

Recombinant Annexin A5 Alleviates Aluminum Chloride-Induced Cytotoxicity, Mitochondrial Dysfunction, and Apoptotic Signaling in SH-SY5Y Cells

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What's Known

- Aluminum chloride (AlCl_3) is a neurotoxic agent.
- Annexin A5 has neuroprotective effects.

What's New

- Annexin A5 reduced AlCl_3 -induced cell death in SH-SY5Y cells.
- Annexin A5 suppressed the apoptotic effects of AlCl_3 in SH-SY5Y cells.

Abstract

Background: Aluminum contributes to neurodegeneration by inducing oxidative stress, disrupting mitochondrial membrane potential (MMP), and triggering apoptotic signaling cascades. Annexin A5 (ANXA5) is implicated in membrane repair, ion transport regulation, and neuroprotection. This study aimed to evaluate whether ANXA5 can mitigate aluminum-induced cytotoxicity in SH-SY5Y cells.

Methods: This *in vitro* study was conducted at Shiraz University of Medical Sciences, Shiraz, Iran, during 2023-2024. A recombinant ANXA5 was expressed in *Escherichia coli* and purified. The functional integrity of the purified ANXA5 was evaluated by assessing its ability to recognize apoptotic cells. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Rhodamine 123 staining, DNA fragmentation assay, and real-time polymerase chain reaction (real-time PCR) were used to assess the effects of aluminum chloride (AlCl_3) and ANXA5 on cell viability, MMP, DNA damage, and gene expression, respectively. Statistical analysis was conducted using SPSS software (version 16). One-way analysis of variance followed by Tukey's *post-hoc* test was employed for data analysis. Statistical significance was determined at $P < 0.05$.

Results: Electrophoresis confirmed ANXA5 expression and purification. Purified ANXA5 successfully detected apoptotic cells, confirming the preservation of its functional activity after purification. AlCl_3 reduced the viability of SH-SY5Y cells in a dose-dependent manner ($\text{IC}_{50} = 450 \mu\text{M}$). ANXA5 ($1 \mu\text{g/mL}$) reduced the cytotoxic effects of AlCl_3 . In addition, ANXA5 ($1 \mu\text{g/mL}$) reduced the percentage of cells with low MMP, decreased DNA fragmentation, and *Bax* expression induced by AlCl_3 ($P < 0.001$).

Conclusion: These findings demonstrated that ANXA5 provided significant protection against AlCl_3 -induced cellular damage, including cytotoxicity, mitochondrial dysfunction, DNA fragmentation, and the expression of pro-apoptotic genes.

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Keywords • Aluminum • Annexin A5 • Apoptosis

Introduction

Neurodegenerative diseases (NDs) constitute an escalating global health challenge, with their prevalence projected to

surpass 150 million cases by 2060.¹ A defining characteristic of NDs is the progressive loss of neuronal cells, primarily driven by oxidative stress, mitochondrial dysfunction, calcium dysregulation, and activation of apoptotic pathways.² The pathogenesis of these disorders is believed to involve a complex interplay between genetic predispositions and environmental factors.³ Among the environmental contributors, aluminum toxicity has garnered considerable attention. Both environmental and occupational exposures to aluminum compounds have been linked to an increased risk of neurodegeneration.⁴ Notably, elevated aluminum accumulation has been detected in the brains of patients suffering from NDs. Experimental studies using cellular and animal models have further demonstrated that aluminum contributes to neuronal death by inducing oxidative stress, disrupting mitochondrial membrane potential (MMP), dysregulating intracellular Ca^{2+} homeostasis, and triggering apoptotic signaling cascades.⁵⁻⁷ Collectively, these findings supported a contributory role for aluminum in the pathophysiology of neuronal loss observed in neurodegenerative conditions.

Annexin A5 (ANXA5) is a multifunctional calcium-binding protein implicated in diverse cellular processes, including membrane repair, ion transport regulation, apoptosis, and inflammatory responses.⁸ Beyond these functions, ANXA5 exerts cytoprotective effects by attenuating the accumulation of reactive oxygen species, stabilizing mitochondrial function, and modulating apoptotic signaling pathways to promote cell survival.⁶ Importantly, ANXA5 binds to phosphatidylserine residues exposed on the outer leaflet of the plasma membrane of apoptotic cells, which has facilitated its widespread use as a sensitive probe for apoptosis detection.⁹ In recent years, researchers have increasingly characterized ANXA5 as a neuroprotective protein capable of ameliorating neuronal damage in models of Alzheimer's disease and traumatic brain injury.^{10,11} Its protective potential has been demonstrated in other models of neuronal toxicity, where ANXA5 restored mitochondrial function and reduced apoptosis.¹²

Building on these findings, the present study investigated whether ANXA5 could attenuate aluminum-induced cytotoxicity in SH-SY5Y neuroblastoma cells. To better understand putative neuroprotective mechanisms of ANXA5, we examined its effects on DNA fragmentation, mitochondrial membrane integrity, and apoptosis.

