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Experimental Research Article

Insulin and SB-216763, a GSK3β inhibitor, reduce methamphetamine toxicity in human neuroblastoma and rat primary midbrain cells

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ABSTRACT

Introduction: Methamphetamine (MA) induces cell death through several mechanisms. Insulin has an important role in cell proliferation and apoptosis via GSK3β inactivation. Here, we evaluated the effect of insulin and SB-216763, a selective GSK3β inhibitor, on MA-induced cell death in neuroblastoma SH-SY5Y, and rat primary midbrain cells.

Methods: Human SH-SY5Y and rat primary midbrain cells extracted from E14.5 rat embryo were treated with insulin (0.005-0.15U) or SB-216763 (0.5-9μM) with or without MA (5mM). The cell viabil-ity was assessed after 24, 48, and 72 h. TNFα, Bax, Bim, and Bcl2 genes expression were exam-ined in primary midbrain cells after 72 h of treatment with 5mM MA, insulin (0.05U), and SB-216763 (3μM).

Results: MA significantly decreased the viability of human SH-SY5Y and rat primary midbrain cells, and insulin and SB-216763 could increase it. In addition, elevated expression of TNFα and Bax fol-lowing MA was attenuated by insulin and SB-216763 in primary midbrain cells.

Conclusion: These findings demonstrated that MA decreases the cell viability of rat primary midbrain cells, at least in part, by upregulation of inflammatory and apoptotic factors, and treatment with insulin and SB-216763 could attenuate MA toxicity.

Keywords:
Methamphetamine
Insulin
SB-216763
Human neuroblastoma SH-SY5Y cells

Introduction

Methamphetamine (MA) can induce long-term deficits in monoaminergic, especially dopaminergic, systems. Reduction in dopaminergic neuron markers and degeneration of neuronal and glial cells in various brain regions have been shown in animal models and humans following MA administration (Ares-Santos et al., 2014; Ferrucci et al., 2022; Kousik et al., 2014). MA-induced neurotoxic effects are mediated through several neurotoxic mechanisms like, neuroinflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress (Marshall and O'Dell 2012). Several in-vitro studies

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also reported MA-induced cell death in M213 (Deng et al., 2002), N27 (Kanthasamy et al., 2006; Kanthasamy et al., 2011; Lin et al., 2012) and PC12 cells (Abbasi et al., 2022; Iravanpour et al., 2021; Mirakabad et al., 2021; Wu et al., 2015) cell lines, and also using primary striatal (Liao et al., 2021) and midbrain dopaminergic neurons (Valian et al., 2019). MA could induce neuronal cell death through several mechanisms such as the JNK pathway, caspase-3 activation, apoptosis, autophagy inhibition, neuroinflammation, mitochondrial dysfunction, and oxidative stress (Iravanpour et al., 2021; Pitaksalee et al., 2015; Wang et al., 2008). Insulin, for many years, has been considered a hormone that cannot cross blood blood-brain barrier. Nowadays, there is evidence that insulin is synthesized in the neurons and plays critical roles in brain functions, including regulation of cell proliferation, apoptosis, and neurodegeneration (Chen et al., 2022; Duarte et al., 2012). Insulin receptors exist in the cortex, hippocampus, hypothalamus, olfactory bulb, cerebellum, midbrain, and striatum (Figlewicz et al., 2003; Pomytkin and Pinelis 2021). Insulin binding to its tyrosine kinase receptor recruits the insulin receptor substrates (IRSs), activates the PI3K/Akt pathway, and inactivates glycogen synthase kinase 3β (GSK3β), finally resulting in apoptotic suppression and neuronal survival (Ghasemi et al., 2013). The anatomical overlapping of insulin and dopamine receptors reflects the importance of insulin signaling in the survival and functions of dopaminergic neurons (Figlewicz et al., 2003). Insulin protection has also been reported in an animal model of Parkinson's disease (PD) against motor impairments and dopaminergic neuron loss induced by 6-hydroxy dopamine (6-OHDA) (Chen et al., 2022; Iravanpour et al., 2021; Pang et al., 2016). Insulin signaling disruption causes neuronal dysfunction, especially in dopaminergic neurons in the CNS (Duarte et al., 2012).

GSK3β, a serine/threonine kinase, has critical roles in the regulation of metabolism, protein degradation, neuronal polarity, function, plasticity, migration, proliferation, survival, and cell death (Beurel et al., 2015; Salcedo-Tello et al., 2011). GSK3β activity is inhibited in response to pro-survival signaling pathways like insulin and Wnt signaling pathways (Pomytkin et al., 2018). It has been indicated that GSK3β inactivation by basic fibroblast growth factor (bFGF) treatment prevented 6-OHDA-induced motor impairments, dopaminergic neuron loss, and tau phosphorylation in a rat model of PD (Yang et al., 2016). It has been shown that GSK3β inhibitors protect the PC12 cells against rotenone toxicity in vitro. Rotenone significantly decreased PC12 cell viability, which was restored by lithium and SB-216763, selective small molecule inhibitor of GSK3β, through stimulation of Wnt signaling pathway, GSK3β inactivation, and increase in Nurr1 expression (Zhang et al., 2016).

These evidences propose that GSK3β inactivation, by insulin or GSK3β inhibitors, can be considered a promising approach to attenuate toxicity and cell death in the context of various damages. So, the present study evaluated the effect of insulin and SB-216763 on MA-induced toxicity in human SH-SY5Y neuroblastoma cells and primary midbrain neurons of rats. Furthermore, the expression of some inflammatory and apoptotic factors was examined in primary midbrain cells.

