

Aluminum Activates PERK-EIF2 α Signaling and Inflammatory Proteins in Human Neuroblastoma SH-SY5Y Cells

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Abstract Aluminum is the third most abundant element present in the earth's crust and human exposure to it is possible due to industrialization, utensils, medicines, antiperspirants, etc. Evidences suggest involvement of aluminum in a variety of neurodegenerative disorders including Alzheimer's disease. Endoplasmic reticulum (ER) stress has been implicated in various neurological disorders. ER stress may be a result of impaired calcium homeostasis due to perturbed redox balance and is known to elicit inflammation through the activation of unfolded protein response (UPR). In the present study, we aimed to investigate the role of aluminum in ER stress-mediated activation of inflammatory responses in neuroblastoma cells. Lactate dehydrogenase (LDH) release assay revealed that aluminum compromised the membrane integrity of neuroblastoma cells, probably due to membrane damage, as indicated by enhanced levels of lipid peroxidation (LPO). Besides this, our results clearly demonstrated elevated reactive oxygen species (ROS) levels and a weakened antioxidant defence system manifested by decrease in catalase (CAT) activity and cellular glutathione (GSH). Moreover, we studied the expression of key apoptosis-related proteins, ER stress-

mediated activation of UPR, and its downstream inflammatory pathway. It was observed that aluminum potentially enhanced protein levels of PERK, EIF2 α , caspase 9, caspase 3, and inflammatory markers like NF- κ B, NLRP3, HMGB1, and nitric oxide (NO). Furthermore, aluminum altered TNF α , IL1 β , IL6, and IL10 mRNA levels as well. The overall findings indicated that aluminum mediates UPR activation through ER stress, which results in induction of inflammatory pathway and apoptotic proteins in neuronal cells.

Keywords ER stress · Unfolded protein response · Oxidative stress · Inflammation

Introduction

Aluminum is the third most abundant element present in the earth's crust. This metal has no known role in normal physiological function, but its exposure is very common to human beings by ways like industrialization, utensils, medicines, antiperspirants, etc. [1, 2]. Though dietary intake of aluminum from food is small, the use of aluminum-containing antacids may provide doses of 50–1000 mg/day [3]. The presence of aluminum in parenteral nutrition solutions and injectable medications also presents potential risk to humans [4, 5]. It has been reported that as one ages, aluminum accumulates in the brain and other organs resulting in their dysfunction and toxicity [6]. There is an increasing amount of evidence suggesting the involvement of Al³⁺ ions in a variety of neurodegenerative disorders such as Alzheimer's disease, Parkinsonism, etc. [6]. Studies have demonstrated that degenerating neurons in Alzheimer's disease show high local aluminum concentrations [7]. In demented and aging brain, the aluminum concentration tends to increase because aluminum is the only common neurotoxicant known to accumulate in the brain with

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increasing age, even in non-demented humans albeit at lower rates [8–11]. Thus, Alzheimer's disease (AD) patients contain more aluminum than that of age-matched non-demented controls. This finding has been confirmed by a number of reports using a variety of analytical techniques [6, 12–21]. Aluminum values in gray matter of brains from aged, non-demented humans typically range between 1 to 2.5 μg aluminum/gram brain tissue (dry weight) [7, 18] while gray matter of AD patient's brain has been detected to contain threefold higher aluminum, generally around 3 to 7 μg aluminum/gram brain tissue [7, 18]. Aluminum levels in the brains of untreated dialysis encephalopathy (DES) patients have been found to exceed 25 $\mu\text{g/g}$ in brain tissues with death occurring within months to several years [21]. Further, Itoh et al. shown progressive leukoencephalopathy linked with aluminum accumulation in myelin sheath [22]. Recently, a case study published by Exley and Vickers [23] reported death of a 66-year-old Caucasian man suffering from Alzheimer's disease with significantly elevated aluminum content in the brain. A number of epidemiological reports also stipulate a relation between aluminum intake and the prevalence of Alzheimer's disease. The aluminum content of drinking water has been suggested to be an important factor related to degree of incidence of Alzheimer's disease in elderly population [24–27]. Moreover, increased risk of AD has been noticed in people residing in areas with on high aluminum concentrations in the municipal drinking water supplies [28, 29]. Environmental factors like acid rain may also contribute to enhanced solubility and bioavailability of aluminum since flow of acid rain may result in release of aluminum from soils into the water located in the watershed [30, 31]. Besides, certain occupational epidemiological studies have also indicated a relation between aluminum exposure and AD incidence. Giorgianni et al. reported deficit in complex attention and memory performance in a group of aluminum welders [32].

Aluminum exposure is known to be associated with oxidative stress and cognitive decline in experimental animals [33]. Furthermore, in addition to its neurotoxicity, aluminum is a potent stimulator of the immune responses. For almost a century, aluminum hydroxide (alum) has been widely used and accepted as vaccine adjuvant. Alum has been injected into billions of people of which children are at utmost risk due to the vaccination programs. On the other hand, in adults, long-term persistence of aluminum through vaccine adjuvants may result in cognitive dysfunction and autoimmunity [34, 35]. A study by Guillard et al. [36] reported that subcutaneous pseudolymphoma, which developed at the site of vaccine injection, was linked to an aluminum overload. Moreover, the use of hydroxyl-aluminum gel in chronic renal patients also increases the aluminum burden in the brain [37]. Campbell et al. [38] observed that when animals were exposed to aluminum lactate through drinking water, considerable inflammation of the brain could be detected. Some reports have also

revealed that aluminum in drinking water could specifically activate TNF α [39] and genes responsible for immune activation and inflammation [40].