Materials and Methods

This *in vitro* study was conducted in the Department of Clinical Laboratory Sciences at Shiraz University of Medical Sciences, Shiraz, Iran, between January 2023 and December 2024. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1399.29).

ANXA5 Expression and Purification

Recombinant human ANXA5 was expressed and purified as described previously.⁶ In brief, the pET28a vector containing the codon-optimized ANXA5 coding sequence (Shinegene; China) was transformed into *Escherichia coli* BL21(DE3) cells (Invitrogen, USA) via the heat shock method.⁶ Transformed bacteria were grown in Luria-Bertani broth (Difco, BD, UK) supplemented with 70 µg/mL kanamycin (Sigma-Aldrich, USA) for 16 hours at 37 °C. To promote protein expression, 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; CinnaClon, Iran) was added, and the culture was incubated at 25 °C for 20 hours. After induction, the bacterial cells were centrifuged and lysed by sonication in phosphate-buffered saline (PBS) lysis buffer containing 2 M urea, 15 mM imidazole, 10% glycerol, 1% Triton X-100, and 5% isopropanol (pH=8.0). The lysate was centrifuged to remove insoluble debris, and the supernatant containing soluble proteins was purified with Ni-NTA affinity chromatography (QIAGEN, Netherlands). The purified ANXA5 protein was then dialyzed against 1,000 mL of carbonate-bicarbonate buffer (pH=9.09.6) using a SnakeSkin dialysis membrane (Thermo Scientific, Germany) to remove impurities and exchange the buffer. The purity and concentration of ANXA5 were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and a Bradford assay.⁶

Assessment of the Apoptosis-Detecting Capability of ANXA5

To evaluate the functional ability of the purified ANXA5, its capacity to bind to apoptotic cells was assessed as previously described.⁹ For this purpose, purified ANXA5 was conjugated with fluorescein isothiocyanate (FITC; SigmaAldrich, USA). The reaction was carried out by incubating ANXA5 in 0.1 M sodium bicarbonate buffer (pH=8.5) with an FITC solution dissolved in DMSO overnight at room temperature in the dark. The unreacted FITC was removed by dialysis, and the ANXA5-FITC conjugate was purified using size-exclusion chromatography.

The human leukemia cell line NALM6 was used

as an established model for evaluating ANXA5–phosphatidylserine binding activity.¹³ Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBBS; Gibco, USA) at 37 °C in a humidified atmosphere containing 5% CO₂. Apoptosis was induced by exposing the cells to 56 °C for 10 min to promote phosphatidylserine externalization. After induction, the cells were washed with binding buffer and incubated with FITClabeled ANXA5 under standard calciumdependent binding conditions in the dark for 10 min. The binding of ANXA5 to apoptotic cells was then assessed using flow cytometry (FACSCalibur, BD Biosciences, USA).

SH-SY5Y Cells Culture

SH-SY5Y cells (Pasteur Institute, Tehran, Iran) were cultured in high-glucose Dulbecco's modified Eagle Medium (DMEM; Invitrogen, USA) containing 10% fetal bovine serum FBS and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Gibco, USA). Cells were maintained in a humidified incubator at 37°C with 5% CO₂.⁶

Cell Viability Assay

SH-SY5Y cells were treated with various concentrations of AlCl₃ and/or ANXA5 for 24 hours. After treatment, 10 µL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) was added to each well, and the cells were incubated for an additional 4 hours at 37°C. Following incubation, the medium was gently removed, and 100 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to the untreated control cells.¹⁴

Mitochondrial Membrane Potential Assessment

Rhodamine 123 (Rh123) staining was used to detect changes in MMP.¹⁴ Rh123 accumulates in mitochondria with intact membranes and high MMP, where it fluoresces upon excitation. In this method, the cells were categorized into two groups with low MMP (low fluorescence) and high MMP (high fluorescence). In brief, SH-SY5Y cells (3×10⁵) were treated with different concentrations of AlCl₃, either alone or in combination with ANXA5 (1 µg/mL), for 24 hours. After treatment, the cells were washed with PBS. Then, the cells were stained with Rh123 solution (2 µg/mL) in the dark at 37°C for 30 min. After incubation, the cells were washed again with PBS, and fluorescence intensity was measured with excitation at 490 nm and

emission detection at 520 nm (FL-1 channel) on a FACS Calibur (BD).

