Original Article

# Annexin A5 Protects SH-SY5Y Cells against L-Glutamate-Induced Cytotoxicity

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## Abstract

**Background:** L- glutamate- induced neurotoxicity is linked to neuronal loss in neurodegenerative diseases and stroke. Annexin A5 (ANXA5) is a cytosolic protein that binds calcium in eukaryotic organisms. This study aimed to evaluate the protective effects of a recombinant ANXA5 against L-glutamate-induced cell death and mitochondrial dysfunction in SH-SY5Y cells.

**Methods:** ANXA5 was expressed in *E. coli* and subsequently purified using affinity chromatography. The effect of L-glutamate (0-300 mM) alone or in combination with ANXA5 (0-5µg/mL) on the viability of the SH-SY5Y cells was assessed using the MTT assay. Mitochondrial membrane potential (MMP) dissipation was detected by rhodamine 123 staining and the flow cytometry method. The expressions of *Bax*, *Bcl-2*, and *NF-E2-Related Factor 2* (*Nrf-2*) genes were determined by real-time polymerase chain reaction. GraphPad Prism 8.0 was used to analyze the data using either one-way ANOVA or the Kruskal-Wallis test. $P<0.05$ was considered statistically significant.

**Results:** The findings revealed that L-glutamate reduced the cell viability of SH-SY5Y cells in a dose-dependent manner ($P<0.001$) (IC50=165 mM). Moreover, treating SH-SY5Y cells with 165 mM of L-glutamate increased MMP dissipation, enhanced *Bax* expression, and reduced the expression of *Bcl-2* and *Nrf-2*, compared to the control group. ANXA5 alone had no significant effects. However, it reversed the effects of L-glutamate on cell death, MMP dissipation, and gene expression in the SH-SY5Y cells.

**Conclusion:** The data suggest that ANXA5 can protect SH-SY5Y cells against glutamate-induced cell death and mitochondrial dysfunction, indicating its possible protective effect against glutamate-induced neurodegeneration.

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**Keywords** ● Neurotoxicity ● Mitochondria ● Annexin A5 ● Apoptosis ● NF-E2-related factor 2

## What's Known

- L-glutamate induces neurodegeneration by increasing oxidative stress.
- Annexin A5 exhibits anti-apoptotic and membrane protective roles in eukaryotic cells.

## What's New

- Annexin A5 reduced L-glutamate-induced cell death.
- Annexin A5 blocked L-glutamate-induced mitochondrial dysfunction.
- Annexin A5 reversed L-glutamate-induced Bax expression.
- Annexin A5 increased nuclear factor-2 expression.

## Introduction

L-glutamate (L-Glu) serves as the primary excitatory amino acid in the human central nervous system, where it plays essential roles in diverse physiological functions by activating its metabotropic and ionotropic receptors.[1] However, elevated levels of L-Glu are neurotoxic, leading to cell death by damaging cellular components. This neurotoxicity is linked to neuronal loss in various neurological conditions, including cerebrovascular

accident, physical injury, Alzheimer's disease (AD), Parkinsonian syndrome, and Huntington's disease,[2] making it crucial to find protective strategies to combat these injuries.

The research has proposed several molecular mechanisms for L-Glu-induced neurotoxicity. Oxidative stress (OS), $Ca^{2+}$ homeostasis dysregulation, mitochondrial damage, and apoptosis are amongst these mechanisms.[3] *In vitro* studies have shown that high L-Glu triggers OS in neurons by dysregulating cysteine glutamate antiporters and reducing cellular glutathione (GSH) levels,[4] resulting in the buildup of reactive oxygen species (ROS) and OS. OS induces $Ca^{2+}$ ion influx, resulting in high cellular $Ca^{2+}$ concentration, which activates apoptosis by activating caspases and inducing mitochondrial dysfunction.[5] Therefore, antioxidant agents such as vitamin E and plant-based antioxidants can mitigate L-Glu neurotoxicity.[6]

Annexin A5 (ANXA5) is a calcium-binding protein that exhibits antioxidant and anti-apoptotic characteristics.[7] Recent studies have shown that this protein can prevent further cellular membrane damage by binding to damaged membrane phospholipids[8] and act as a neuroprotective agent.[9] Additionally, ANXA5 can prevent neuronal cell death by inhibiting inflammatory signaling pathways such as NF-κB and reducing ROS production.[10] However, the possible protective role of ANXA5 against glutamate-induced damage has not been investigated yet.