Materials and Methods

SH-SY5Y cells culturing

Human neuroblastoma cells (SH-SY5Y) (Iranian Biological Resource Center, Tehran, Iran) were seeded in 96-well plates (15×10^3 cells/well) in DMEM/Ham's F12 medium (Gibco, USA) containing 10% FBS and 1% penicillin/streptomycin (Gibco, USA), in a humidified incubator at 37°C with 5% CO2 (An et al., 2019). On day in-vitro 4 (DIV4), the confluent SH-SY5Y cells were treated with pharmacological agents.

Animals

Adult Wistar rats (male and female) from the Neuroscience Research Center breeding colony were kept under a 12/12 h light/dark cycle, 50-60% humidity at 24-25°C, and had free access to standard laboratory chow and water. The experiments were approved by the ethics committee for animal research of Shahid Beheshti University of Medical Sciences (IR.SBMU.PHNS.REC.1400.126).

Primary midbrain cell preparation

Male and female rats were mated for 12 h, and then the male rat was removed from the cage. Fourteen days later, the pregnant rats were used for the primary midbrain cell extraction from the rat embryo, as previously described (Choi et al., 2013). In brief, after anesthetization with CO_2, the embryos were extracted from the uterus and washed in phosphate-buffered saline (PBS). The

TABLE 1: Primer Sequences for qPCR

Gene	Forward primer (5' 3')	Reverse primer (5' 3')
TNFα	ACTGAACTTCGGGGTGATCG	CGCTTGGTGGTTTGCTACG
Bax	AAGTCCAGTGTCCAGCCC	TGGTTGCCCTCTTCTACTTTGC
Bim	AGATAATGGTTGAAGGCCTGG	ACAGAATCGCAAGACAGGAG
Bcl2	AGATAATGGTTGAAGGCCTGG	ACAGAATCGCAAGACAGGAG
β-actin	AACGCAGCTCAGTAACACTCC	TCTATCCTGGCCTCACTGTC

isolated ventral midbrain tissues were washed in Ca^{2+} and Mg2+-free Hank's Balanced Salt solution (HBSS; Sigma Aldrich, USA). They then incubated with 0.05% trypsin solution for 20 min at 37°C. Trypsin action was inactivated by HBSS containing 10% fetal bovine serum (FBS; Gibco, USA). The tissues were dissociated into single cells using fire-polished Pasteur pipettes. The dissociated cells were seeded in 0.01% Poly-L-lysine-coated plates at the density of 6×10^3 cells/well in 96-well (for MTT assay) and 1.2×10^6 cells in T25 flasks (for RNA extraction) in DMEM/Ham's F12 medium (Gibco, USA) with 1% glutamine (Gibco, USA), 10% FBS, and 1% penicillin/streptomycin (Gibco, USA). The half of medium was changed on day 3 and was replaced by serum-free DMEM containing 2% B27 on day 5 (Gibco, USA). The cells were treated with pharmacological agents on day 7.

Treatments

Methamphetamine hydrochloride (School of Pharmacy, Tehran University of Medical Sciences, Iran) was dissolved in 0.9% normal saline. SH-SY5Y cells were treated with MA (0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, and 5mM) for 24 h, or normal saline, and the cell morphology was observed under a light microscope. MA at 5mM concentration reduced the viability of SH-SY5Y cells, so this concentration was selected for co-treatment with insulin or GSK3β inhibitor (SB-216763). Regarding the rat primary midbrain cells, we have previously reported 5mM MA as the toxic concentration to reduce the cells' viability (Valian et al., 2019), so this concentration was also selected for evaluating the effect of insulin and SB-216763 on MA toxicity in rat primary midbrain cells. SH-SY5Y and midbrain cells were treated with insulin (0.005, 0.01, 0.015, 0.02, 0.025, 0.05, 0.1 and 0.15U) (Human recombinant insulin; Exir pharmaceutical company, Iran) or SB-216763 (0.5, 1, 2, 3, 4, 5, 7 and 9μM in dimethyl sulfoxide (DMSO)) (Sigma, USA) without or with 5mM MA. Normal saline and DMSO were used as the corresponding controls. Treatments were repeated 2-4 times with 3 wells/repeat. To evaluate the gene expression, the rat primary midbrain cells were treated with 0.05U insulin or 3μM SB-216763 without or with 5mM MA for 72 h.

MTT assay

The viability of the cells was measured using MTT, after 24, 48, and 72 h (Collins et al., 2016). MTT assay is a test for measuring the dehydrogenase enzyme activity that reduces 3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide to insoluble formazan crystals, giving a purple color. After removing the medium, MTT reagent (5mg/ml in DMEM) was added, and then incubated at 37°C for 4 h. Insoluble crystals were dissolved in DMSO (100μl/well) for 30 min on a shaker at 37°C. Spectrophotometric absorbance was evaluated by a microtiter plate reader at 570nm wavelength.