DNA Fragmentation Assay

SH-SY5Y cells were grown on 6-cm plates with a density of 6×10⁶ cells per plate and subjected to 450 µM AlCl₃, with or without the addition of 1 µg/mL ANXA5, for 24 hours. After treatment, the cells were collected, washed with PBS, and lysed with 500 µL of saponin for 30 min at room temperature. The genomic DNA was then extracted with a 25:24:1 (v/v/v) solution of phenol, chloroform, and isoamyl alcohol, followed by ethanol precipitation. The DNA pellet was resuspended in Tris-EDTA buffer containing 20 µg/mL RNase. DNA concentration was determined using spectrophotometry. The purified genomic DNA samples were separated on a 1.0% agarose gel with 1 µg/mL safe dye. The DNA bands were visualized under UV light.⁶

Gene Expression Evaluation

The cells were treated with AlCl₃ (450 µM) or ANXA5 (1 µg/mL), either individually or in combination, for 24 hours. RNA extraction was carried out using TRIzol (CinnaClone, Iran). The synthesis of cDNA was executed utilizing the CinnaClone cDNA kit. Real-time polymerase chain reaction (real-time PCR) was performed with the cDNA and specific primers.⁶ Real-time PCR using the Rotor-Gene Q system (QIAGEN, USA) was utilized to investigate the effects of the treatments on the expression of *Bax* and *Bcl-2*. The PCR conditions comprised an initial denaturation step at 95 °C for 30 seconds, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 30 seconds. A final extension step was performed at 72 °C for 30 seconds. The housekeeping TATA binding protein (*TBP*) gene was used to calculate fold change using the 2^{-ΔΔCT} method.

Statistical Analysis

The normal distribution of the data was evaluated using the Shapiro-Wilk test. The presence of significant differences between the groups for variables with a normal distribution was evaluated using a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Interaction effects between AlCl₃ and ANXA5 were evaluated using a two-way ANOVA. Significant interactions identified by two-way ANOVA were further analyzed using Tukey's *post hoc* test. P values less than 0.05 were considered statistically significant. SPSS software (version 21, IBM, USA) was used for data analysis.

Results

Expression and Purification of ANXA5

Recombinant ANXA5 was overexpressed using 0.5 mM IPTG at 37°C for 4 hours. The expressed protein was purified using Ni-NTA affinity chromatography. SDS-PAGE verified that ANXA5 was successfully expressed and purified, with a clear band at the predicted molecular weight of approximately 35 kDa (figure 1). The final concentration of purified ANXA5 was 1.1 mg/mL.

Evaluation of the Apoptotic Cell Detection Capability of Purified ANXA5

To confirm the functional integrity of the purified ANXA5, its capacity to recognize apoptotic cells, a well-characterized property, was assessed. As illustrated in figure 2, purified ANXA5 detected a significantly higher proportion of apoptotic cells in the heat-treated group (Panel B) than the untreated control group, indicating that the protein retained its apoptotic cell-binding activity post-purification.

MTT Assay to Evaluate the Neuroprotective Effect of ANXA5 on SH-SY5Y

As indicated in figure 3A, AlCl_3 decreased the viability of SH-SY5Y cells in a dose-dependent manner. The findings were fitted to a logarithmic scale, and the IC_{50} was determined to be 450 μM . ANXA5 did not significantly affect the viability of SH-SY5Y cells at concentrations up to 20 $\mu\text{g/L}$. Two-way ANOVA, followed by Tukey's multiple comparison *post hoc* test, was used to determine the interaction between AlCl_3 and ANXA5. When the cells were treated with AlCl_3 (450 μM) and ANXA5 (1 $\mu\text{g/L}$) for 24 hours, ANXA5 significantly attenuated the cytotoxic

effects of AlCl_3 ($F=9.329$, $P=0.012$; figure 3B).

ANXA5 Protected Against AlCl_3 -Induced Mitochondrial Membrane Potential Loss

The impact of the treatments on the MMP of SH-SY5Y cells is illustrated in figure 4. Flow cytometry plots demonstrated the categorization of the SH-SY5Y cells into low MMP (N1) and high MMP (N2) cells. In the control and ANXA5 groups, the mean proportion of N2 cells was substantially higher than that of N1 cells, indicating a greater number of cells with high MMP.

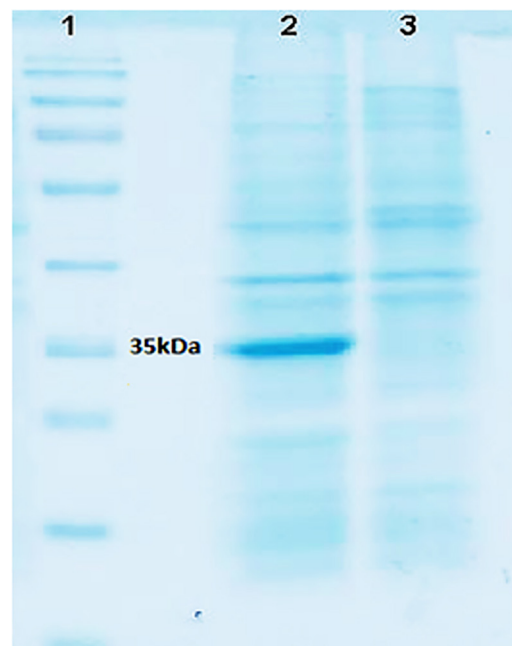


Figure 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed the expression of Annexin A5. The expression of Annexin A5 was induced by IPTG (1 mM). Lane 1: molecular weight marker, Lane 2: IPTG-induced condition, Lane 3: Uninduced condition. IPTG: isopropyl β -D-1-thiogalactopyranoside