The neuroblastoma-derived SH-SY5Y cell line is a well-known neuronal cellular model and serves as an appropriate tool for studying neurotoxicity.[11, 12] This cellular model is frequently used to study L-Glu-induced neurotoxicity.[2] This study aims to evaluate the protective effects of a recombinant ANXA5 against L-Glu-induced toxicity in SH-SY5Y cells.

## Materials and Methods

This study was conducted in the Diagnostic Laboratory Sciences and Technology Research Center of Shiraz University of Medical Sciences between April and December 2024. This research received approval from the Ethics Committee at Shiraz University of Medical Sciences (IR.SUMS.REC.1403.152).

### *Expression and Purification of ANXA5 Protein*

A pET28a plasmid harboring a codon-optimized human ANXA5-coding sequence with a His-tag sequence at its N-terminal domain, supplied by ShineGene Bio-Technologies Company (Shanghai, China), was used to overexpress ANXA5 protein in competent *BL21 (DE3)* cells (Invitrogen), as previously described.[9] The bacteria were transformed with the plasmid and cultured in LB broth (Difco, BD, UK) containing 70 µg/mL kanamycin (Sigma-Aldrich, USA) at 37 ˚C overnight. The expression of ANXA5 was examined in the presence (induced condition) and absence (non-induced) of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; CINNAclone, IRAN) at 25 ˚C for 20 hours. This temperature was the optimum temperature for the expression of ANXA5, according to our previous study.[9] The cell pellet was harvested from the bacterial culture and lysed by ultrasonication in lysis buffer (300 mM NaCl, 50 mM $NaH_2PO_4$, pH 8.0, 2 M urea, 15 mM imidazole, 10% glycerol, 1% Triton, and 5% isopropanol). The supernatant was collected, and ANXA5 was purified using Ni-NTA chromatography (QIAGEN, Netherlands). The purified protein was filtered through a sterile syringe filter (0.22 µm, Membrane Solutions, USA) to remove contaminants. The protein concentration was measured using the Bradford method with bovine serum albumin (Sigma-Aldrich, USA) as a standard protein, as previously described.[13] The purity of the recombinant protein was confirmed using SDS-PAGE.

### *SH-SY5Y Cell Culture*

SH-SY5Y cells were purchased from the Pasteur Institute (Tehran, Iran) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Inc., USA) with high glucose concentration (4.5 g/L) containing 10% Fetal Bovine Serum (FBS, Gibco), 100 units/mL penicillin, and 100 µg/mL streptomycin. Cultures were maintained in a humidified incubator at 37 °C with 5% $CO_2$.

### *MTT Assay*

Cell viability was measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA) assay. In brief, the SH-SY5Y cells ($1\times10^4$ cells) were cultured in each well of a 96-well plate. After 24 hours, cells were treated with L-Glu (0-300 mM) and ANXA5 (0-5 µg/mL) for 24 hours. The cell viability was then evaluated using the MTT assay. Briefly, the cells were incubated with MTT solution (5 g/L) for 4 hours at 37 °C. Dimethyl sulfoxide was added, and the absorbance was measured at 545 nm using a microplate reader.[14] The results were expressed as percentages relative to the control group, and calculations were performed using Prism 10.2.3 software. The results were expressed as a percentage of the control group.

*Mitochondrial Membrane Potential Assay*

Rhodamine 123 (Rh123) staining was used to determine mitochondrial membrane potential (MMP) dissipation. This method is based on the accumulation of Rh123 in the mitochondria with an intact membrane and high MMP.[15, 16] In brief, SH-SY5Y cells ($3\times10^5$) were seeded in each well of 6-well plates. The cells were exposed to L-Glu (165 mM) alone or in combination with ANXA5 (2.5 µg/mL) for 24 hours. After completing the treatment, the cells were washed with phosphate buffer saline (PBS). Then, Rh 123 solution (2 µg/mL) was added to the cells in the dark. The cells were incubated for 30 min at 37 °C and washed with PBS buffer. The stained cells were stimulated at a wavelength of 490 nm, and the resulting fluorescent light emitted at 520 nm was recorded in the FL-1 channel of a FACS Calibur (BD).[15]

*Real-time PCR*

Real-time PCR was used to investigate the effects of the treatments on the expression of *Bax*, *Bcl-2*, and *Nrf-2* genes. The cells were treated with L-Glu (165 mM) or ANXA5 (2.5 and 5 µg/mL) alone or in combination for 24 hours. RNA was extracted using TRIzol (CINNA clone, Iran). cDNA synthesis was performed using the Sinnaclon kit (Sinnagene, Iran). Real-time PCR was conducted using cDNA and specific primers (table 1). The Rotor-Gene Q system (QIAGEN, USA) was used. PCR conditions included a denaturation step at 95 °C for 30 sec, followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 30 sec, and extension at 72 °C for 30 secs. A final extension step was conducted at 72 ºC for 30 sec. The TATA binding protein (*TBP*) gene was employed as a reference housekeeping gene,[17] and fold change was calculated using the $2^{-\Delta\Delta CT}$ method.

