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Research Article

Protective Effects of Dendrobium Alkaloids against Manganese-Induced Cytotoxicity in SH-SY5Y Cells

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Abstract

Excessive environmental exposure to manganese (Mn) is associated with neurotoxicity and can produce Parkinson's disease (PD)-like neurological disorders. Although the molecular mechanism is not fully described, Mn-induced neuronal cell death is central to Mn neurotoxicity. The present study was designed to determine the effects of Dendrobium alkaloids (DNLA), a Chinese medicinal herb extract, on Mn-induced cell toxicity, and to investigate whether modulation of the expression of apoptotic regulator proteins is involved in the mechanism of DNLA-mediated cell protection against Mn toxicity. The human neuroblastoma SH-SY5Y cell line was utilized as an *in vitro* model of Mn cell toxicity. It was found that the treatment of the SH-SY5Y cells with Mn for 48h resulted in a concentration-dependent cell death, whereas pretreatment of cells with DNLA significantly alleviated cell toxicity induced by Mn. Examination of apoptosis regulator proteins Bax and Bcl-2 revealed that Mn treatment caused a marked increase in the protein expression of Bax and a decrease in the expression of Bcl-2 protein. On the contrary, DNLA increased Bcl-2 expression, and concomitantly decreased Bax/Bcl-2 ratio. These results demonstrate that DNLA is able to suppress Mn induced cell toxicity, which may be mediated through modulating the expression of the apoptotic regulatory proteins Bax and Bcl-2.

Keywords: *Manganese; Dendrobium Alkaloids; SH-SY5Y; Apoptosis; Bcl-2*

Introduction

Manganese (Mn) is an essential trace element maintained at an optimal level in human body for proper function of brain [1], but excessive Mn exposure can result in its accumulation in basal ganglia, striatum and substantia nigra, and thereby can cause manganism which manifests the Parkinson's disease (PD)-like movement such as gait imbalance, rigidity, tremors, dystonia, and bradykinesia [2-5]. The molecular basis behind the development of these neurological disorders has been attributed to the accumulation of Mn in brain triggering a cascade of cellular events, consequently the loss of dopaminergic cell death [6,7]. Environmental and occupational exposure is a major risk factor of the development of PD [5,8,9]. There is a variety of environmental sources of Mn such as pesticides (maneb and mancozeb) application, and use of gasoline containing methylcyclopentadienyl manganese [10,11], which results in the release of Mn into the environment and consequently human exposure. Though a relatively lower level of exposure, the environmental exposure to Mn is receiving increasing concern on human health due to the chronic and lasting toxic effect [12]. On the other hand, the occupational exposure is frequently linked to high level of Mn, and is the primary source of clinically identified Mn intoxication occurred mainly in occupational workers such as welders and miners [13,14]. Despite growing awareness of the Mn exposure and Mn-induced neurotoxicity, however, the exact mechanisms underlying the pathogenesis of the specific Mn neurotoxicity remains unclear.

It has been well demonstrated that Mn causes a wide range of effects on mitochondrial bioenergetics including depolarizing mitochondrial membrane potential, inhibiting ATP synthase and decreasing AP content [15,16]. Studies also show that Mn exposure elevates

intracellular reactive oxygen species (ROS) and disrupt Ca^{2+} homeostasis with resultant neuronal cell death in rat brain [15,17]. It has been further demonstrated that Mn induces ER stress-mediated apoptosis in rat striatum, which is involved in Mn neurotoxicity [18,19]. A growing evidence has established that the initiation of oxidative stress resulting from discrepancy between ROS and antioxidant generation is one of the key events of Mn neurotoxicity in the cells, which plays critical role in triggering a cascade of signaling leading to apoptosis and/or necrotic cell death in neuronal or glial cells [20-22]. Although the underlying mechanisms are not fully understood, oxidative stress is proposed to be key cellular events involved in Mn neurotoxicity [23], and the use of antioxidants has become a strategy combating the neurotoxicity of Mn.

Dendrobium nobile is a traditional Chinese herbal medicine. In our previous studies, alkaloids extract from *Dendrobium nobile* Lindl. (DNLA) showed neuro-protective activity. For example, DNLA can prevent neuronal damages induced by LPS as well as oxygen-glucose deprivation and reperfusion [24-26], inhibit hyperphosphorylation of tau protein and Aβ deposition, and decrease neuronal apoptosis in rat brain [27,28]. In the present study, we utilized a cellular model of Mn-induced cytotoxicity of dopaminergic SH-SY5Y cells and investigated the protective role of DNLA in Mn induced apoptotic cell death.