Certain recent studies demonstrated that alum activates the cytoplasmic NOD-like receptor family, pyrin domain containing 3 (NLRP3) protein in macrophages, and human peripheral blood mononuclear cells (PBMC) [41, 42]. NLRP3 inflammasome has recently been recognized as an innate immune signaling receptor that plays key role in mediating cell responses to various endogenous and exogenous signals. Furthermore, alum has been reported to synergize with toll like receptors (TLR) agonists in vitro to stimulate IL-1 β production from human PBMC [43]. Brain injury evokes inflammatory responses by the activation of NF- κ B transcription factor which is the major signaling pathway that induces inflammatory responses in conjugation with TLRs and NOD-like receptor [44, 45]. Endoplasmic reticulum is the site of protein synthesis, protein folding, synthesis of lipids and sterols, and maintaining calcium homeostasis, etc. [46, 47]. Genetic or environmental insults can alter the function of ER which may culminate in ER stress. ER senses stress mainly by three stress sensors PERK, IRE1, and ATF6 transducers. These sensors induce unfolded protein response (UPR) after the recognition of misfolding of proteins [48, 49]. Recent studies have demonstrated that UPR is activated in neurons [50, 51] and induces inflammatory responses through different UPR transducers [52–54]. During ER stress, neurons can send alarmin signals like HMGB1 which is also a potent stimulator of neuroinflammation and microglia activation [55, 56].

We have already demonstrated the involvement of aluminum in the induction of ER stress [57]; however, there is no report till date which has demonstrated the role of ER stress-mediated inflammatory response in neuronal cells following aluminum exposure. In the present study, we have investigated the role of ER stress in inflammation using SH-SY5Y neuron like cells following aluminum maltolate [Al(mal) $_3$] exposure.

Material and Methods

Materials

Aluminum chloride hexahydrate, Maltol, Tox 7 kit, Neutral Red (NR) dye, and 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). DMEM/F12 cell culture medium, trypsin-EDTA, fetal bovine serum (FBS), CMFDA, and 100 \times antibiotic and antimycotic solution were purchased from Invitrogen Corporation (Van Allen Way, Carlsbad, California, USA). PERK, EIF2 α , NLRP3, HMGB1, NF- κ B antibodies, and nitric oxide colorimetric kit were purchased from Abcam (Cambridge, MA, USA), while caspase 9 and

caspase 3 were procured from Santa Cruz (Finnell Street, Dallas, Texas, USA). All other chemicals were obtained from Merck (Billerica, Massachusetts, USA) unless otherwise mentioned.

Preparation of Al(mal)₃

Al(mal)₃ was prepared from aluminum chloride hexahydrate and maltol (3-hydroxy-2-methyl-4H-pyran-4-one) according to the method described by Bertholf et al. [58]. Briefly, for 10–15 g of complex, 15.5 g (122.8 mM) of maltol and 9.9 g (40.9 mM) of AlCl₃·6H₂O were dissolved in 160 ml of deionized water. The mixture was gently heated to ease dissolution. When the particulate substance had dissolved, the pH was adjusted to 8.3. Next, the mixture was heated to a temperature of 65 °C following which a thin precipitate was obtained, the formation of which was enhanced by stirring the solution. Off-white crystals of Al(mal)₃ were obtained after cooling. The crystals were filtered, washed a number of times with the help of acetone, and dried overnight in vacuum desiccator. Yields of 75–85 % of the theoretical 16.5 g of product were obtained. The aluminum maltolate salt was freshly dissolved in media before exposure to neuroblastoma cells. The aluminum concentration was taken from previous published paper Johnson et al. [59]. Neuroblastoma cells were exposed to different concentrations of Al(mal)₃ (100 and 200 μM) for 24 h. A 3× concentration of maltol alone was also included as control for each concentration of Al(mal)₃ for 24 and 48 h.

Neutral Red Uptake (NRU) Assay

The neutral red uptake assay was done according to the method of Borenfreund and Puerner [60]. Briefly, cells were seeded in 96-well tissue culture plates (10,000 cells/well) in complete DMEM F-12 medium, followed by incubation in 5 % CO₂-95 % atmosphere for 24 h at 37 °C. Cells were exposed to different concentrations of Al(mal)₃ (100, 200 μM) followed by addition of 100 μl of neutral red (NR) dye (50 mg/ml) to each well. After incubation at 37 °C for 3 h, cells were washed with a solution of 0.5 % formaldehyde and 1 % CaCl₂. The NR dye taken up by cells was dissolved in a medium containing 50 % ethanol and 1 % acetic acid in Milli-Q water. Absorbance was taken at 540 nm in a SYNERGY-HT multiwell plate reader, Bio-Tek (Winooski, USA) using KC4 software.

Lactate Dehydrogenase (LDH) Release

Cells were seeded as described above for the NRU assay. LDH released in cell culture medium due to cell membrane damage was assessed by Tox-7 kit (Sigma-Aldrich Inc., St. Louis, MO, USA) as per manufacturer's protocol. The resulting colored compound was measured

spectrophotometrically at 490 nm in a SYNERGY-HT multiwell plate reader, Bio-Tek (Winooski, USA).

Measurement of Reactive Oxygen Species (ROS)

Cells were seeded as described above for the NRU assay. Intracellular ROS generation was estimated by the method of Wan et al. [61] using 2',7'-dichlorofluorescein diacetate (DCFH-DA) dye by measuring the conversion of non-fluorescent DCFH-DA to fluorescent dichloro fluorescein (DCF) within the cell using SYNERGY-HT multiwell plate reader (Bio-Tek, Winooski, USA). Briefly, cells seeded in black 96-well plate at a density of 10,000 cells/well were incubated with 10 μM DCFH-DA for 30 min at 37 °C followed by incubation with desired treatments of Al(mal)₃ for 24 h. The measurement of intracellular ROS was carried out at 485 nm excitation and 535 nm emission wavelengths.