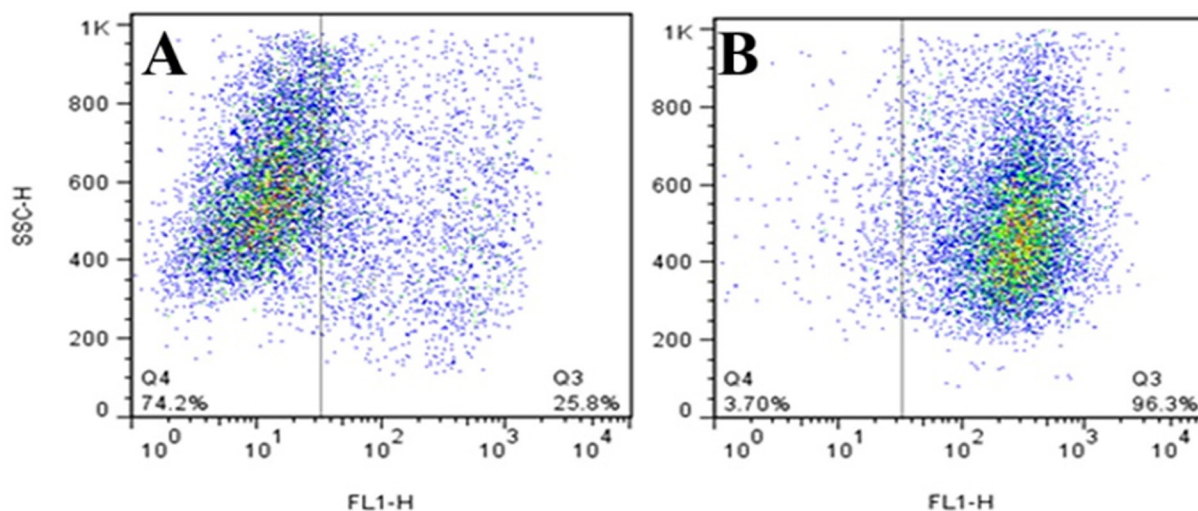


Figure 2: Flow cytometry revealed the binding of FITC-labeled ANXA5 to apoptotic cells. Apoptosis was induced by exposing NALM-6 cells to 56 °C for 10 min. The extent of apoptosis and the binding of FITC-labeled ANXA5 were assessed using flow cytometry (FACSCalibur, BD Biosciences). Panel A shows the control (untreated) group, while panel B represents the heat-treated apoptotic group. FITC: Fluorescein isothiocyanate; ANXA5: Annexin A5