RNA extraction and qPCR

RNA was isolated using YTzol RNA kit (Yekta tajhiz azma, Tehran, Iran) from the primary midbrain cells. After determination of the RNA concentration using Nanodrop (Thermo Fisher Scientific, USA), cDNA was synthesized from 1μg total RNA (cDNA) by PrimeScript First-Strand cDNA Synthesis Kit (Takara, Japan). Briefly, RNA, random hexamer and oligo dT primers, RT Enzyme Mix, PrimeScript Buffer, and DEPC-treated water were incubated at 37°C for 15 min followed by 85°C for 5 sec. The gene expression was quantitatively assessed by ABI System (USA) using SYBR Green Real-Time PCR Master Mix (Ampliqon, Denmark) reagents. Relative expression of TNFα, Bax, Bim, and Bcl2 was quantitatively calculated by the $2^{-\Delta\Delta Ct}$ method, and β-actin gene expression was considered as an

internal control. RNA was extracted from 1.2×10^6 cells of the control, insulin, and SB-216763 groups. For the other groups, because of cell number reduction following MA, 2.4×10^6 cells were used for RNA extraction. The sequences of primers used for qPCR are shown in Table 1.

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Statistical analyses were performed in SPSS (version 16.0), with the $p<0.05$ level as the statistical significance level. One-way ANOVA analysis with Dunnett's and Tukey's post hoc tests was used to determine the differences between groups.

Results

MA-induced changes in the morphology and the viability of SH-SY5Y cells

The morphology of human neuroblastoma cells was observed by light microscopy following MA treatment. Low to moderate MA concentrations did not affect the cell's morphology; however, the higher concentrations (2-5mM) changed the morphology to a spherical form with obvious cell death after 4 and 5mM MA (Figure 1). Statistical analysis indicated that MA significantly changed the viability of the cells [$F_{(9, 110)} = 17.101$, $p<0.001$]. Although the low to moderate concentrations (0.2-3mM) did not affect the cells' viability ($p>0.05$), the higher concentrations (4 and 5mM) significantly decreased it compared to the control ($p<0.05$ and $p<0.001$, respectively) (Figure 1).

The effect of insulin and SB-216763 on SH-SY5Y cell viability following MA

The viability of SH-SY5Y cells was evaluated after 24 h of treatment with insulin and SB-216763 without or with 5mM MA (Figure 2). ANOVA analysis demonstrated that none of the insulin concentrations changed the viability [$F_{(8, 45)} = 1.193$, $p=0.324$] (Figure 2A). As shown in Figure 2B, MA decreased the cell survival compared to control [$F_{(9, 50)} = 4.312$, $p<0.001$], and insulin (0.05 and 0.1U) significantly increased it in comparison to MA ($p<0.01$). No significant change was also observed after treatment with SB-216763 [$F_{(9, 50)} = 1.277$, $p= 0.273$] (Figure 2C). Statistical analysis indicated that the viability of the cells was significantly decreased following MA treatment in comparison to control and DMSO [$F_{(10, 55)} = 7.388$, $p<0.001$], and treatment with SB-216763 (4 and 5μM) elevated it ($p<0.05$) (Figure 2D).

The effect of insulin on MA-induced cell death in primary midbrain cells

The survival of primary midbrain cells was evaluated after treatment with normal saline and different concentrations of insulin for 48 and 72 h without or with 5mM MA (Figure 3). Statistical analysis indicated that none of the insulin concentrations changed the viability of the cells after 48 h [$F_{(8, 45)} = 0.934$, $p=0.498$], and 72 h [$F_{(8, 72)} = 1.042$, $p=0.413$] (Figure 3A-B). ANOVA analysis indicated that 5mM MA significantly decreased cell viability after 48 h [$F_{(9, 110)} = 2.277$, $p<0.05$], and insulin could not elevate it (Figure 3C). However, insulin (0.025, 0.05, and 0.1U) significantly enhanced cell survival after 72 h [$F_{(9, 110)} = 11.448$, $p<0.001$] in comparison to MA ($p<0.01$, $p<0.01$, and $p<0.05$, respectively) (Figure 3D).

The effect of SB-216763 on MA-induced toxicity in primary midbrain cells

The viability of primary midbrain cells was measured 48 and 72 h after treatment with normal saline, DMSO (vehicle), and SB-216763 without or with 5mM MA (Figure 4). SB-216763 changed the cells' survival after 48 h [$F_{(9, 110)} = 3.797$, $p<0.001$] and 72 h [$F_{(9, 110)} = 3.829$, $p<0.001$]. In both time points, 7 and 9μM concentrations significantly decreased the cell viability compared to control (48 h; $p<0.01$ and $p<0.001$, respectively, 72 h; $p<0.001$ and $p<0.01$, respectively) (Figure 4A-B). Therefore, these concentrations were excluded to treat the cells in the presence of MA. ANOVA analysis demonstrated that MA induced cell death after 48 [$F_{(8, 99)} = 8.602$, $p<0.001$] and 72 h [$F_{(8, 99)} = 12.049$, $p<0.001$]. None of the SB-216763 concentrations affected MA-induced toxicity after 48 h ($p>0.05$ compared to MA); however, SB-216763 at 3μM concentration could significantly ($p<0.05$ in comparison to MA), and at 4μM partially ($p>0.05$ compared to control) attenuate the MA effect on the cell viability (Figure 4C-D).