Measurement of Lipid Peroxidation

Cells were seeded as described above for the NRU assay. Lipid peroxidation (LPO) was estimated by measuring the formation of malondialdehyde (MDA) using the method of Ohkawa et al. [62]. Mixture of 0.1 ml cell lysate and 1.9 ml of 0.1 M sodium phosphate buffer (pH 7.4) was incubated at 37 °C for 1 h. Mixture was precipitated with 5 % TCA then centrifuged (2300×g for 15 min at room temperature) and the supernatant was collected. Then, 1.0 ml of 1 % TBA was added to the supernatant and placed in the boiling water for 15 min. Mixture was cooled to room temperature. The absorbance of mixture was taken at 532 nm and expressed in nanomolar MDA/hour/milligram protein using molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Measurement of GSH Levels

Cells were seeded as described above for the NRU assay. Cells were incubated with fluorescent probe Cell Tracker Green CMFDA (Molecular Probes, Invitrogen Corporation, Van Allen Way, Carlsbad, CA, USA) and treated with the different concentration of aluminum. CMFDA was solubilized in DMSO (Sigma, St. Louis, MO, USA) to form a 10 mM stock concentration and used at a final concentration of 1 μM. CMFDA was added for 30 min in black 96-well tissue culture plate prior to treatment. Fluorescence was measured 24 h after Al(mal)₃ treatment at 517 nm emission with excitation at 492 nm. The level of reduced glutathione (GSH) in control cells was set to 1.

Measurement of Catalase Activity

Cells were seeded as described above for the NRU assay. Catalase (CAT) activity was measured by following its ability

to split hydrogen peroxide (H₂O₂) within 1 min of incubation time. Dichromate/acetic acid reagent was added to stop the reaction and the remaining H₂O₂ was determined by measuring chromic acetate at 570 nm which is formed by reduction of dichromate/acetic acid in the presence of H₂O₂ as described earlier by Aebi [63]. CAT activity was expressed as micromolar H₂O₂ decomposed/minute/milligram protein.

Real-Time PCR

Cells (2.5 \times 10⁵) were plated in six-well tissue culture plates. RNA was isolated from control and Al(mal)₃-treated cells using RNAqueous kit (Ambion Inc., Austin, TX, USA) following the manufacturer's protocol. RevertAidTM First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany) was used to synthesize cDNA from 100 ng of RNA. Real-time PCR was performed using Power SYBR green PCR master mix (ABI, Foster City, CA, USA) on ABI 7500 Fast Real-Time PCR System (ABI, Foster City, CA, USA) following the fast thermal cycling conditions: 95 °C for 5 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Sequences of primers are listed in Table 1. Expression levels were calculated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control.

Isolation of Total Cellular Protein

Cells were seeded in 25 cm² tissue culture flasks (8 \times 10⁵ cells/flask). Cells were treated with different concentrations of aluminum, and after specified exposure time, cells were pelleted, washed with ice-cold PBS, and lysed in radioimmunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 1 % NP-40, 0.25 % sodium dodecyl sulphate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium orthovanadate in 50 mM Tris-Cl (pH 7.4). Protease inhibitor cocktail was added fresh prior to lysis. Following incubation in lysis buffer for 1 h, the lysate was gently vortexed for 15 s and supernatant was collected by centrifugation at 17,000 \times g for 15 min and stored in aliquots at -80 °C. Protein content was quantified using Lowry's method.

Immunoblot Analysis

Thirty to 40 μ g of protein sample was separated on 10 % SDS-polyacrylamide gel and electro-blotted on PVDF membrane. The membrane was incubated for 2 h with specific polyclonal IgG antibodies of NLRP3, NF- κ B, HMGB1, PERK, EIF2-alpha, caspase 9, and caspase 3. After 30 min washing with Tris-buffered saline-Tween 20 (TBS-T), respective, HRP-conjugated secondary antibodies were added for 1 h at room temperature. Immunoblot was revealed using ImmobilonTM Western Chemiluminescent HRP substrate kit (Millipore Corporation, MA, USA). β -Actin was used as internal standard. PageRulerTM Prestained Protein Ladder (5 μ l) (Thermo, EU) was used to determine molecular weight of the protein bands. Densitometry of the protein bands was done using NIH software Image J version 1.41 (USA) and results were expressed as arbitrary units for each experimental band.

Nitric Oxide Assay

Nitric oxide (NO) was assessed by measuring the levels of oxidized forms (nitrites and nitrates) in samples using the Nitric Oxide Colorimetric Assay Kit (Abcam) as per manufacturer protocol. Briefly, 2 \times 10⁶ cells were plated in 25 cm² flasks. Control and treated cells were washed with cold PBS, resuspended in ice cold assay buffer (provided with kit), cells were homogenized by pipetting up and down for few times, centrifuged the samples for 5 min at 4 °C at top speed, collected supernatant, and transferred to a clean tube to finally performed deprotenization. Obtained protein samples were exposed to nitrate reductase and cofactor after which plate was incubated at room temperature for 1 h to transform nitrate to nitrite. Five microliters of enhancer was added to each well and incubated for 10 min. Griess reagents were applied to convert nitrite to a deep purple azo compound and the color was developed for 10 min at room temperature. Absorbance was recorded at 540 nm using a multimode-detection microplate reader synergy HT (Biotek).

Statistical Analysis

All experiments were performed for a minimum of three times and results have been presented as mean \pm SE. Statistical analysis was performed using SPSS 14.0 statistical package (SPSS

Table 1 List of primer sequences used in real time PCR

TNF α	F: 5'CCCAGGGACCTCTCTCTAATCA	R: 5'AGCTGCCCTCAGCTTGAG
IL1 β	F: 5'-ACAGATGAAGTGCTCCTTCCA-3'	R: 5'-GTCGGAGATTCGTAGCTGGAT-3'
IL6	F: 5'-AAATGCCAGCCTGCTGACGAAC-3'	R: 5'-AACAACAATCTGAGGTGCCCATGCTAC-3'
IL10	F: 5'-CTTTAAGGGTTACCTGGGTTGC-3'	R: 5'- CCTTGATGTCTGGGTCTTGGT-3'
GAPDH	F: 5'-GTCAACGGATTTGGTCGTATTG-3'	R- 5'-TGGAGGGATCTCGCTCCTGGAAGAT-3'

Inc., Chicago, IL, USA). Statistical significance of the results was determined using one-way ANOVA by Tukey's multiple comparison tests. Differences were considered statistically significant at $P < 0.05$.