Materials and Methods

Drugs and Reagents

Dendrobium nobile Lindl. alkaloids (DNLA) was collected from Dendrobium planting regions of Xintian Traditional Chinese Medicine Industry Development co., LTD of Guizhou Province in 2014.

$MnCl_2$ and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Reagents for cell culture and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Beijing Solarbio Science and Technology Co., Ltd (Beijing, China). Antibodies for Bax, Bcl2 and β-actin were purchased from Beyotime Biotechnology (Beijing, China). ECL Western blot detection kit was purchased from 7Sea Biotech (Shanghai, China).

Cell Culture and Treatment

Human neuronal (SH-SY5Y) cells were obtained from ATCC, USA. SH-SY5Y cells were grown in Dulbecco's Modified Eagle Medium/F-12 nourished with fetal bovine serum (10%) and antibiotics (100 Unit/mL) in CO_2 (5%) incubator at 37°C. The SH-SY5Y cells were pre-treated with DNLA (5 and 10 μg/mL) for 2h before exposure to $MnCl_2$, and were then subjected to further experiments.

MTT Reduction Assay

SH-SY5Y cells were seeded in culture plates (6-well and 6×10^5 cells per well) and cultured for 24h. The cells were then pre-treated with DNLA (5 and 10 μg/mL) for 2h, before exposed to various concentration of MnCl2 (0, 50, 100, 200, 400, 600 or 800 μmol/L) for 48h in DMEM medium containing 10% (v/v) FBS. At the end of the treatments, the cells were incubated with 80 μL of 5 mg/mL MTT solution for 4h. Following this, the medium was discarded, the formazan crystal products were dissolved in 200 μL dimethyl sulfoxide (DMSO) and stirred for 10 min, and optical density (OD) was read on a Microplate Reader at 570 nm. The results were expressed as percentage viability compared to the untreated control. The relative cell viability (%) related to control was calculated by [OD]test/[OD]control × 100%. For each concentration triplet of determinations was run. The representative data shown in this study were obtained from three to five independent experiments.

Western Blotting

SH-SY5Y cells (6×10^5) were seeded in 6-well culture plates and 24h later, and were pre-treated with DNLA (5 and 10 μg/mL, respectively) for 2h before exposure to $MnCl_2$. After 48h, cells were collected and homogenized in RIPA lysis buffer (1:5, w/v). Protein concentrations were determined by BCA protein assay kit. Cell extracts containing 5 or 10 μg of proteins were separated on 12% (w/v) SDS-PAGE gels and transferred to the polyvinylidene difluoride (PVDF) membrane. After 1.5h, the PVDF membrane was blocked with 5% (w/v)

nonfat dry milk in TBST (1.5 mol/L of NaCl, 20 mmol/L of Tris-HCl, 0.05% (v/v) Tween-20, pH 7.4), and the membrane was incubated with Bax, Bcl-2, or actin primary antibodies (at 1:1000 dilutions) overnight at 4°C, followed by incubation with secondary antibodies (at 1:2000 dilutions) at room temperature for one hour. The blots were visualized using the enhanced ECL Western blot detection kit and scanned to Gel Imaging. The band intensity was quantified using Quantity One software (Bio Rad).

Statistical Analysis

Data were expressed as mean ± SD of at least three individual experiments. One-way analysis of variance (ANOVA) and t-test were used to determine statistical differences between the groups. All analyses were carried out using SPSS 17.0 software. A value of $P < 0.05$ was considered significant difference.

Results

$MnCl_2$ induced cytotoxicity of SH-SY5Y cells

SH-SY5Y cells were incubated with various concentrations of $MnCl_2$ (0, 50, 100, 200, 400, 600 or 800 μmol/L) for 48h, and then MTT assay was carried out to determine the cell growth in response to $MnCl_2$ treatment. As shown in figure 1, a slight inhibitory effect was observed in cells exposed to 200 μM of $MnCl_2$. The cell growth was markedly inhibited from the concentration of 400 μM onwards, and displayed a concentration dependent toxic response to the treatment of $MnCl_2$. According to these results, a concentration of 400 μM was chosen for $MnCl_2$ to treat cells in the following experiments.