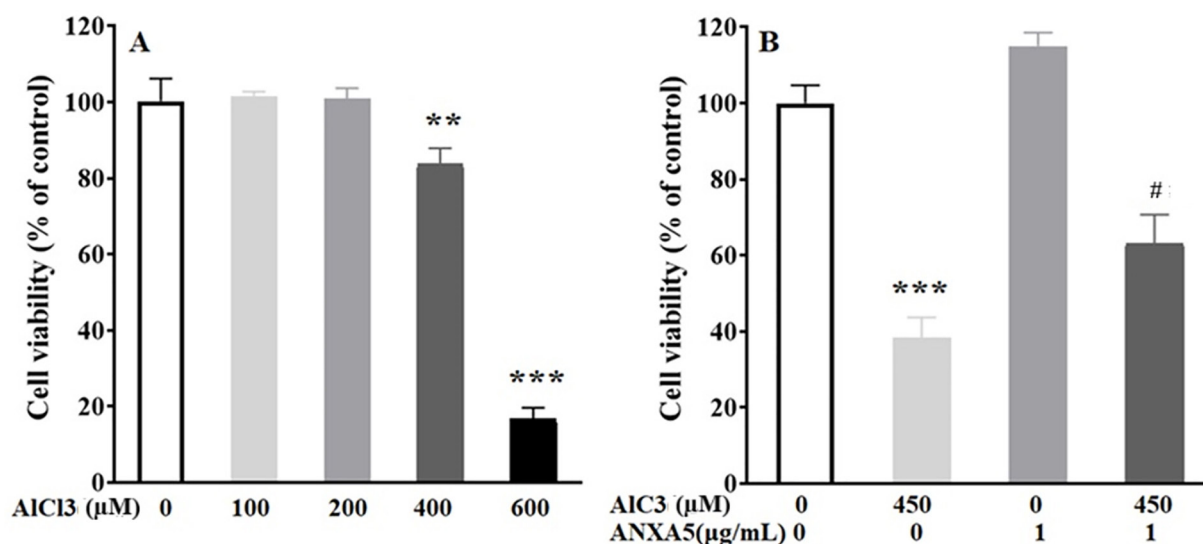


Figure 3: (A) SH-SY5Y cells were treated with different concentrations of AlCl₃ for 24 hours, and the cell viability was measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To determine the presence of a significant difference between groups, a one-way ANOVA test was employed, followed by a Tukey's multiple comparisons test. **P<0.01 and ***P<0.001 compared to the control group. (B) To determine the interaction between Annexin A5 (ANXA5) and AlCl₃ on cell viability, the cells were treated with AlCl₃ (450 μM) in the presence or absence of ANXA5 (1 μg/mL) for 24 hours, and the cell viability was measured using the MTT assay. A two-way ANOVA, followed by Tukey's multiple comparisons *post hoc* test, was used to determine the interaction between AlCl₃ and ANXA5. ***P<0.001 compared to the control group and #P=0.012 compared to the SH-SY5Y cells treated with AlCl₃ (450 μM) alone. ANOVA: Analysis of variance; ANXA5: Annexin A5; AlCl₃: Aluminum chloride; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

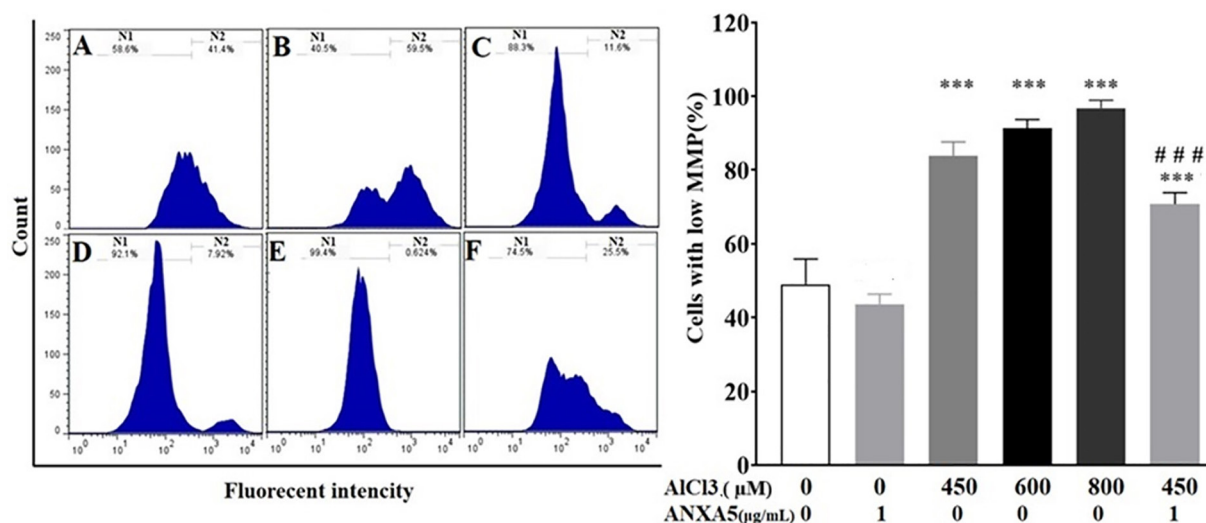


Figure 4: ANXA5 reversed the effects of AlCl₃ on mitochondrial membrane potential in SH-SY5Y cells. The cells were treated with various concentrations of AlCl₃ in the presence and absence of ANXA5, and the number of cells with low MMP (N1) and high MMP (N2) was counted using Rh123 staining. Panel A: Control, B: ANXA5 (1 μg/mL), C: AlCl₃ (450 μM), D: AlCl₃ (600 μM), E: AlCl₃ (800 μM), and F: ANXA5 (1 μg/mL) and AlCl₃ (450 μM). The effects of various concentrations of AlCl₃ on the MMP were analyzed using a one-way ANOVA, followed by Tukey's multiple comparisons *post hoc* test. ***P<0.001 compared to the control group. The interaction between ANXA5 and AlCl₃ on the MMP was evaluated using a two-way ANOVA, followed by Tukey's multiple comparisons *post hoc* test. ###P=0.007 compared to AlCl₃ groups. ANOVA: Analysis of variance; ANXA5: Annexin A5; MMP: Mitochondria membrane potential; AlCl₃: Aluminum chloride

In SH-SY5Y cells treated with different concentrations of AlCl₃, the percentage of N1 cells exceeded that of N2 cells in a dose-dependent manner, indicating an increased number of cells experiencing MMP loss compared to the control group (P<0.001). When SH-SY5Y cells were treated with ANXA5 (1 μg/mL) and AlCl₃ (450 μM), two-way ANOVA analysis revealed that the proportion of N1 cells

dropped significantly compared to those treated with AlCl₃ alone (F=10.984, P=0.007), indicating the protective properties of ANXA5 against AlCl₃-induced MMP loss.