Results

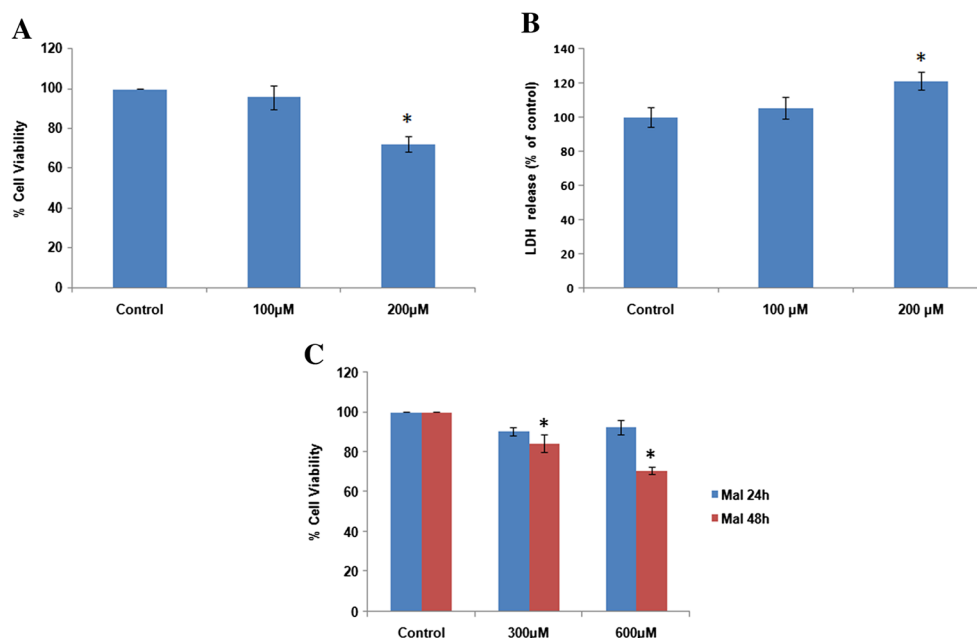
Al(mal)₃ Reduces Cell Viability

We exposed neuroblastoma cells to different concentrations of Al(mal)₃ (100 and 200 μ M) for 24 h and a 3 \times concentration of maltol alone was also included as control for each concentration of Al(mal)₃ for 24 and 48 h. Neutral Red Uptake assay showed dose-dependent toxicity of Al(mal)₃ that is at 100 and 200 μ M concentration, cell viability reduced to 98.3 and 74.5 %, respectively at 24 h (Fig. 1a). Thus, significant cell death could be observed at 200 μ M at 24 h. No significant cell death was observed upon treatment with its respective maltol control (200 and 400 μ M maltol) at 24 h (Fig. 1c). Further, we also performed total LDH release assay which revealed that 200 μ M concentration of Al(mal)₃ induced significant leakage of LDH (Fig. 1b). This again reflects the dose dependent cytotoxicity of Al(mal)₃.

Al(mal)₃ Induces Oxidative Stress and Disturbs the Antioxidant Defenses Within Neuroblastoma Cells

ROS generation was assessed at 24 h after treatment with Al(mal)₃ (100 and 200 μ M concentration). Significant elevation in ROS levels could be observed at both the doses; however, maximum ROS levels were detected at 200 μ M

Fig. 1 Al(mal)₃ induced cell death of neuroblastoma cells. Cells were exposed to Al(mal)₃ at 100 and 200 μ M concentrations and a 3 \times concentration of maltol alone was also included as control for each respective concentration of Al(mal)₃. Viability of cells (10,000 cells/well) was evaluated by **a** NRU assay; **b** LDH assay in 96-well tissue culture plates at 24 h. The data are represented as means \pm SE of three independent experiments. * $P < 0.05$ vs. control



(Fig. 2a). Peroxidation of membrane lipids may be one of the mechanisms by which ROS contributes to the cascade of events leading to damage of cell membrane and intracellular cytoplasmic bodies. Therefore, we also measured MDA levels as a marker of LPO and found that MDA levels increased significantly as the Al(mal)₃ concentration increased, reaching 1.7-fold at 200 μ M (Fig. 2c). Moreover, we also measured catalase activity and cellular glutathione levels since they are considered as first line of defence against deleterious effect of ROS. Our data showed that catalase activity and cellular glutathione was significantly suppressed as the Al(mal)₃ concentration increased (Fig. 2b, d).

Al(mal)₃ Induces Unfolded Protein Response (UPR)

Treatment of neuroblastoma cells with Al induced expression of key proteins involved in UPR and that also play critical role in propagating inflammation. Western blot analysis demonstrated the changes in expression level of PERK and EIF2 α which were observed to be considerably enhanced following aluminum exposure with respect to untreated control (Fig. 3a). These proteins have downstream targets of inflammatory response like NF- κ B.

