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Original Research Article

Endoplasmic reticulum stress and apoptosis induced by manganese trigger α-synuclein accumulation

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Abstract

Purpose: *To explore whether α-synuclein aggregation is linked to endoplasmic reticulum (ER) stress and apoptosis induced by manganese (Mn) on CATH.a dopaminergic cell lines.*
Methods: *Western blot analysis for the expression of 78 kDa glucose-regulated protein (GRP78), phosphorylated eukaryotic initiation factor 2α (p-eIF-2α), eIF2α, inositol requiring enzyme 1(IRE-1α), cleaved caspase-3, and C/EBP homologous protein (CHOP) was performed, including overexpression of recombinant adenovirus-mediated α-synuclein on CATH.a dopaminergic cell line.*
Results: *It was observed that cell viability ($p < 0.05$) was significantly reduced by 250 μM exposed for 3 h and 1,000 μM of $MnCl_2$ exposed for 24 h. The expression of p-eIF-2α, IRE-1α, and GRP78 was especially induced by 1,000 μM of $MnCl_2$ exposed at 3, 6, and 12 h, respectively ($p < 0.05$). Twenty four-hour exposure of 250 uM of $MnCl_2$ and the 3 h exposure of 1,000 uM of $MnCl_2$ significantly induced CHOP, active caspase 3 and α-synuclein expression ($p < 0.05$). α-Synuclein combined with recombinant adenoviral transduction increased GRP78, IRE-1α and eIF2a, CHOP and caspase 3 expression at longer times and at higher concentrations of manganese exposure on CATH.a dopaminergic cells.*
Conclusion: *Based on these findings, Mn is a risk factor for diseases associated with α-synuclein accumulation. Furthermore, α-synuclein accumulation is associated with apoptosis via ER stress induced by Mn.*

Keywords: *Manganese (Mn), α- Synuclein, Endoplasmic reticulum (ER) stress, Apoptosis*

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INTRODUCTION

Neurodegenerative diseases result in the progressive loss of structure or function of neurons, including the death of neurons. They are caused by genetic mutation or by extracellularly excreted proteins such as amyloid-β, or by damage to the membranes of organelles by monomeric or oligomeric proteins such as α-synuclein [1], or by aggregation of misfolded proteins [2].

α-Synuclein is a protein that is expressed in abundant amounts in the human brain during human fetal development[3]. It is found at the tips of neurons in the presynaptic terminals which release neurotransmitters from synaptic vesicles [4]. In experiments with mouse models for learning and working memory, α-synuclein function has been reported as potentially playing an important role in the development of cognitive function [5]. This means that α-synuclein may be strongly related to the progressing of Alzheimer's disease (AD). It has also been reported that α-synuclein precipitates predominantly in the cytoplasm of neurons in Lewy-body disorders, and has a critical role in maintaining supply of synaptic vesicles in presynaptic terminals [6] as well as regulating dopamine, a neurotransmitter related to voluntary movements [7]. Movement disorders such as rigidity, tremor, bradykinesia, postural instability and dyskinesia are usually classified as related to problems in Parkinson's disease (PD) [8].

One of the risk factors for PD, is associated with exposure to heavy metals like manganese (Mn) [9]. Appropriate amount of Mn can play critical roles in growth, metabolism, and antioxidant system in the human body. But excessive Mn causes Mn toxicity that induces manganism, the symptom of neurodegenerative disorders such as motor impairment and dementia. How neurodegenerative disorders are related to Mn toxicity is not well known.

In our study, the mechanism of Mn toxicity and the relationship between α-synuclein aggregation as well as ER stress and apoptosis, were investigated.

EXPERIMENTAL

Materials

Roswell Park Memoria Institute (RPMI) 1640 medium was purchased from Grand Island Biological Company (GIBCO, New York, USA). Fetal bovine serum (FBS), trypsin, and other tissue culture reagents were purchased from Life Technologies Inc. (Gaithersburg, MD, U.S.A). $MnCl_2$ and other reagents were purchased from either Sigma or Aldrich (St. Louis, MO, U.S.A) and stored according to the manufacturer's instructions. All reagents were of analytical grade, and plastic wares such as cell culture dishes were obtained from Falcon Inc. (Franklin, NJ, U.S.A). 78 kDa glucose-regulated protein (GRP78), C/EBP homologous protein (CHOP), ß-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A). Phosphorylated eukaryotic initiation factor 2α (p-eIF-2α) was provided by Cell Signaling Technology Inc. (Danvers, MA, U.S.A). CATH.a (mouse neuroblastoma) cells were obtained from the American Type Culture Collections (Manassas, VA, USA).