ANXA5 Decreased AlCl₃-Induced DNA Damage

To investigate the effect of ANXA5 on AlCl₃-induced DNA fragmentation, SH-SY5Y cells were treated with AlCl₃ (450 μM),

ANXA5 (1 $\mu\text{g/mL}$), or a combination of both for 24 hours. Analysis by agarose gel electrophoresis demonstrated that AlCl_3 treatment led to significant DNA fragmentation, indicative of apoptosis. In contrast, treatment with ANXA5 alone did not cause noticeable DNA fragmentation. Importantly, co-treatment with ANXA5 (1 $\mu\text{g/mL}$) substantially reduced the DNA fragmentation induced by AlCl_3 , suggesting a protective effect of ANXA5 against AlCl_3 -mediated apoptotic DNA damage (figure 5).

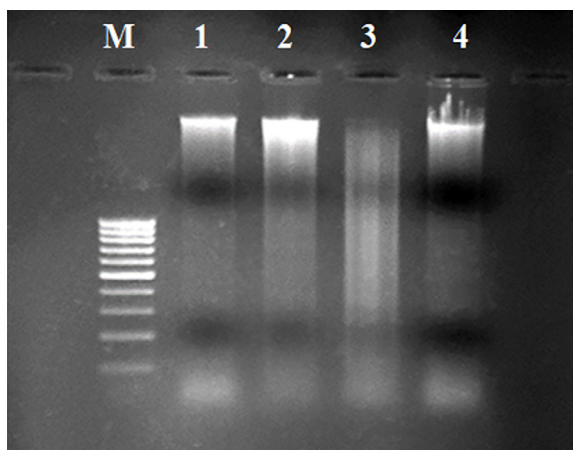


Figure 5: ANXA5 reversed the effects of AlCl_3 on DNA damage in SH-SY5Y cells. A DNA fragmentation assay was performed on SH-SY5Y cells (6×10^6) treated with 1 $\mu\text{g/mL}$ ANXA5 with or without 450 μM AlCl_3 . Lane 1: Untreated control group; Lane 2: ANXA5 group; Lane 3: AlCl_3 group; Lane 4: AlCl_3 +ANXA5 group; M: Molecular weight marker; ANXA5: Annexin A5; AlCl_3 : Aluminum chloride

ANXA5 alters AlCl_3 -Induced changes in apoptotic gene expression

The interaction between ANXA5 and AlCl_3 on apoptotic markers, *Bax* and *Bcl-2* mRNA, was evaluated using two-way ANOVA. As shown in figure 6, a strong significant effect of AlCl_3 on increasing *Bax* expression ($F=34.328$, $P=0.001$) and an ANXA5 $\times\text{AlCl}_3$ interaction on decreasing *Bax* ($F=20.76$, $P=0.004$) were observed, indicating inhibitory effects of ANXA5 on AlCl_3 -induced *Bax* expression. In contrast, analysis of the *Bcl-2* expression revealed no significant main effects of ANXA5 ($F=0.002$, $P=0.966$), AlCl_3 ($F=0.002$, $P=0.966$), and ANXA5 $\times\text{AlCl}_3$ interaction ($F=0.993$, $P=0.358$) on *Bcl-2* expression.

Discussion

The present study demonstrated that purified recombinant ANXA5 exerted significant protective effects against AlCl_3 -induced neurotoxicity in SH-SY5Y neuroblastoma cells. Exposure to AlCl_3 induced cytotoxicity, MMP loss, DNA fragmentation, increased *Bax* expression, and cell death. In contrast, ANXA5 markedly attenuated AlCl_3 -induced injury by preserving mitochondrial function, reducing DNA fragmentation, suppressing *Bax* expression, and improving overall cell viability, suggesting a strong neuroprotective role against aluminum-induced cellular damage. These findings were consistent with previous