Al(mal)₃ Induces Expression of Inflammatory Proteins and Transcription of Inflammatory Genes

Neuroinflammation is an important event in neurodegenerative processes and aluminum potentially induces proteins involved in inflammation. We demonstrated protein level of key inflammatory signaling proteins by western blotting. The protein level of NF- κ B, a transcription factor, was detected to be

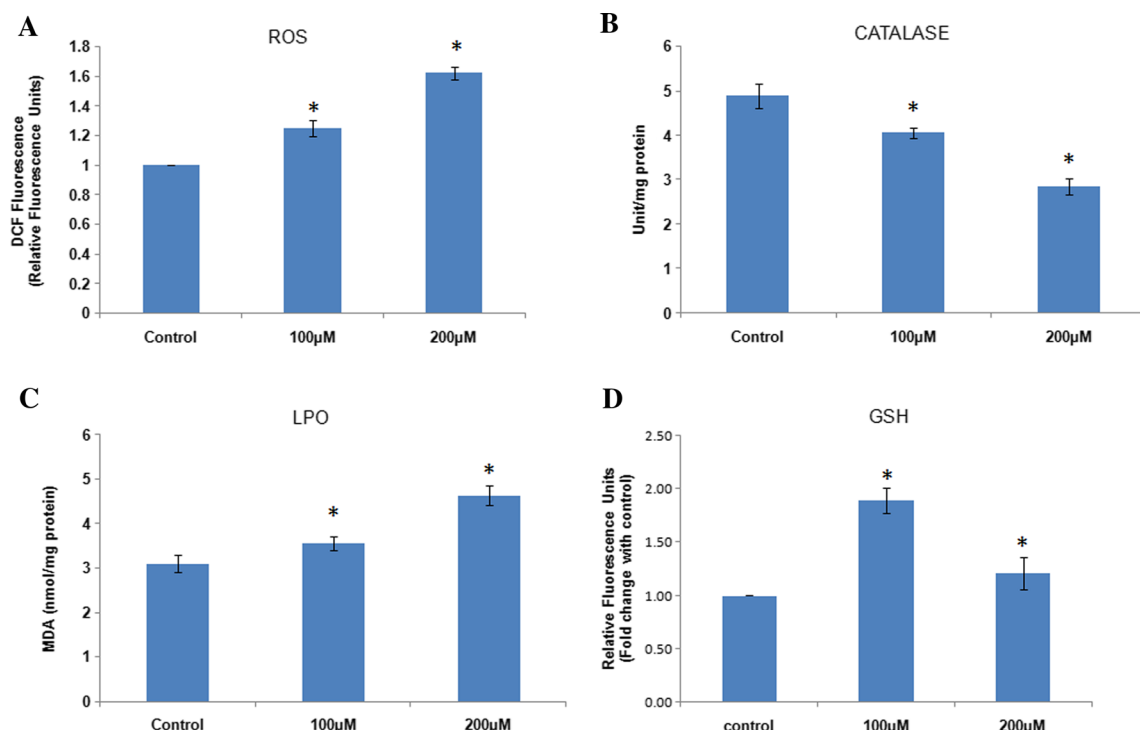


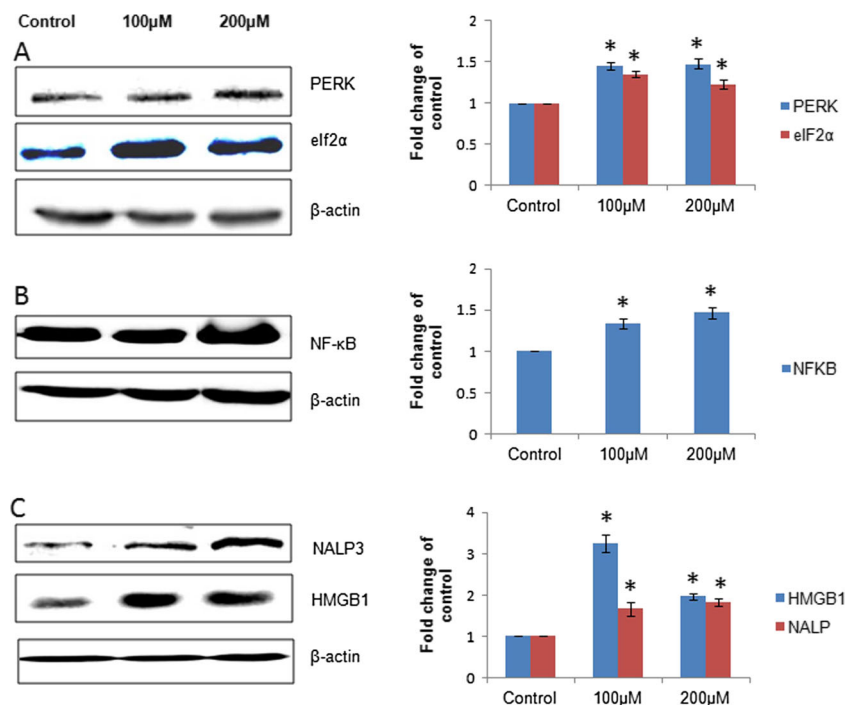
Fig. 2 Al(mal)₃ induced oxidative stress and compromised antioxidant defenses of neuroblastoma cells. **a** ROS generation was assessed in terms of relative fluorescence units using 10 μ M DCFH-DA in neuroblastoma cells at 24 h of Al(mal)₃ exposure in black-bottomed 96-well plates. **b**

Catalase activity; **c** LPO levels; and **d** GSH levels were assessed using 5 μ M CMFDA dye at 24 h of Al(mal)₃ exposure. The data are represented as means \pm SE of three independent experiments. **P*<0.05 vs. control

significantly enhanced in response to aluminum exposure at both 100 and 200 μ M concentrations by 1.4- and 1.5-fold, respectively, with respect to control (Fig. 3b). HMGB1 which

plays important role in inflammatory responses was also up-regulated along with the enhanced expression of inflammasome NLRP3 at both the concentration of aluminum

Fig. 3 Al(mal)₃ induced ER stress-mediated activation of UPR and inflammation in neuroblastoma cells. Western blot analysis of **a** EIF2 α and PERK; **b** NF- κ B; **c** NLRP3 and HMGB1 at 24 h of Al(mal)₃ treatment (100–200 μ M). Band intensities were calculated by densitometry and change in protein expression (Al(mal)₃ treated) was calculated with respect to controls and expressed as fold change in graph. Results were normalized to β -actin. The data are represented as means \pm SE of three independent experiments. **P* < 0.05 vs. control