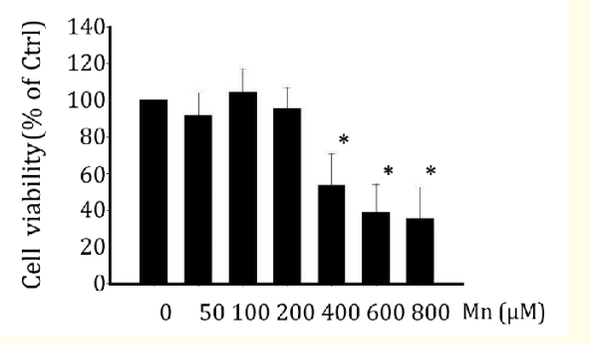

Figure 1: *$MnCl_2$ induced cytotoxicity of SH-SY5Y cells. Cells were treated with 50, 100, 200, 400, 600 or 800 μM $MnCl_2$ for 48h, then cell viability was monitored by MTT assay. Values represent mean ± SD of three individual experiments. *p < 0.01 compared with control.*

Protective effects of DNLA on cytotoxicity induced by $MnCl_2$ in SH-SY5Y cells

SH-SY5Y cells were pre-treated with DNLA (5 or 10 μg/mL) for 2h before exposure to $MnCl_2$ (400 μM) and 48h later, MTT assay was carried out to determine the protective effects of DNLA on Mn-elicited cytotoxicity. As shown in figure 2, DNLA was able to significantly inhibit Mn-induced cell toxicity.

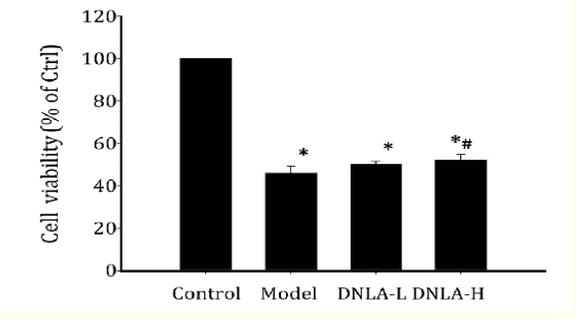

Figure 2: *Effects of DNLA on Mn-induced cytotoxicity in SH-SY5Y cells. Cells were pre-incubated with DNLA (5 and 10 μg/mL, respectively) for 2h, then exposed to 400 μM Mn (Model) for 48h. The relative cell viability was determined using MTT assay. DNLA reduced Mn-induced cytotoxicity. Values represent mean ± SD of three individual experiments. DNLA-L and DNLA-H respectively stand for 5 and 10 μg/mL of DNLA plus 400 μM Mn. *p < 0.01 compared with control; # p < 0.05 compared with Mn alone.*

Effects of DNLA on the apoptosis-related protein expression of Bax and Bcl-2 in SH-SY5Y cells

Bax and Bcl-2 are two important members of the Bcl-2 family of apoptosis regulator proteins, which regulate apoptotic cell death by inducing and inhibiting apoptotic cascades, respectively [36-38]. To determine the role of apoptosis regulator proteins in Mn-induced cytotoxicity and in DNLA-mediated protection mechanism, we examined the protein expression level of Bax and Bcl-2 by Western blotting. The results showed that Mn treatment caused marked increase in the expression of apoptosis inducing protein Bax, while down-regulating the expression of anti-apoptosis protein Bcl-2 (Figure 3). On the contrary, pretreatment with DNLA significantly attenuated the effect of Mn on the protein expression of both Bax and Bcl-2. As shown in figure 3, DNLA increased Bcl-2 expression, and concomitantly dramatically decreased Bax/Bcl-2 ratio, suggesting an inhibitory effect of DNLA on apoptosis.

