty of Sciences, Science and Research Branch, Islamic Azad University, Tabriz, Iran.

Availability of data and materials

All data generated during this study are included in this published article. Further data supporting the findings of the current article are available from the corresponding author (Dr. Hossein Zhaleh, Email: h.zhaleh @basu.ac.ir) on reasonable request.

Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

Abbreviations

PC12: pheochromocytomacell-12; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH: Lactate Dehydrogenase; IL-1β: Interleukin-1β; IL-6: Interleukin-6; INFγ:Interferon gamma; NO: nitric oxide; TNF-α: Tumor necrosis factor; FCS: Fetal Calf Serum; DMEM: Dulbecco's Modified *Eagle Medium.*

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