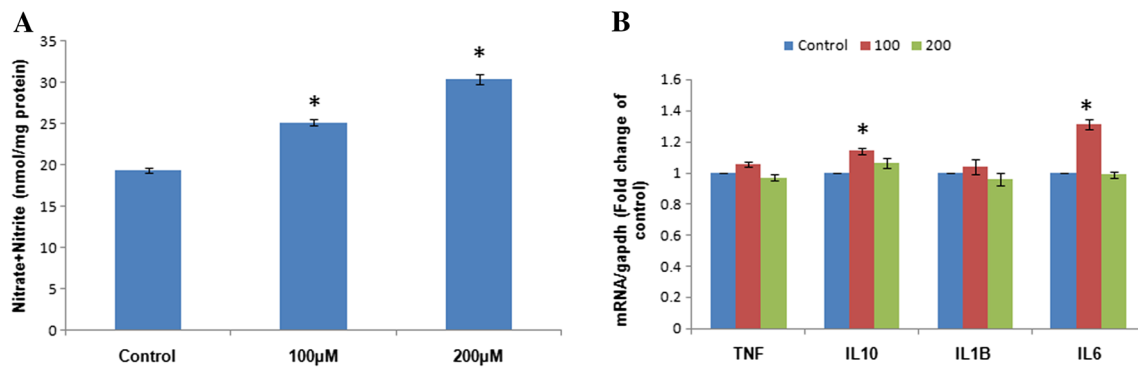


Fig. 4 Al(mal)₃ disturbed the transcriptional status of inflammatory genes and NO levels. Real-time PCR of **a** NO levels; **b** TNF α , IL1 β , IL6, and IL10. The data are represented as means \pm SE of three independent experiments.* P <0.05 vs. control

(Fig. 3c). Further, ELISA used to measure the level of NO, a potent mediator of inflammatory responses, revealed significant enhancement in NO levels after aluminum exposure (Fig. 4a). The study of mRNA expression of TNF α , IL1 β , IL6, and IL10 revealed no significant alteration in expression of these inflammatory genes except IL6 and IL10 which were observed to be significantly up-regulated at 100 μ M concentration of aluminum. Moreover, insignificant increase in TNF α and IL1 β was also detected at 100 μ M concentration of aluminum (Fig. 4b).

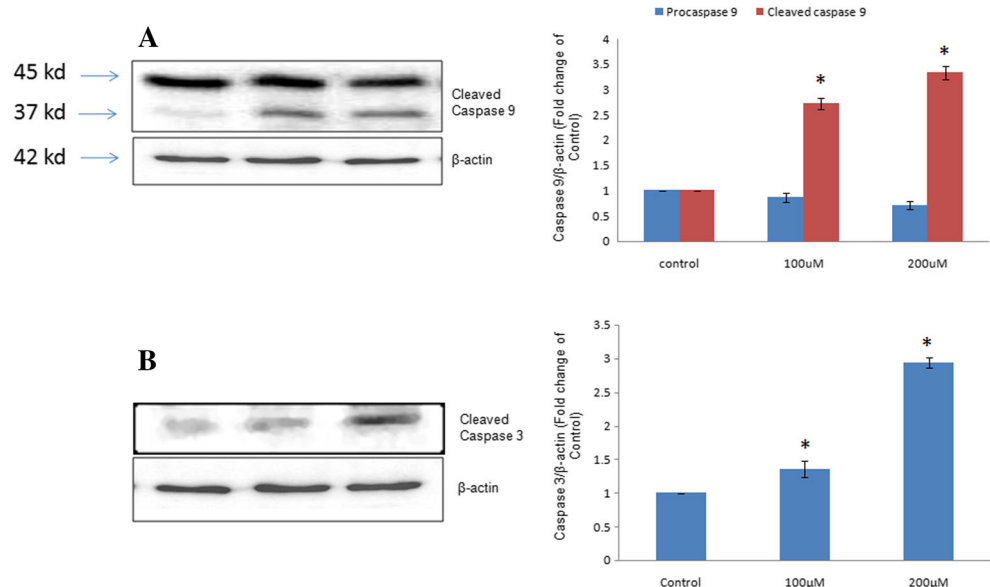
Al(mal)₃ Induces Expression of Apoptosis-Related Proteins

It is well noted that prolonged inflammatory responses induce apoptosis which was supported by increased expression of caspase 3 (by 3-fold) and caspase 9 (by 3.3-fold) following aluminum exposure (Fig. 5a, b) to neuroblastoma cells.

Discussion

Aluminum and its compounds are neurotoxic, and animals are routinely exposed to this metal by natural as well as manmade sources. In this study, we have employed Al(mal)₃, as it is electroneutral complex which provides significant amount of free aqueous aluminum at physiological pH [64]. Further, maltol is a naturally occurring organic compound commonly present in human diet that has been shown to increase the absorption of aluminum through gastrointestinal tract. A study by Noritsugu Kaneko et al. [65] showed greater accumulation of aluminum in the brain, liver, kidney, and spleen of mice who receives long-term (90 days) administration of the complex aluminum–maltolate. A number of studies have been carried out exploring the role of aluminum in neurodegenerative disorders especially in Alzheimer's [23, 24]. Aluminum reaches the brain via blood, bound to transferrin. Transport across the blood–brain barrier (BBB) is possible through carriers of aluminum identified at the BBB. Transferrin-mediated

Fig. 5 Al(mal)₃ induced expression of apoptotic proteins. Western blot analysis of **a** cleaved caspase 9 and **b** caspase 3 at 24 h of Al(mal)₃ treatment (100–200 μ M). Band intensities were calculated by densitometry and change in protein expression (Al(mal)₃ treated) was calculated with respect to controls and expressed as fold change in graph. Results were normalized to β -actin. The data are represented as means \pm SE of three independent experiments.* P <0.05 vs. control



transport of aluminum has been suggested to be one of the mechanisms [66]. Monocarboxylate transporter (MCT), a proton co-transporter which is located at both the luminal and abluminal surfaces of the BBB, is another important carrier for brain aluminum influx [66]. Individuals older than 70 years of age have been reported to show a potentially pathological accumulation of aluminum in their brain. Oxidative stress and inflammatory reactions both play important roles in neuronal disorders including Alzheimer's and Parkinsonism. Here, we have attempted to understand the role of aluminum in neuroinflammation by evaluating oxidative and ER stress-mediated UPR activation and inflammatory responses following aluminum exposure in human neuroblastoma cells.