Cell culture and cell viability

CATH.a (mouse neuroblastoma) cells were maintained at 37 °C in a humidified atmosphere with 5 % CO_2. The cell lines were cultured in RPMI 1640 medium which supplemented with 8 % horse serum, 4 % FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine. Cell viability was assessed with the trypan blue exclusion assay and calculated by dividing the non-stained (viable) cell count by the total cell count ($3x10^6$).

Western blot analysis

Proteins extracted from CATH.a cells were analyzed by Western blot as described in the previous method [10, 11] . Cells were lysed in RIPA buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate, 1 mM phosphatase inhibitor cocktail, 1 mM protease inhibitor cocktail) left on ice for 30 min. Proteins were extracted and they were separated on 7-12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and electro transferred to 0.22 µm polyvinylidene difluoride (PVDF) membrane using a Bio-Rad mini-transfer tank. Then, membranes were probed with the primary antibodies: anti-cleaved Caspase-3, anti-p-eIF2α, anti-eIF2α (Cell Signalling, Danvers, MA), anti-IRE-1α (Abcam, Cambridge, UK) anti-GRP78, anti-CHOP, anti-β-actin and anti-α-synuclein (Santa Cruz Biotechnology, Santa Cruz, CA). The antibodies were recognized using secondary antibodies of anti-mouse or anti-rabbit IgG_1 linked to horseradish peroxidase at room temperature for 60 min. The immune reactive bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham, Buckinghamshire, UK) and exposed to LAS 3000 (Fujifilm, Japan).

Expression of α-synuclein and induction of aggregation

For α-synuclein expression in CATH.a. cells, a recombinant adenoviral vector containing human α-synuclein cDNA (ad.α-synu), and recombinant adenoviral vector containing β-galactosidase (BD Biosciences, USA) were used as previously described [12]. Recombinant adenoviral vector containing β-galactosidase was described as adenoviral non-specific vector (ad.NS) for α-synuclein. Adenoviruses kindly were provided by

Dr. S.J. Lee. CATH.a. cells were infected with ad.α-synu and ad.NS for 3 days. After infection, cells were incubated in a humidified, 5 % CO_2 atmosphere at 37 °C. Incubation with adenovirus continued for 16 h. $MnCl_2$ was treated at a concentration of 1,000 µM for 0 (control), 3, 6, 12, 24 h. It was treated with concentration of 0 (control), 100, 250, 500, 1,000 µM of $MnCl_2$ for 24 h for induction of α-synuclein aggregation at the same day when the infected cells were split.

Protein determination

Protein concentrations of the homogenates were determined using the method of with bovine serum albumin (BSA) as standard.

Statistical analysis

All the data were expressed as mean ± standard error (SE) and one-way analysis of variance (ANOVA) followed by Dunnett's test using SPSS software (version 16, Chicago. IL. USA). The criterion for significance *($p < 0.05$)* was set as stated in Figure legends.

RESULTS

Cell toxicity

Cells were treated with 1,000 µM of $MnCl_2$ for different durations (3, 6, 12 and 24 h) and with different doses: 100, 250, 500 and 1,000 µM of $MnCl_2$ for 24 h. The longer the time and the higher the dose of exposure of $MnCl_2$ induced less cell viability (Figure 1) and more toxicity.

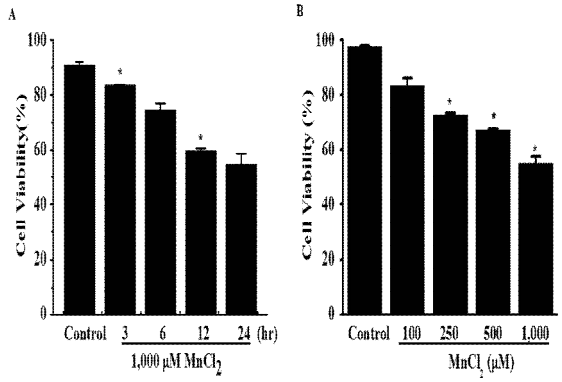

Figure 1: Effects of different time and different concentration of $MnCl_2$ exposure. Cell viability was accessed by tryphan blue assay. (A) Cells were treated with 1,000 µM of $MnCl_2$ for 3, 6, 12, 24 h and the cell viability was assessed by trypan blue assay.(B) Cells were treated 100, 250, 500, 1,000 µM of $MnCl_2$ for 24 h and the cell viability was assessed by trypan blue assay. Data represent mean ± SE (n = 3); *$p < 0.05$ significantly different from control group