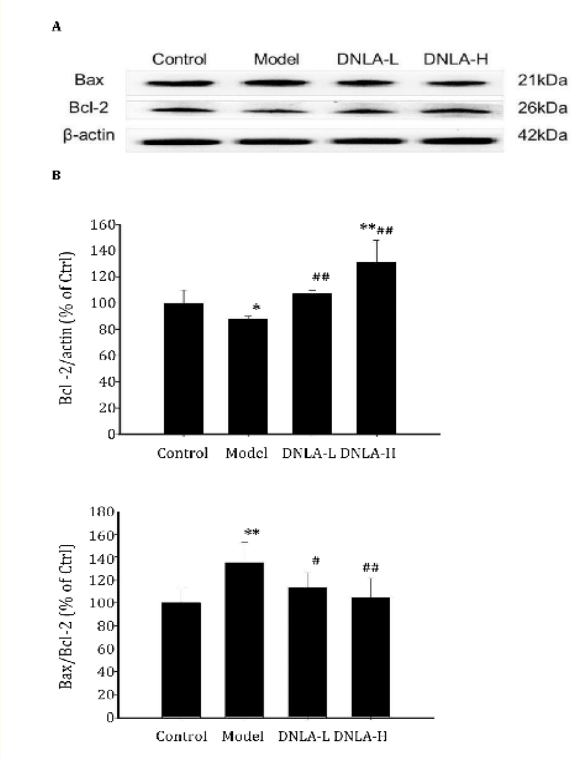

Figure 3: *Effects of DNLA on the protein expressions of Bax and Bcl-2 in SH-SY5Y cells. Cells were pre-incubated with DNLA (5 and 10 μg/mL, respectively) for 2 h, then exposed to 400 μM Mn (Model) for 48h. The expression levels of apoptosis-related proteins Bax and Bcl-2 were determined by Western blotting, with β-actin as a loading control. (A) Representative Western blot of Bax and Bcl-2. (B) Quantification of Bcl-2 expression and Bax/Bcl-2 Ratio, respectively. Values represent mean ± SD of five individual experiments. DNLA-L and DNLA-H respectively stand for 5 and 10 μg/mL of DNLA plus 400 μM Mn. *p < 0.05 compared with control; **p < 0.01 compared with control; # p < 0.05 compared with Mn alone; ## p < 0.01 compared with Mn alone.*

Discussion

Mn is a naturally occurring element and abundantly present in environment. While essential to human health, excessive accumulation of Mn in the body can cause manganism, a motor syndrome similar to PD [6,7]. Studies have shown that mitochondria are the targets of Mn-induced toxicity both *in vitro* and *in vivo* models [18,29-31]. Mn can induce mitochondrial dysfunction such as mitochondrial membrane potential depolarization [13,16], as well as cell death [31,32]. One important consequence of mitochondrial dysfunction is increase in the formation of ROS, which in turn can cause oxidative stress and lead to the release of cytochrome c triggering apoptotic cascade [33-

35]. In the present study, we showed that Mn treatment caused toxic effect in SH-SY5Y cells. By MTT assay it was found that Mn induced significant cytotoxicity in SH-SY5Y cells in a dose-dependent manner. The pretreatment of cells with DNLA markedly reversed the cell toxicity induced by Mn treatment, indicating a protective role of DNLA over Mn cell toxicity.

It is well documented that Mn causes apoptosis in cell lines [36]. Studies have demonstrated that DNLA is capable of protecting LPS-induced neuronal damage by inhibiting apoptotic signaling [27,28]. To further understand the mechanism of DNLA mediated cell protection over Mn toxicity, therefore, we investigated the protein expression of Bax and Bcl-2, which participate in the regulation of apoptotic event in the cell by inducing and inhibiting apoptotic cascades, respectively [37-39]. Consistent with the result of cell toxicity, we observed that Mn decreased the expression level of Bcl-2 protein, an important regulator of apoptotic signaling pathways [40]. Moreover, Mn increased the protein level of Bax, a pro-apoptotic protein. The increase in Bcl-2 expression with concomitant decrease in Bax/Bcl-2 ratio indicates the role of DNLA in regulating Mn-induced apoptosis in SH-SY5Y cells. Although apoptosis plays an important role in Mn cell toxicity, the results from the present study did not rule out other mechanism of cell death implicated in Mn mediated neurotoxicity, which requires further investigation for better understanding the mechanism of DNLA mediated cell protection. Nevertheless, we demonstrate that DNLA modulates the expression of the key apoptosis regulatory proteins Bax and Bcl-2.

Conclusion

In conclusion, the present study demonstrates that Mn impairs apoptotic signaling resulting in cytotoxicity in cultured dopaminergic neuron cell line SH-SY5Y. DNLA confers a protective effect on Mn-induced cell death through modulating at least partially the protein expression of apoptosis regulator proteins Bax and Bcl-2.

Acknowledgements

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Conflicts of Interests

The authors declare no competing financial interest.

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