Our results demonstrated that aluminum-induced cytotoxicity in SH-SY5Y cells is dose dependent, as indicated by NRU assay and total LDH release (Fig. 1). Lysosome functions are a membrane-dependent process and the stability of lysosomal membrane, through neutral red uptake (NRU) assay, has been used to determine their integrity and cell function. When the lysosomal membrane is destabilized under stress conditions, the neutral red dye leaks into the cytosol of the cell much more quickly. On the other hand, LDH is released from the injured cells as a result of loss in membrane integrity. Both lysosomal and membrane integrity are directly related to the inflammatory responses in the cells.

Neurons are very sensitive to oxidative stress and it is the primary event in any neurological disorder. We observed that aluminum caused redox imbalance in neuronal cells as evident from a significant surge in ROS generation at both 100 and 200 μ M concentrations. These results were further supported by elevation detected in levels of MDA, a product of lipid peroxidation, and significant decline in catalase activity and GSH levels of neuronal cells (Fig. 2b, d) in response to aluminum exposure.

ER is a multifunctional organelle and is implicated in many neurological disorders including Alzheimer's. It maintains calcium homeostasis, posttranslational modifications, protein folding, synthesis of lipids, etc. Besides these functions, ER is a sensor of different types of insults like calcium imbalance, redox imbalance, and protein misfolding, each of which can disturb ER functioning and induce ER stress. To avoid these situations, ER activates stress sensor pathways through complex signaling network of PERK-eIF2 α , IRE1-XBP, and ATF6-CREBH transducers. This signaling network further activates the UPR which subsequently initiates changes in the expression of hundreds of genes to restore cellular homeostasis. However, if the ER stress is prolonged, then UPR activates apoptotic signaling.

In this study, we have evaluated the expression of PERK-eIF2 α pathway and observed increase in levels of PERK and eIF2 α proteins (Fig. 3a) along with the increased expression of apoptotic proteins caspase 3 and caspase 9 (Fig. 5a, b) following aluminum exposure. These results confirm that

aluminum induces UPR in neuronal cells which leads to the activation of apoptotic proteins. Hoozemans et al. [67] and Unterberger et al. [68] have previously demonstrated increased PERK and eIF2 α levels in hippocampus neurons of AD brain. Moreover, in an earlier study, we have reported ER stress-mediated apoptosis evoked due to Al(mal)₃ and silica nanoparticles, [57, 69] through the activation of caspase 12. Al(mal)₃ also induced oligomerization of A β in neuronal cells in vitro apart from enhancing CCAAT-enhancer-binding protein homologous protein (CHOP), caspase 12, and intra cellular calcium levels. The present study further strengthens and validates our previous findings indicating that ER stress generates signals to alert nearby cells and draw inflammatory responses to prevent tissue damage [70, 71].

ER stress and inflammatory responses are linked to the pathogenesis of many diseases [53, 72–74]. The inflammatory reactions are generally initiated through the TLR or NOD like receptors (NLRs) and these pathways mostly act on the NF- κ B signaling pathway to induce inflammatory responses. Besides this, stressed cells secrete alarmins to recruit immune cells [55, 56]. On the basis of these findings, it is clear that ER stress pathways are linked to the generation of the inflammatory responses. We also evaluated the NLRP3 inflammasome and NF- κ B pathway and found that aluminum activates NF- κ B, which is the key signaling pathway that induces pro-inflammatory responses in conjugation with the NLRP3 inflammasome (Fig. 3b, c). NF- κ B activates pro-IL1 β which is converted to the mature IL1 β by the NLRP3, ASC, and caspase 1 complex. Moreover, ROS and lysosomal damage also activate NLRP3 inflammasome pathway and induce pro-inflammatory reactions. We observed that aluminum increased protein expression of the NLRP3. We also assessed protein expression of the alarmin, HMGB1, which is present in neurons and is also a potent stimulator of the inflammatory reactions. HMGB1 was significantly up-regulated in response to aluminum exposure at 100 μ M, while at 200 μ M it was decreased when compared to the 100 μ M concentration. The levels of GSH were also observed to alter in a similar manner with significant decrease observed at 200 μ M compared to 100 μ M concentration of Al(mal)₃. GSH is the first line of defense against the deleterious effects of ROS, and HMGB1 is the alarmin protein which signals nearby cells to protect them from stress or release cytokines to counteract the stress. At 100 μ M, aluminum induced rise in the levels of GSH and HMGB1 which could indicate that at a low dose (i.e., 100 μ M) cells try to protect themselves by increasing the synthesis of GSH and HMGB1 to cope with stress, after the available cellular content is utilized. However, at high dose (i.e., 200 μ M), the cells may be unable to cope with the increased demand beyond the threshold levels which may have led to overall depletion of GSH and HMGB1. Furthermore, reports have demonstrated NLRP3-dependent HMGB1 release. The increased HMGB1 levels observed in our study

may be a prelude to activation of the NLRP3 by the aluminum exposure.