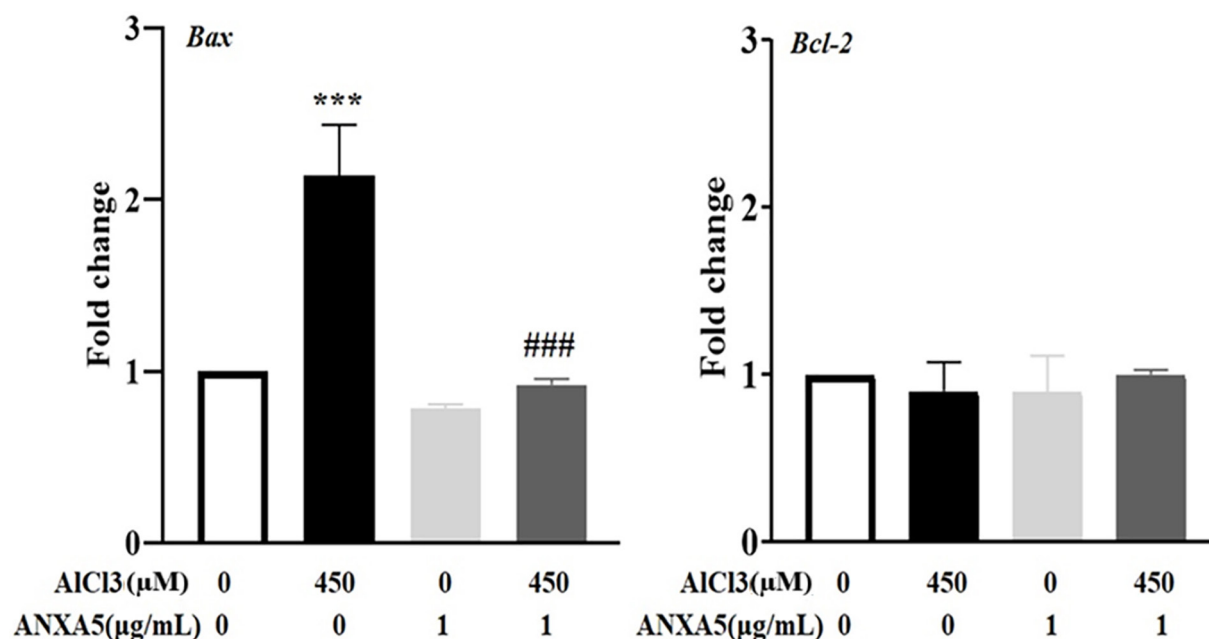


Figure 6: ANXA5 reversed the effects of AlCl_3 on *Bax* Expression. SH-SY5Y cells were treated with AlCl_3 and ANXA5 alone or together, and the levels of *Bax* and *Bcl-2* mRNA were measured using real-time PCR. Statistical comparisons were performed using two-way ANOVA with ANXA5 and AlCl_3 as fixed factors. Tukey *post hoc* test was used for multiple comparisons. Data are reported as mean $\pm$ SD. *** $P=0.001$ compared to the control group, ### $P=0.004$ compared to AlCl_3 group. ANOVA: Analysis of variance; ANXA5: Annexin A5; AlCl_3 : Aluminum chloride; PCR: Polymerase chain reaction

studies, which indicated that aluminum-associated neurotoxicity was mediated through several interrelated mechanisms, including mitochondrial impairment and activation of pro-apoptotic pathways.¹⁵⁻¹⁸ This further supported the robustness of AlCl_3 -induced cellular damage in SH-SY5Y cells as an *in vitro* model of neurotoxicity.¹⁹

In the present study, recombinant ANXA5, a calcium-binding protein well-known for its ability to recognize and bind to phosphatidylserine exposed on the cell surface of apoptotic cells, was produced and purified.²⁰ Therefore, this property was used to verify the activity of the purified ANXA5. The results demonstrated that FITC-ANXA5 bound to phosphatidylserine exposed on the outer membrane of the apoptotic cells. This confirmed that ANXA5 maintained its biological integrity and functionality post-purification, which is essential for its use in downstream functional assays.

AlCl_3 reduces the viability of SH-SY5Y cells with an IC_{50} of 450 μM , consistent with its known neurotoxic properties, likely mediated via mitochondrial damage and oxidative damage to biomolecules.²¹ These changes closely resemble the pathological alterations observed in NDs. Rather and colleagues reported that AlCl_3 exposure induced DNA damage and mitochondrial impairment in neuroblastoma cell lines.²² The observed DNA fragmentation in our study was consistent with these findings and suggested that oxidative DNA damage was a critical step in aluminum-induced cytotoxicity. Importantly, ANXA5 alone did not negatively impact cell viability, indicating a non-toxic profile, and it significantly reversed the effects of AlCl_3 . This protective effect might be linked to its membrane-stabilizing, anti-apoptotic, or anti-inflammatory properties.²⁰