ER stress is dependent on time and dose of Mn exposure

ER stress markers, GRP 78, IRE-1α and p-eIF2α, were used in the experiment to determine whether ER stress was affected by the time and dose of $MnCl_2$ exposure in CATH.a cell line. The longer the exposure time of $MnCl_2$, the more GRP 78 expressed. Exposure of $MnCl_2$ for more than 6h induced significant expression of IRE-1α and exposure of $MnCl_2$ for more than 3 h induced remarkable expression of p-eIF2α (Figure 2 A).

GRP 78 was significantly expressed at exposure of $MnCl_2$ over 500 µM, IRE-1α was remarkably expressed at exposure of $MnCl_2$ of over 250 µM, and p-eIF2α expressed at 1,000 µM of $MnCl_2$ exposure (Figure 2 B).

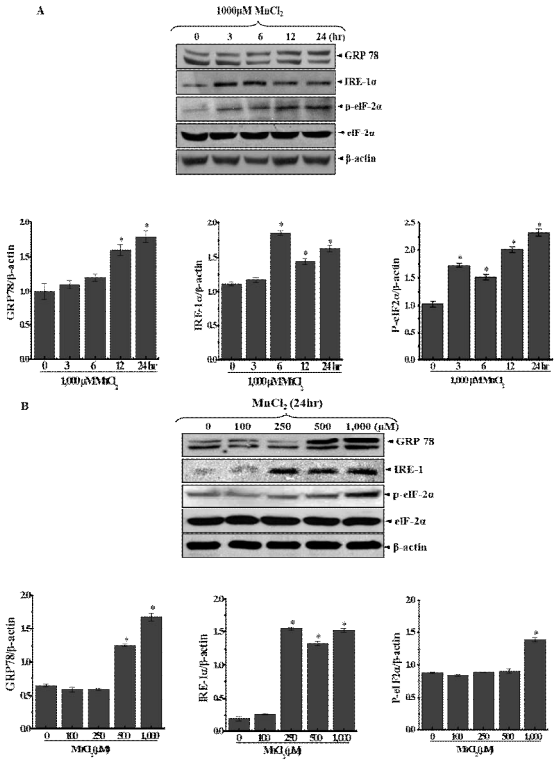

Figure 2: Exposure time and dose to $MnCl_2$ were associated with ER stress expression. (A)Cells treated with 1,000 µM of $MnCl_2$ for 3, 6, 12 and 24 h were lysed. SDS-PAGE and immunoblotting were performed with anti-GRP78, IRE-1, p-eIF-2α, eIF-2α, and β-actin. (B)Cells treated with 100, 250, 500, 1,000 µM of $MnCl_2$ for 24 h were lysed. SDS-PAGE and immunoblotting were performed with anti-GRP78, IRE-1, p-eIF-2α, eIF-2α, and β-actin. Data represent mean ±SE (n = 3); *$p < 0.05$ significantly different from control group (0 denotes control)

Apoptosis is regulated by the time and the dose of $MnCl_2$ exposure

CHOP of the apoptotic markers gradually increased expression when cells exposed to

1,000 uM of Mn at over 3 h, and showed a significant expression from over 250 µM of Mn for 24 hours exposure. Another of the apoptotic markers, caspase 3 was time and dose dependent (Figure 3), suggesting that Mn causes apoptosis.