Cytokines play an important role in maintaining the functions of neurons; while on the other hand, any disturbance in the homeostasis of cytokines is implicated in many neurological disorders including Alzheimer's and Parkinsonism. Studies reveal that TNF and IL1 receptors are present on the neurons, and it has been reported that TNF and IL1 mRNA accumulate in neurons. Moreover, the respective proteins have also been shown to be released by neurons. TNF also play a role in maintaining physiologic levels of neurotransmitter release through regulation of adrenergic auto-receptor activity [75]. Additionally, changes in TNF levels correlate with the neuronal survival or apoptosis that dictates cell survival or cell death [76]. IL-6 is produced locally in the brain and reports indicate that increased brain IL-6 is associated with severe cognitive impairments and that it may contribute to the behavioral deficits and neuronal loss [77–80]. On the basis of the above reports and our findings, we can say that aluminum is somehow responsible for the altered cytokine expression and signaling which can be correlated with its toxicity and its mediated neuroinflammation and cell death. Our results, indicating significant increase of IL6 and NO and non-significant increase in IL1 β and TNF α in vitro at 100 μ M concentration, further corroborate the potential of aluminum to induce

neuroinflammation and activate innate immune responses by the activation of ER stress.

In order to curtail the harmful effects of aluminum, every attempt should be made to minimize the exposure to this metal at the first place. Keeping into account the significant involvement of ER stress in the pathogenesis of aluminum-induced neurotoxicity, as documented by present findings as well as our earlier study [57] (Fig. 6), inhibition of apoptotic signals evoked as a result of ER dysfunction may be considered as an attractive strategy to counteract aluminum-induced cytotoxicity in neuronal cells. CHOP is a 29 kDa protein with 169 amino-acid residues in human. CHOP is ubiquitously expressed at very low levels. Under unstressed conditions, CHOP is present in the cytosol; however, stress induces its translocation and accumulation in the nucleus. Recent microarray studies demonstrate that CHOP is one of highest inducible genes during ER stress. During prolonged ER stress condition, CHOP activates apoptotic signaling through the deactivation of antiapoptotic bcl2 protein. Further, it also promotes the translocation of bax to the mitochondrial membrane which result into the release of cytochrome c (cyt c), a marker of mitochondrial-mediated apoptosis. CHOP is reported to be a critical mediator of ER stress-induced apoptosis in a number of disease models. Silencing CHOP in tunicamycin-treated SH-SY5Y cells was observed to significantly decrease the

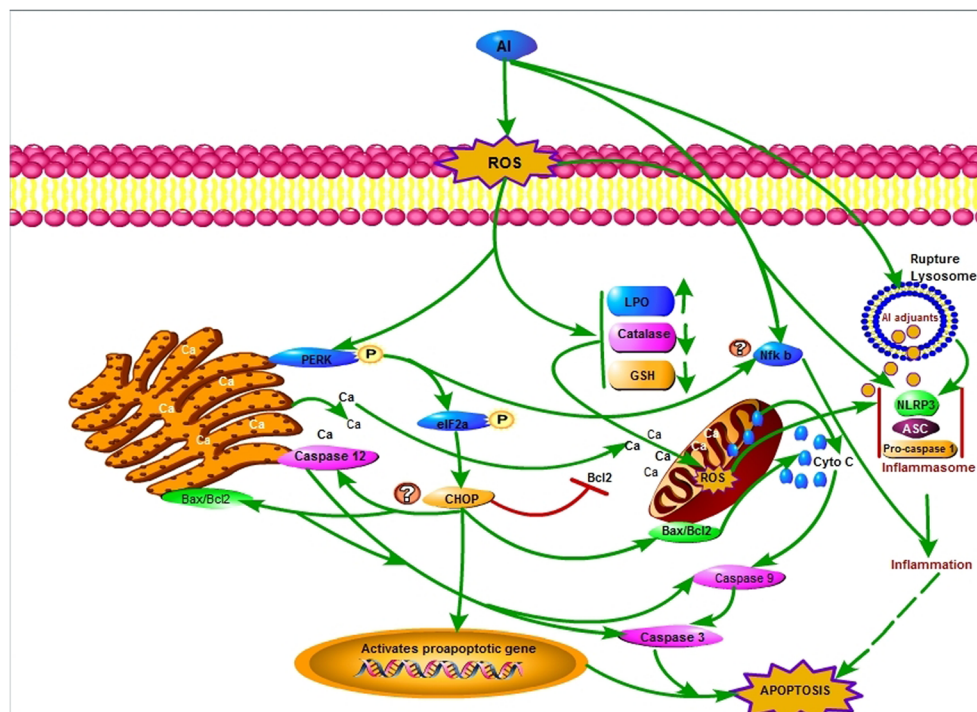


Fig. 6 Schematic diagram illustrating possible crosstalk involved during aluminum toxicity in human neuroblastoma cell line. Aluminum exposure induces oxidative stress and results in perturbation of endoplasmic reticulum function. This leads to release of ER stress-related proteins like PERK, CHOP, and caspase 12 as well as calcium which additionally disturb mitochondrial membrane permeability.

Cytochrome c released into the cytosol activates caspase cascade. Caspase 12 may evoke initiation of apoptosis through direct activation of caspase 3 or by caspase 9. Activation of CHOP also disturbs the ratio of Bax/Bcl2. Al further activates NLRP3 inflammasome and NF- κ B which play a significant role in inflammation and apoptosis

expression of pro-apoptotic proteins Bim and Puma [81]. Further, the attenuation of mitomycin C-induced apoptosis in fibroblasts by CHOP-knockdown was demonstrated to accompany increased Bcl-2/Bax ratio and decreased Bim expression [82]. Similar ameliorative effects were obtained by targeting CHOP to counteract neuronal apoptosis induced by endoplasmic reticulum stress [83]. Thus, CHOP may serve as a potential therapeutic target to address neurodegenerative conditions exacerbated due to aluminum-induced ER stress.

Conclusion

In the present study, we report that aluminum induces oxidative and ER stress and these events promote the signaling of UPR which in turn activates the inflammatory responses through the activation of NF- κ B and NLRP3 inflammasome pathway. These observations demonstrated a relationship between aluminum and neurodegenerative disorders, especially Alzheimer's, where pro-inflammatory responses and ER-evoked protein misfolding are the major hallmarks of the disease.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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