The effects of insulin and SB-216763 on gene expression following MA

The gene expression of inflammatory and apoptotic factors was evaluated in primary midbrain cells, 72 h

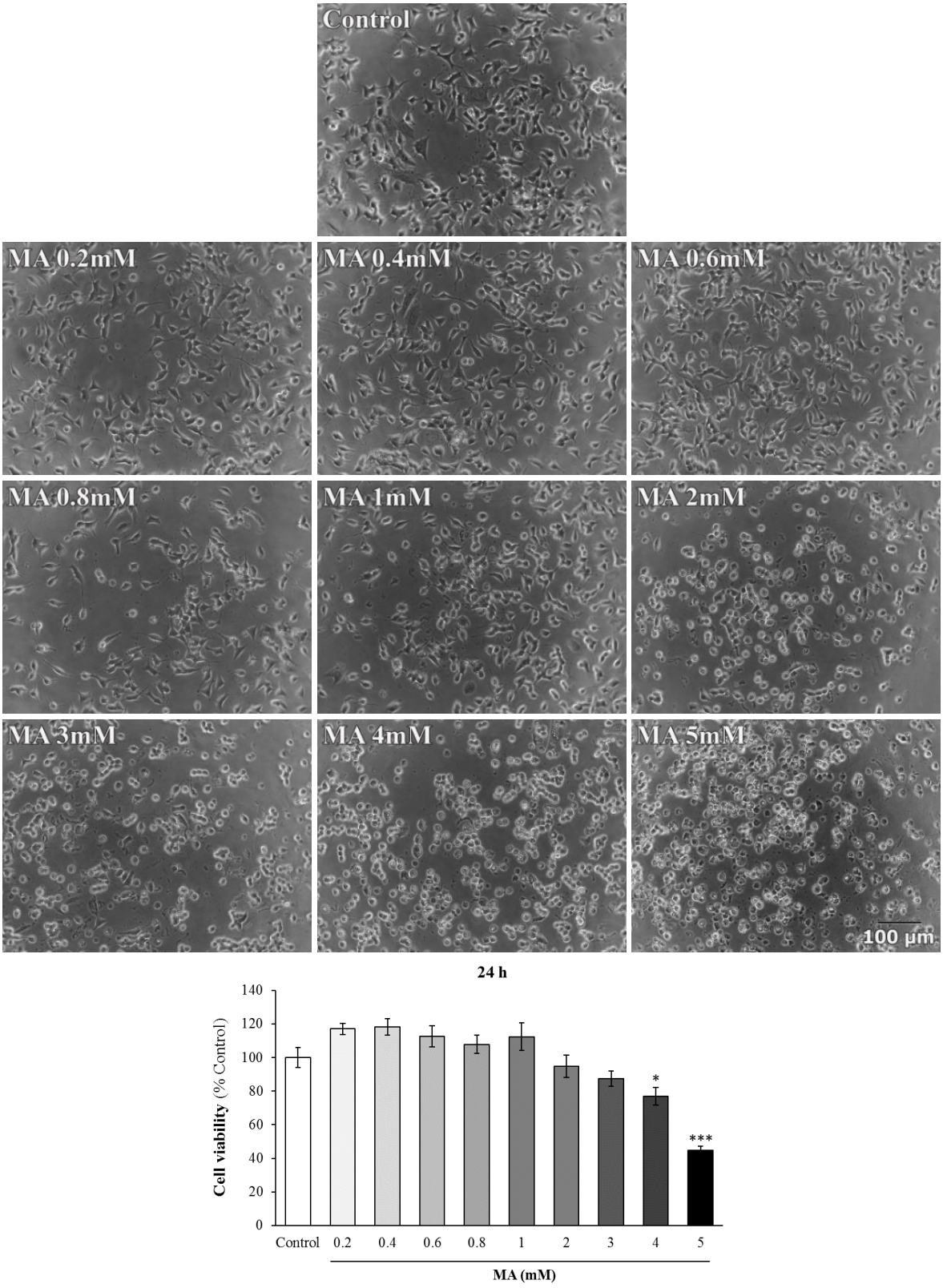

FIGURE 1. Morphology of the neuroblastoma cells following MA treatment. The neuroblastoma cells were treated with normal saline and MA (0.2-5mM) for 24 h. Low to moderate MA doses did not change cell morphology. In contrast, the morphology was changed following higher doses (2-5mM) with obvious cell death after 4 and 5mM MA. Treatments were repeated 4 times and there were 3 wells in each repeat. *p<0.05, ***p<0.001 vs. Control