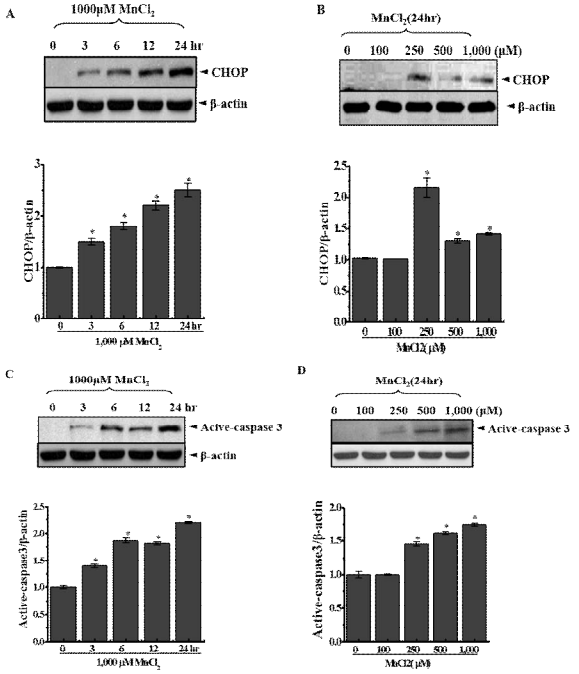

Figure 3: Exposure of $MnCl_2$ induced apoptosis. (A) Cells treated with 1,000 µM of $MnCl_2$ for 3, 6, 12 and 24 h were lysed. SDS-PAGE and immunoblotting were performed with anti-CHOP and β-actin. (B) Cells treated with 100, 250, 500, 1,000 µM of MnCl2 for 24 h were lysed. SDS-PAGE and immunoblotting were performed with anti-CHOP and β-actin. (C) Cells treated with 1,000 µM of $MnCl_2$ for 3, 6, 12 and 24 h were lysed. SDS-PAGE and immunoblotting were performed with anti-Active caspase 3 and β-actin. (D) Cells treated with 100, 250, 500, 1,000 µM of $MnCl_2$ for 24 h were lysed. SDS-PAGE and immunoblotting were performed with anti-active caspase 3 and β-actin. Data represent mean ±SE (n = 3); *p < 0.05 significantly different from control group (0 denotes control)

More α-synuclein was expressed by longer time and higher dose of $MnCl_2$ exposure

To determine whether $MnCl_2$ can cause the accumulation of α-synuclein, western blotting with α- synuclein protein was performed. Interestingly, expression of α-synuclein was dependent on the time and the dose of $MnCl_2$ exposure in the CATH.a cell (Figure 4 and 5). As the concentration of $MnCl_2$ increased by 100, 250 and 500 µM, the amount of α-synuclein expression gradually increased and it was increased significantly at 1,000 µM of $MnCl_2$. The data showed the longer the exposure time to Mn, the more accumulation of α-synuclein (Figure 4). α-Synuclein also showed more oligomerization with increasing time and concentration of Mn in cells treated with recombinant adenovirus vector containing α-synuclein cDNA (Figure 5).

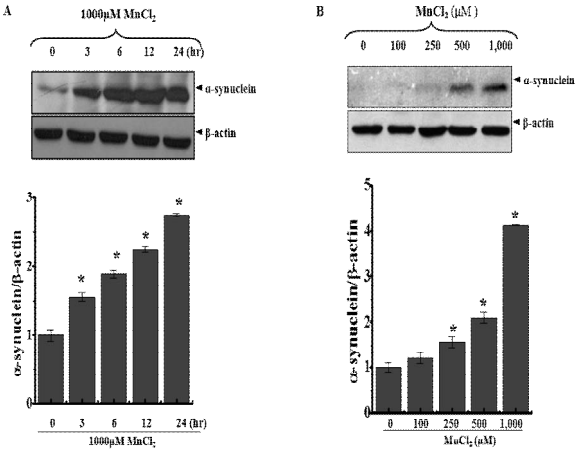

Figure 4: Effect of $MnCl_2$ on endogenous α-synuclein levels in CATH. a cells. (A) Cells treated with 1,000 µM of $MnCl_2$ for 3, 6, 12 and 24 h were lysed. SDS-PAGE and immunoblotting were performed with anti-α-synuclein and β-actin. (B) Cells treated with 100, 250, 500, 1,000 µM of $MnCl_2$ for 24 h were lysed. SDS-PAGE and immunoblotting were performed with anti- α-synuclein and β-actin. Data represent mean ±SE (n = 3); *p < 0.05 significantly different from control group (0 denotes control)