*Statistical Analysis*

GraphPad Prism software (version 8.0, GraphPad Software, Inc., USA) was used to analyze the data. The Shapiro-Wilk test was used to determine whether the data followed a normal distribution. The data with a normal distribution were analyzed using one-way ANOVA, followed by the Tukey *post-hoc* test. The Kruskal-Wallis test was used to analyze the non-parametric data. Results are presented as mean±standard deviation. P-values less than 0.05 were considered statistically significant.

## Results

*ANXA5 Expression and Purification*

Figure 1 shows the SDS-PAGE of ANXA5 in the induced (IPTG; 0.1 mM) and non-induced conditions. IPTG (0.1 mM) increased the expression of ANXA5 compared to the non-induced condition. The presence of the His-tag sequence facilitated the purification of ANXA5 using affinity chromatography. The purity of ANXA5 was confirmed using SDS-PAGE. Based on the Bradford protein assay, the concentration of purified ANXA5 in the induced condition was 3.4 mg/mL.

*The Cytotoxic Effects of L-Glu on the Cell Viability of SHSY-5Y Cells*

The SHSY-5Y cells were exposed to various concentrations of L-Glu (0 to 300 mM) for 24 hours, and the cell viability was evaluated using the MTT assay. As shown in figure 2A, the treatment with L-Glu decreased the viability of SH-SY5Y cells dose-dependently. The data were fitted to the sigmoidal dose-response model, and an IC50 value of 165 mM was calculated for L-Glu (figure 2B).

*The Protective Effect of ANXA5 against L-Glu-Induced Toxicity in SHSY-5Y Cells*

The SH-SY5Y cells were treated with ANXA5 alone (1.0, 1.5, 2.0, 2.5, and 5.0 µg/mL) or in combination with L-Glu (165 mM), and the cell viability was evaluated using the MTT assay (figure 3). As shown in figure 3A, ANXA5 had no significant effects on the viability of the SH-SY5Y cells. Furthermore, the findings revealed that ANXA5 protected the SH-SY5Y cell against L-Glu-induced cell death in a dose-dependent manner (figure 3B).

**Table 1:** The list of primers used for real-time PCR

| Primers | Sequences | Primers |
|---|---|---|
| *TBP* (F) | 5′-GTGCCCGAAACGCCGAAT-3′ | *TBP* (F) |
| *TBP* (R) | 5′-GTCTGGACTGTTCTTCACTCTTGG-3′ | *TBP* (R) |
| *Bax* (F) | 5′-CCGAGAGGTCTTTTTCCGAG-3′ | *Bax* (F) |
| *Bax* (R) | 5′-AAGTCCAATGTCCAGCCCA-3′ | *Bax* (R) |
| *Bcl-2* (F) | 5′-GCGACTCCTGATTCATTGGG-3′ | *Bcl-2* (F) |
| *Bcl-2* (R) | 5′-CTACTTCCTCTGTGATGTTGTATT-3′ | *Bcl-2* (R) |
| *Nrf-2*(F) | 5′-AGATGACAATGAGGTTTCTTCGG-3′ | *Nrf-2*(F) |
| *Nrf-2*(R) | 5′-AGTTTGGCTTCTGGACTTGG-3′ | *Nrf-2*(R) |

*TBP*: TATA binding protein; F: forward primer; R: reverse primer; *Bax*: BCL2 Associated X; *Bcl-2*: B-cell leukemia/lymphoma 2 protein; *Nrf-2*: NF-E2-Related Factor 2