Loss of MMP is a key event in initiating apoptosis.²³ AlCl_3 exposure increased the number of cells with low MMP, indicating mitochondrial damage. Previous studies demonstrated that AlCl_3 disrupts mitochondrial integrity, leading to cytochrome *c* release and caspase activation.²⁴ Similarly, increased Bax expression indicated activation of the intrinsic apoptotic pathway, which was previously reported as a hallmark of AlCl_3 neurotoxicity.²⁴ Conversely, co-treatment with ANXA5 significantly reduced MMP loss, suggesting that ANXA5 helps maintain mitochondrial integrity under toxic stress. This effect might underlie the improved viability observed and highlights the importance of ANXA5 in preserving mitochondrial function, which is central to cell survival and energy homeostasis.²³

DNA fragmentation, a hallmark of late-stage apoptosis,²⁵ was prominently induced by AlCl_3 treatment, as observed in agarose gel electrophoresis. ANXA5 alone did not induce DNA fragmentation, confirming its non-apoptotic nature. More importantly, co-treatment with ANXA5 significantly reduced DNA fragmentation, reinforcing its anti-apoptotic effect. This suggested that ANXA5 might interfere with the execution phase of apoptosis, potentially by stabilizing membranes or modulating apoptotic signaling pathways. At the molecular level, AlCl_3 significantly upregulated the expression of *Bax*, a pro-apoptotic gene that promotes cell death, while having no significant effect on *Bcl-2*, an anti-apoptotic gene that inhibits cell death. The selective upregulation of *Bax* contributes to mitochondrial permeabilization and apoptosis initiation.²⁶ Interestingly, ANXA5 alone did not alter the expression of either gene but effectively reversed the AlCl_3 -induced *Bax* upregulation. This indicates that ANXA5 acts specifically under stress conditions, potentially inhibiting upstream apoptotic signals triggered by AlCl_3 . These findings suggested a targeted modulatory role of ANXA5 on the Bax-dependent apoptotic pathway.

In addition to its anti-apoptotic and membrane-stabilizing effects, ANXA5 might protect against aluminum-induced neurotoxicity through its ability to modulate intracellular calcium homeostasis. Alpha-synuclein plays a central role in aluminum-induced toxicity, contributing to mitochondrial dysfunction and neuronal apoptosis.²⁷ In that context, lithium and nimodipine were shown to mitigate aluminum toxicity, partly by regulating calcium influx and preventing calcium-mediated damage.⁷ Given that ANXA5 interacts with calcium, it might similarly help stabilize calcium levels under aluminum stress. Dysregulated calcium signaling is closely associated with α -synuclein aggregation, which exacerbates mitochondrial dysfunction and apoptosis. The potential of ANXA5 to regulate calcium homeostasis might interfere with this pathogenic cycle.²⁸ This suggested that calcium regulation could be a key mechanism by which ANXA5 exerts neuroprotection. Further studies are required to investigate the role of ANXA5 in calcium handling, its interaction with synuclein aggregation, and its collective impact on aluminum-induced neurodegeneration.

These data provided evidence that ANXA5 exerted a protective role against AlCl_3 -induced neurotoxicity through multiple mechanisms, including the preservation of mitochondrial integrity, reduction in DNA fragmentation, inhibition of Bax-mediated apoptotic signaling,

and improvement in overall cell viability. The multifaceted action of ANXA5 implied that it did not merely serve as a passive marker of apoptosis but might actively participate in membrane stabilization, signaling modulation, or inhibition of apoptosis progression. While these findings provided valuable insights into the protective effects of ANXA5 against aluminum toxicity, we acknowledge certain limitations. This study primarily focused on the expression of *Bax* and *Bcl-2* at the mRNA level, and further investigation is required to confirm these effects at the protein level. Additionally, the precise molecular pathway mediating the protective effects of ANXA5 was not fully elucidated in this study. Therefore, future research should aim to address these aspects to provide a more comprehensive understanding of the mechanism of action of ANXA5 in this context.

Conclusion

This study highlighted the potential of ANXA5 as a protective agent against aluminum-induced neurotoxicity, which might have implications for neurodegenerative disorders where aluminum accumulation and oxidative stress contribute to pathogenesis. Future studies should focus on mechanistic studies to elucidate the signaling targets of ANXA5, *in vivo* validation in animal models of neurodegeneration, dose optimization, and delivery strategies for therapeutic applications. The non-toxic nature and endogenous origin of ANXA5 further support its candidacy as a therapeutic molecule or delivery system in neuroprotective interventions.

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Authors' Contribution

SM.P: Conceptualization, data gathering, data analysis, study design, investigation, drafting and reviewing the manuscript; M.M: Conceptualization, data gathering, data analysis, investigation, methodology, software, and drafting; M.A.T.: Conceptualization, data

gathering, data analysis, study design, project administration, validation, investigation, drafting and reviewing the manuscript; All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of AI

The authors acknowledge that ChatGPT5.4 was used for English language editing and improving the clarity of the text.

Conflict of Interest: None declared.

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