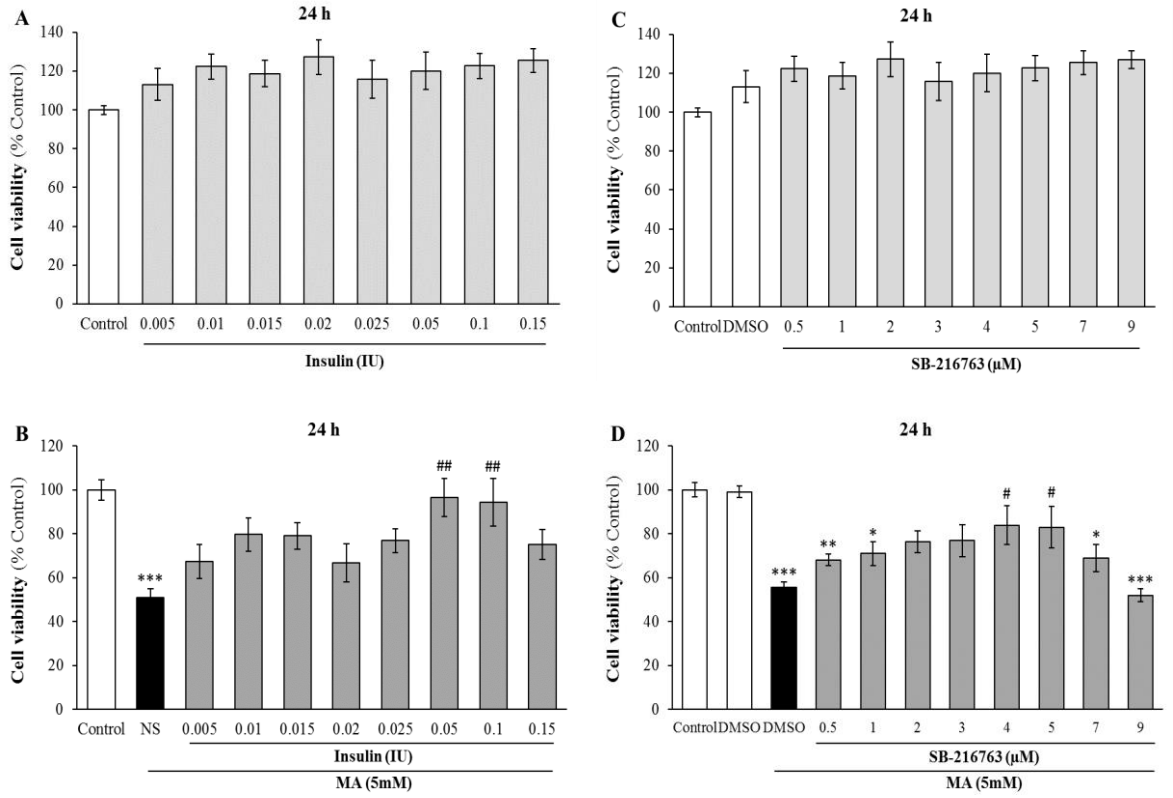

FIGURE 2. The effect of insulin and SB-216763 on MA-induced cell death in SH-SY5Y cells. The neuroblastoma cells were treated with insulin (0.005-0.15U) without (A) or with (B) 5mM MA for 24 h. None of the insulin concentrations changed the cell viability (A). MA decreased the cell viability, and 0.05 and 0.1U insulin restored it to the control level (B). The cells were treated with SB-216763 (0.5-9μM) without (C) and with (D) 5mM MA for 24 h. No significant change was observed after SB-216763 treatment compared to control and DMSO (C). SB-216763 (4 and 5μM) significantly protected the cells against MA-induced reduction in cell viability (D). Data are shown as means ± SEM. Treatments were repeated 2 times with 3 wells/repeat. **p*<0.05, ***p*<0.01, ****p*<0.001 vs. Control
#*p*<0.05, ##*p*<0.01 vs. MA

after treatment with 0.05U insulin or 3μM SB-216763 without or with 5mM MA (Figure 5). MA increased the expression of TNFα [$F_{(5, 12)} = 14.303$, $p<0.001$] and Bax [$F_{(5, 12)} = 6.491$, $p<0.01$] in comparison to control. Treatment with insulin and SB-216763 could significantly decrease them (TNFα: $p<0.001$; Bax: $p<0.01$ and $p<0.05$, respectively) (Figure 5A-B). However, no significant change was observed in the mRNA levels of Bim [$F_{(5, 12)} = 1.384$, $p=0.298$] and Bcl2 between groups [$F_{(5, 12)} = 3.028$, $p=0.054$] (Figure 5C-D).

Discussion

Our findings revealed that insulin and SB-216763 could restore MA-induced reduction in the viability of human SH-SY5Y and rat primary midbrain cells after 24 and 72 h of treatment, respectively. Moreover, MA elevated the expression of TNFα and Bax, which was attenuated by insulin and SB-216763.

In the present study, we aimed to evaluate and compare the effect of insulin and SB-216763 on MA-induced toxicity on both the SH-SY5Y cell line and primary neurons extracted from the rat embryos. SH-SY5Y cells are immortalized and proliferative cells that express markers of immature neurons. They can be differentiated into several types of adult neuronal cells, including cholinergic, adrenergic, and dopaminergic neurons (Lu et al., 2017). It has been shown that undifferentiated SH-SY5Y cells are more susceptible to 6-OHDA and MPTP toxicity than differentiated cells (Carter et al., 2014; Wei et al., 2017). Pro-survival PI3k/Akt signaling pathways are upregulated in retinoic acid-induced differentiated cells (Wei et al., 2017), which is responsible, at least in part, for less susceptibility to toxins in differentiated cells. It has been previously reported that upregulation of elements involved in PI3K/Akt and ERK1/2 pathways can protect neurons against