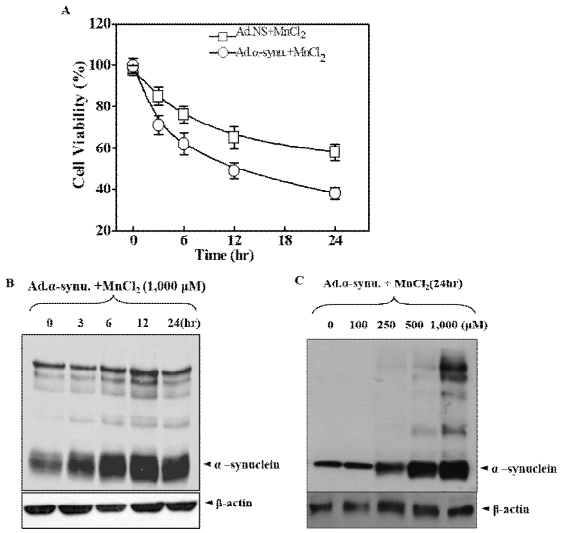

Figure 5: Effects of $MnCl_2$ and α-synuclein on recombinant adenoviral vector with α-synuclein cDNA (Ad. α-synu) and without α-synuclein cDNA (Ad. NS) (A) Cell viability on CATH.a cells. Cell viability was assessed using trypan blue. (B) α-Synuclein oligomerization after 0, 3, 6, 12 and 24 h exposure of 1,000 µM of $MnCl_2$ on recombinant adenoviral vector with α-synuclein cDNA (Ad. α-synu). (C) α-Synuclein oligomerization after 24 h exposure of 0, 100, 250, 500 and 1,000 µM of $MnCl_2$ on recombinant adenoviral vector with α-synuclein cDNA (Ad. α-synu)

α-Synuclein expression is associated with apoptosis via ER Stress

ER stress markers such as GRP 78, IRE-1, p-eIF2a, eIF2 were more expressed in the cells treated with the cDNA recombinant adenovirus vector containing α- synuclein than the cells treated with the recombinant adenovirus vector containing β- galactosidase (non- specific cells, ad.NS) when cells exposed to Mn. CHOP and active caspase 3 expressed more on the cells of recombinant adenoviral vector with α-synuclein cDNA than non-specific cells. These data suggested that apoptosis induced by Mn was significantly declined under non-specific cells of α-synuclein cDNA. Apoptosis increased in cells treated by recombinant adenoviral vector with α-synuclein cDNA. These data implied that α-synuclein overexpression could play an important role in Mn induced ER stress and apoptosis (Figure 6).

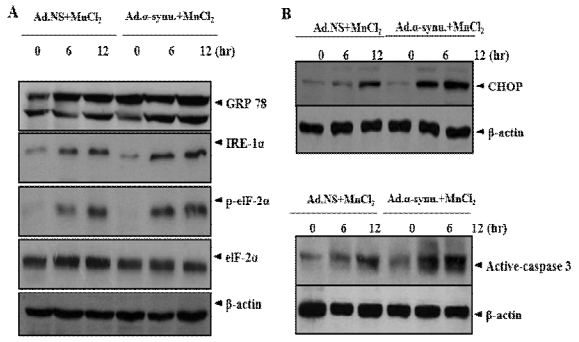

Figure 6: Characterization of $MnCl_2$ and α-synuclein on recombinant adenoviral vector with (Ad. α-synu) and without α-synuclein cDNA (Ad.-NS). (A) Effect of ER stress markers on recombinant adenoviral vector with (Ad. α-synu) and without α-synuclein cDNA (Ad.-NS). (B) Effect of apoptotic markers on recombinant adenoviral vector with (Ad. α-synu) and without α-synuclein cDNA (Ad.-NS). (Ad.α-synu: recombinant adenovirus vector containing α-synuclein cDNA, Ad.NS: non-specific recombinant adenovirus vector)

DISCUSSION

Exposure to high Mn level leads progressive and irreversible symptoms such as rigidity, tremor, bradykinesia, postural instability, dyskinesia, cognitive disorder and neurologic damage [13]. The neuronal damage has been known to be associated with ER stress [11]. However, the exact mechanism of Mn-induced ER stress is not fully understood.

ER stress is activated by mutations in proteins, oxidative stress, calcium depletion, nutrient deprivation, altered glycosylation. And it triggers the unfolded protein response (UPR) [14]. When the UPR fails to restore ER homeostasis with prolonged ER stress, cell progresses from survival condition to apoptosis due to the activated UPR. The UPR is initiated by the activation of three molecular chaperones [15]: protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring kinase 1α (IRE-1α), the mechanism of which is not yet fully clarified. However, 78 kDa glucose regulated protein (GRP 78)/binding immunoglobulin protein (BiP) located in the lumen of the ER and it is one of the most widely accepted models for the recognition of unfolded proteins. It is an important component of the translocation [16].