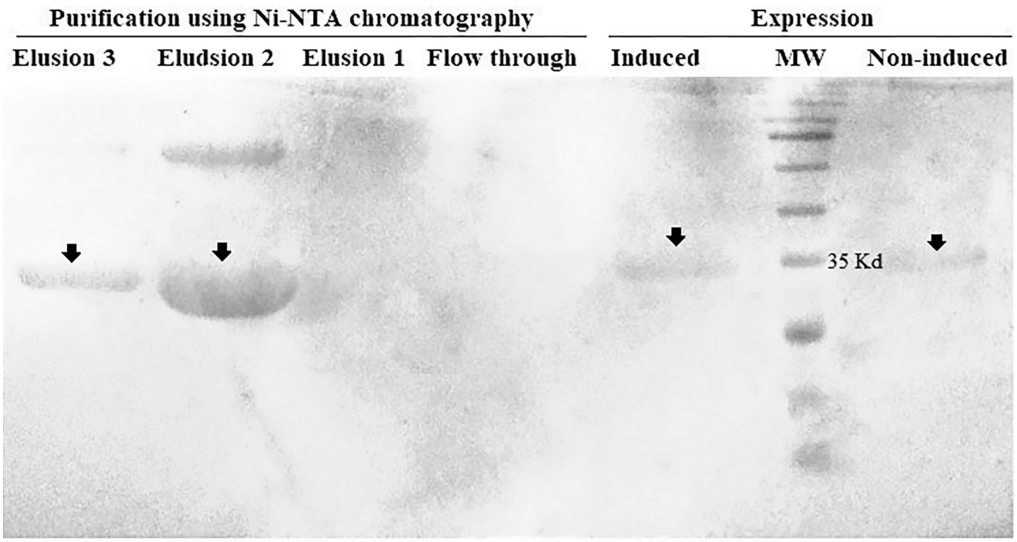


**Figure 1:** Annexin A5 was expressed in *E. coli* BL21 (DE3) in the absence (non-induced) and presence of isopropyl β-D-1-thiogalactopyranoside (induced). Annexin A5 was purified using Ni-NTA column chromatography and run on SDS-PAGE (arrows). MW: Molecular weight marker; Ni-NTA chromatography: Nickel-Nitrilotriacetic acid chromatography

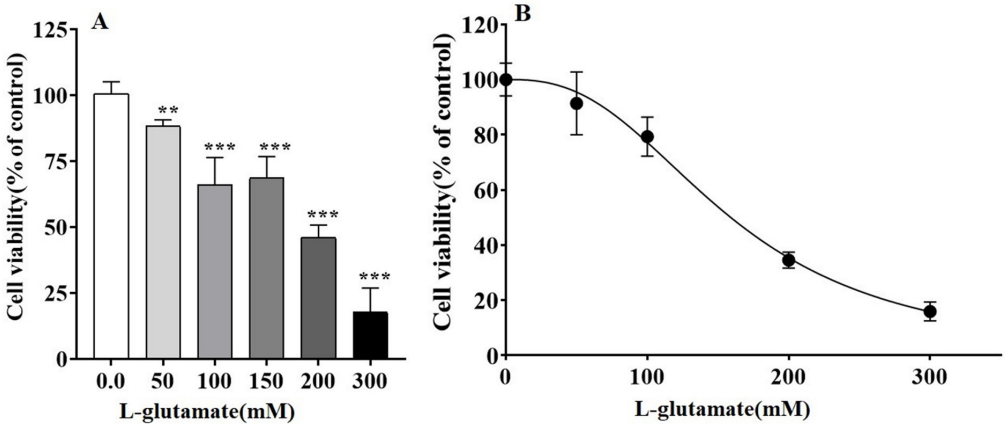


**Figure 2:** L-glutamate reduced the cell viability of SHSY-5Y cells. The cells were treated with the various concentrations of L-glutamate (0 to 300 mM) for 24 hours, and the cell viability was assessed using the MTT assay. The experiments were performed in triplicate. The data were analyzed using the Kruskal-Wallis test (A). To calculate the IC50 for L-glutamate, the data were fitted to the sigmoidal dose-response model (B). Values are as mean±SD. **P<0.01, ***P<0.001 compared to the control group.