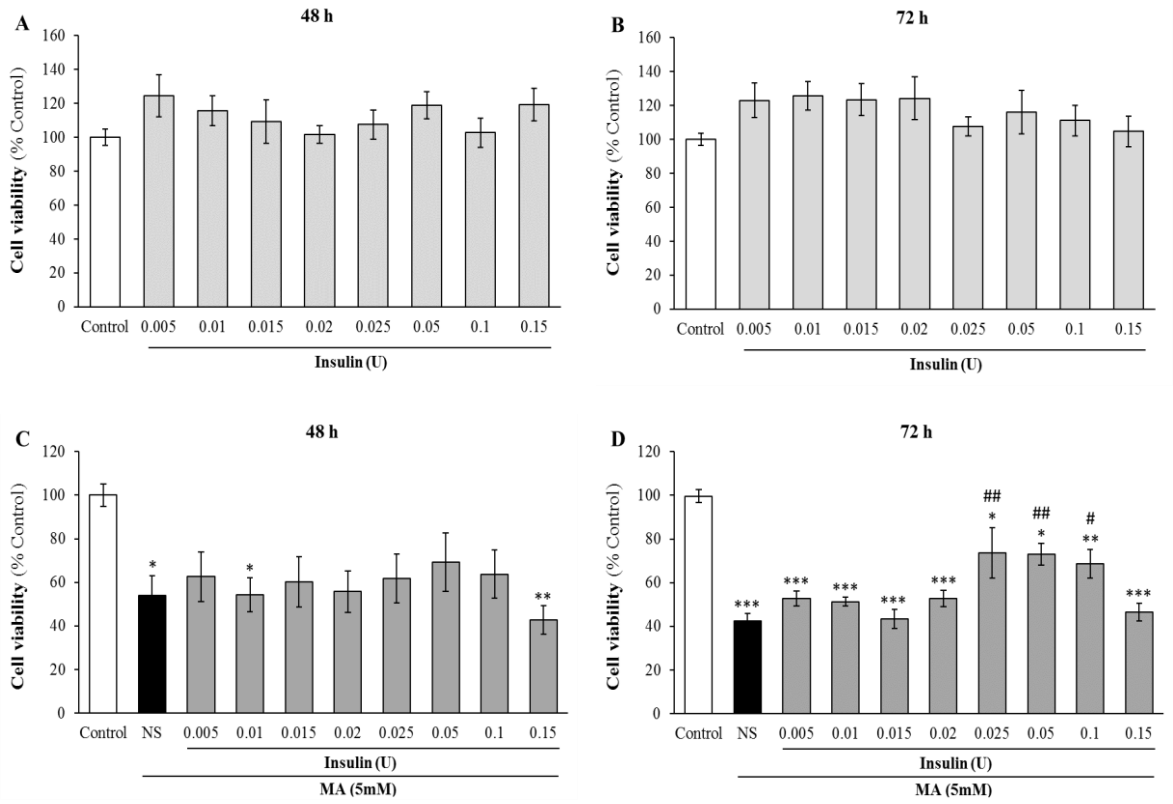

FIGURE 3. The effect of insulin on reduced viability of primary midbrain cells due to MA treatment. The cells were treated with normal saline or insulin (0.005-0.15U) without (A, B) or with 5mM MA (C, D) for 48 and 72 h. None of the insulin concentrations changed the cell viability at both time points (A, B). Although insulin could not prevent MA-induced cell death after 48 h (C), it significantly increased the viability of the cells compared to MA (D). Data are presented as means ± SEM. Treatments were repeated 2-4 times with 3 wells/repeat.
**p*<0.05, ***p*<0.01, ****p*<0.001 vs. Control
#*p*<0.05, ##*p*<0.01 vs. MA

various neurotoxins used for inducing the cellular models of PD (Kunnimalaiyaan et al., 2018; Mathuram et al., 2016; Rani and Goyal 2019). In parallel with these findings, we found that the viability of SH-SY5Y cells was decreased after 24 h of treatment with MA, while the reduction of primary dopaminergic neuron viability was observed following 48 and 72 h (not 24 h, as we previously reported (Valian et al., 2019)). It suggested that SH-SY5Y cells are more sensitive to MA toxicity than primary dopaminergic neurons as fully differentiated cells. However, insulin and SB-216763 could attenuate MA toxicity in both types of cells.

The protective effect of insulin and GSK3β inhibitors has been previously reported in several studies. In-vivo studies have demonstrated that insulin could protect dopaminergic neurons against 6-OH DA (Pang et al., 2016), through the inactivation of GSK3β, downregulation of apoptotic factors, and upregulation of anti-apoptotic factors (Cheng et al., 2010; Duarte et al., 2012). Furthermore, insulin-like growth factor II (IGF-II) has also been shown to protect dopaminergic neurons against 1-methyl-4-phenylpyridinium (MPP^+) by reducing oxidative damage and improving mitochondrial function (Claros et al., 2021). Regarding the role of insulin in the regulation of cell proliferation, it has been demonstrated to induce the proliferation of a breast cancer cell line (MCF-7) (Wei et al., 2017) and colorectal cancer cells (Lu et al., 2017) through IRS1 up-regulation and activation of Ras/Raf/ERK and MAPK pathways (Lu et al., 2017; Wei et al., 2017).

GSK3β activation causes apoptotic cell death by down-regulation of Bcl2 family proteins involved in cell survival (Beurel et al., 2015; Maurer et al., 2014). Insulin and the other neuroprotective agents, like Wnt, are mainly involved in the maintenance of neuronal survival and negatively regulate GSK3β activity through phosphorylation on serine residue, resulting in GSK3β inactivation (Pomytkin et al., 2018). Oxygen-glucose