In this study, it was observed that exposure to $MnCl_2$ increased the expression of GRP78, IRE-1α, p-eIF-2 α and eIF-2α, and it suggests that ER stress plays a salient role in Mn induced toxicity (Figure 2). CCAAT/enhancer-binding protein homologous protein (CHOP) is a pro-apoptotic protein and stimulates cell death by promoting protein synthesis and oxidation in the stressed ER [17]. Caspase-3 is an active effector caspase of the cysteine aspartic acid protease protein family [18] and is activated through proteolytic cleavage in the apoptotic cell death by intrinsic (mitochondria) and extrinsic (death ligand) pathways [19]. The extrinsic activation of caspase-3 plays a salient role in the caspase cascade characteristic of the apoptotic pathway [20]. It was further observed that the longer exposure time and the higher exposure concentration to Mn, the more expression of CHOP and caspase-3 increased and it suggested that ER stress induced by Mn elicits apoptosis in dopaminergic models (Figure 3).

Many studies have been conducted to identify whether the mechanism of α-synuclein as well as the accumulation of α-synuclein can have harmful or beneficial effects. However, the exact function of α-synuclein has not been fully demonstrated.

While previous studies have been focused on the overexpression of α-synuclein in various cell lines [21], our study is hypothesized that Mn can induce α-synuclein directly expression, and that Mn and α-synuclein are closely related to the development of PD. Recent research reported that overexpression of α- synuclein can activate ER stress through the PERK signaling pathway following apoptosis [22]. However, α-synuclein function on cell damage like ER stress and apoptosis, is also still unclear. To explore how α-synuclein could play a critical role in Mn toxicity, experiment was carried out on recombinant adenoviral vector containing human α-synuclein cDNA and non- specific recombinant adenoviral

vector of α-synuclein cDNA to clarify the mechanism of Mn toxicity.

To find the relationship between α-synuclein aggregation and ER stress, we explored α -synuclein aggregation induced by Mn (Figures 4, 5 and 6). Some studies have illustrated the association between pathological α-synuclein and neuron cell death remained so far correlative [16]. However, the experimental results showed that recombinant adenoviral vector containing human α-synuclein cells demonstrated significantly decreased cell viability *in vitro* than non-specific recombinant adenoviral vector cells, suggesting α-synuclein induced cell toxicity. As the time and the dose of Mn exposure increased, α-synuclein gradually aggregated *in vitro* (Figure 5). Chronically exposed to Mn, alpha synuclein aggregated in neurons and glial cells *in vivo* [23]. This suggests that α-synuclein is associated with neuron cell death during acute or chronic exposure of Mn. Whereas a previous study suggested that the molecular chaperone GRP-78 played a neuroprotective role in α-synuclein induced PD-like neurodegeneration [24].

The data would demonstrate that GRP-78 could play an anti-protective role in it, as α-synuclein increased cell toxicity followed by activating more GRP-78 expression as well as IRE-1α, p-eIF-2α, and eIF-2α, leading to the activation of ER stress (Figure 6A). CHOP is a pro-apoptotic protein and CHOP transcription is regulated by ER stress through UPR pathways [25]. Caspase 3 functioned as a dominant role in the apoptotic cells via an extrinsic pathway [20]. Recombinant adenoviral vector containing human α-synuclein expressed more CHOP and caspase 3 protein than non – specific recombinant adenoviral vector (Figure 6B). These data suggest that α-synuclein activated ER stress mediators associated with IRE-1α, p-eIF-2α and eIF-2α pathway, leading to apoptosis (Figure 6).

CONCLUSION

Mn is a risk factor for diseases associated with α-synuclein accumulation. Furthermore, α-synuclein accumulation is associated with apoptosis via ER stress induced by Mn, leading to Mn-induced neurotoxicity.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. H Yoon, GH Lee and HJ Chae who conceived and designed this study. H Yoon, GH Lee, SJ Lee and B Li who collected and analysed the data. SJ Lee provided conceptual technical guidance. H Yoon and GH Lee wrote the manuscript, while HJ Chae has reviewed it. Hyonok Yoon and Geum Hwa Lee contributed equally to the work. Finally, all authors read and approved the manuscript for publication.

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