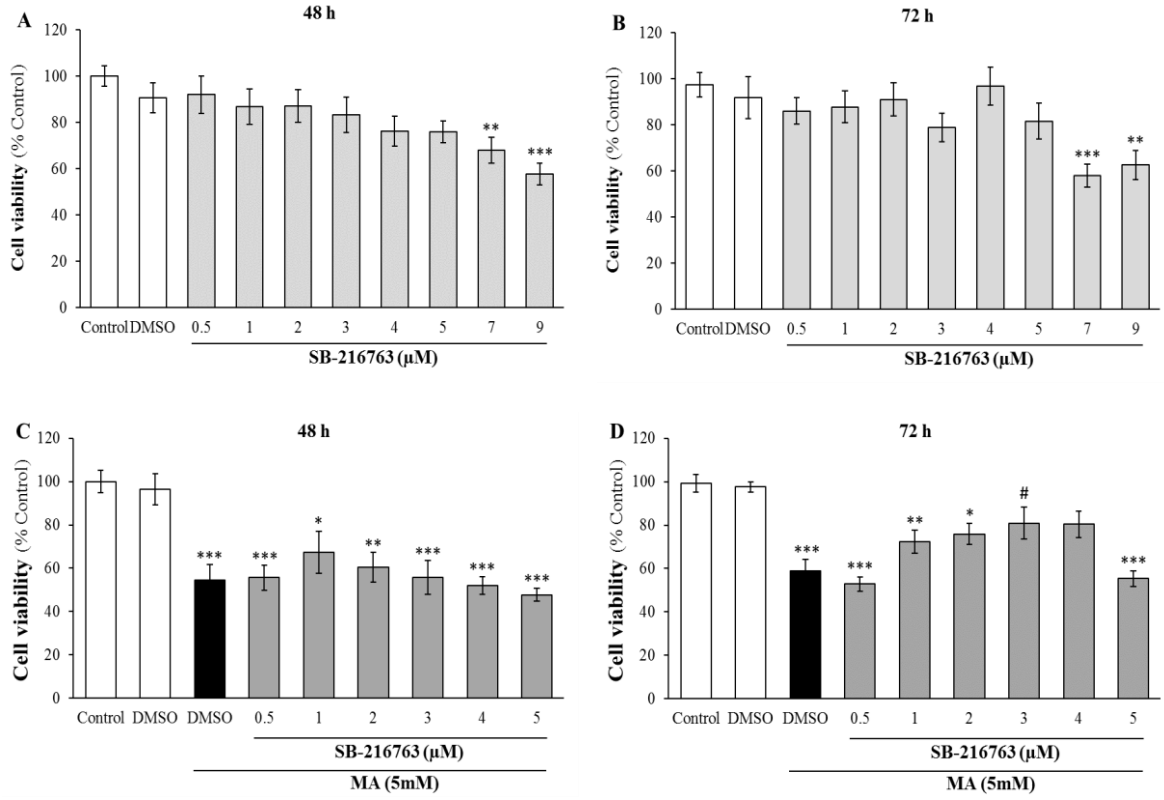

FIGURE 4. The effect of SB-216763 on MA-induced decrease in the viability of primary midbrain cells. The cells were treated with normal saline, DMSO (vehicle), or SB-216763 (0.5-9μM) without (A, B) or with 5mM MA (C, D) for 48 and 72 h. High concentrations of SB-216763 (7 and 9 μM) reduced the viability after 48 and 72 h (A, B). Although none of the SB-216763 concentrations affected MA-induced cell death after 48 h (C), some concentrations (3 and 4μM) increased the viability after 72 h (D). Data are shown as means ± SEM. Treatments were repeated 4 times with 3 wells/repeat.*p<0.05, **p<0.01,***p<0.001 vs. Control
#p<0.05 vs. MA

deprivation/reoxygenation has been shown to decrease the viability of human SH-SY5Y cells through GSK3β activation and enhanced apoptotic factors such as cleaved caspase-3, cleaved caspase-9, p53, p21, and Bax, which were restored by GSK3β inhibition via treatment with emodin (Kunnimalaiyaan et al., 2015). The role of GSK3β activation in cell death has also been shown in the cellular model of traumatic brain injury induced by mechanical stretch (Cheng et al., 2021), and cellular and animal models of PD induced by MPTP (Ahmadzadeh-Darinsoo et al., 2022; Cao et al., 2021; Hu et al., 2020), which was attenuated by GSK3β inactivation. It has been demonstrated that GSK3β inhibitors could prevent mitochondrial dysfunction and restore the viability of SH-SY5Y cells following MPP^+ (Kunnimalaiyaan et al., 2018), and decrease tau phosphorylation and Aβ aggregation through the β-catenin pathway (Lu et al., 2017). Administration of valproate and lithium, which have an inhibitory effect on GSK3β activity, and SB-216763 has been indicated to decrease PC12 cells death following MA (Wu et al., 2015), and prevent MA-induced locomotor sensitization and hyperactivity through GSK3β inactivation in the rodent nucleus accumbens (Enman and Unterwald 2012; Xing et al., 2015; Xu et al., 2011). GSK3β inactivation using curcumin (Moosavi et al., 2018) and rifampicin (Carter et al., 2014) could protect the SH-SY5Y cells against rotenone and 6-OHDA toxicity (Carter et al., 2014; Moosavi et al., 2018). In parallel with these findings, we indicated that insulin and SB-216763 attenuated MA-induced toxicity after 72 h of treatment, but not 48 h, suggesting that they significantly increased the viability of the living cells over time. Furthermore, insulin and SB-216763 attenuated the toxic effect of MA by downregulation of apoptotic (Bax) and inflammatory factors (TNFα) in primary midbrain cells.

Regarding the physiological functions of GSK3β, including the regulatory effects on cell growth, prolif-

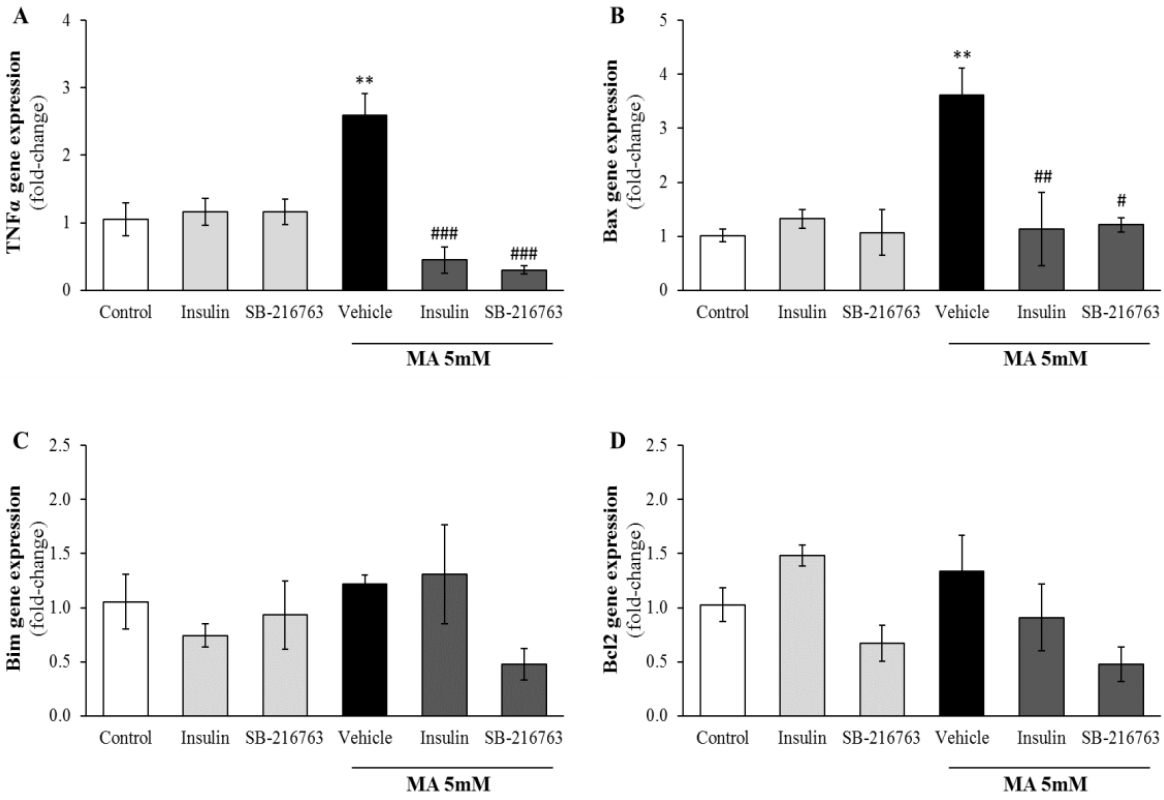

FIGURE 5. The effect of insulin and SB-216763 on gene expression in MA-treated primary midbrain cells. Primary midbrain cells were treated with 0.05U insulin or 3μM SB-216763 with or without 5mM MA for 72 h. The expression of TNFα (A) and Bax (B) was increased following MA, and treatment with insulin and SB-216763 significantly decreased them. No significant change was observed in Bim (C) and Bcl2 mRNA levels (D). Data are presented as means ± SEM (n=3). ***p*<0.01 vs. Control
#*p*<0.05, ##*p*<0.01, ###*p*<0.001 vs. MA

eration, and different aspects of mitochondrial function like permeability, biogenesis, motility, and mitochondrial-dependent apoptosis (Yang et al., 2017), a proper balance between GSK3β activation and inactivation is crucial (Salcedo-Tello et al., 2011). Interestingly, the excessive inactivation of GSK3β activity, similar to its overactivation, could also reduce the viability of the cells depending on the cell type and its cellular localization (Salcedo-Tello et al., 2011). It has been shown that GSK3 inhibitors could induce apoptotic cell death in human neuroblastoma (NGP, SK-N-AS, SH-SY5Y) (Carter et al., 2014; Kunnimalaiyaan et al., 2018; Mathuram et al., 2016; Mathuram et al., 2020) and several cancer cell lines, such as pancreatic (MiaPaCa2, PANC-1, and BxPC-3) (Kunnimalaiyaan et al., 2015), lung (Mathuram et al., 2020), and bladder (Kuroki et al., 2019) cancer cell lines. Enhancement of pro-apoptotic and reduction of anti-apoptotic factors, reactive oxygen species generation, and mitochondrial dysfunction are the underlying mechanisms of apoptotic cell death following GSK3β inhibitors, which are important in treating cancers (Rani and Goyal 2019). Consistent with these reports, we observed a significant reduction in the viability of primary midbrain cells when treated with high concentrations of SB-216763 (7 and 9μM) for 48 and 72 h.

The present study was a preliminary in vitro study evaluating the toxic effects of MA on the primary dopaminergic neurons. We assessed the morphology of the cells under light microscopy, and molecular assessments were only based on gene expression. Immunocytochemistry and protein level measurements can help to achieve a better conclusion. Therefore, more in-depth studies are needed to assess the underlying mechanisms of MA-induced neurotoxicity at both gene expression and protein levels. However, in general, these findings demonstrate that insulin and SB-216763, a selective GSK3β inhibitor, increase the viability of human SH-SY5Y and rat primary midbrain cells following high MA concentrations

exposure, mediated by downregulation of pro-apoptotic and inflammatory factors.

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There is no grant for this project.

Conflict of interest

The authors have no competing interests to declare.

Ethics approval

This study was approved by the Animal Research Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.PHNS.REC.1400.126).

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