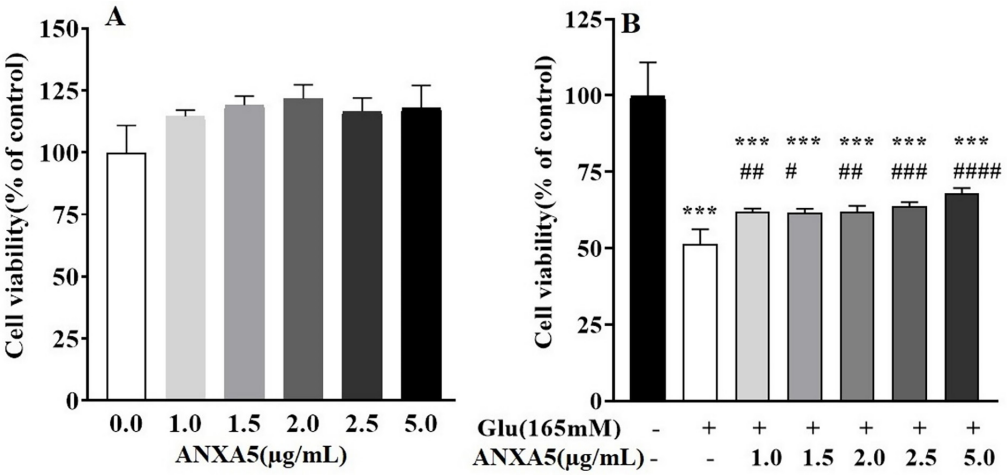


**Figure 3:** Annexin A5 reduced the cytotoxic effects of L-glutamate in SH-SY5Y cells. The SH-SY5Y cells were treated with different concentrations of Annexin A5 alone (A) or in the presence of L-glutamate (165 mM) (B) for 24 hours, and the cell viability was measured using the MTT assay. The experiments were performed in triplicate. A) The Kruskal-Wallis test was used to evaluate the non-parametric data. B) One-way ANOVA test followed by Tukey *post hoc* test was used to analyze the parametric data. The data are as mean±SD. ***P<0.001 *vs.* control group; ##P<0.01 *vs.* L-glutamate group. ANXA5: Annexin A5; Glu: L-glutamate

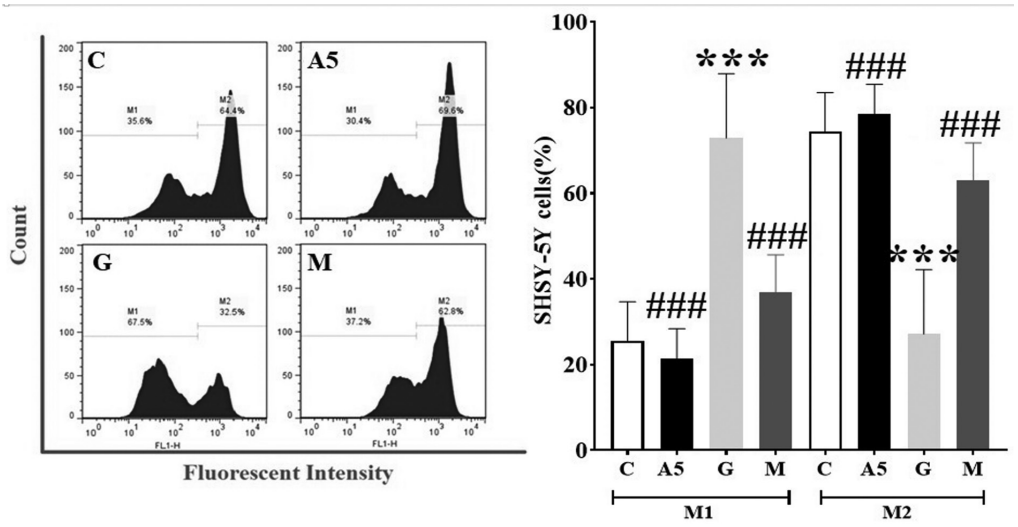


**Figure 4:** Annexin A5 ameliorated L-glutamate-induced mitochondrial membrane potential dissipation of SH-SY5Y cells. The cells were treated with L-glutamate (165 mM) alone or in combination with Annexin A5(2.5 µg/mL) for 24 hours, and mitochondrial membrane potential (MMP) was measured using the Rhodamine-123 flow cytometry method (n=3). The histogram results for each group were obtained after gating cells in the initial phase and then gating healthy mitochondria (FL1-H subset) using FlowJo 7.8 software. In the flowcharts (C: control, A5: Annexin A5, G: L-glutamate, M: L-glutamate Annexin A5 groups), M1 and M2 represent cell populations with low MMP and high MMP, respectively. The significant difference between groups was tested by one-way ANOVA followed by a Tukey *post-hoc* test. The data are mean±SD. ***P<0.001 *vs.* control group; ###P<0.001 *vs.* L-glutamate group

*ANXA5 Reduced Glu-Induced Mitochondrial Membrane Potential Loss in SHSY-5Y Cells*

The effects of the treatments on the MMPs of SHSY-5Y cells are shown in figure 4. As shown in the flowcharts, the SHSY-5Y cells were divided into mean low intensity (M1) and high intensity (M2) Rh-123 fluorescent cells. In the control group, the percentage of M1 cells (25.60±9.06%) was significantly lower than that of M2 cells (74.40±9.06%) (P<0.001), suggesting a lower number of cells with low MMP. Similarly, in the ANXA5 group, the percentage of M1 cells (21.40±6.89%) was significantly lower than that of M2 cells (78.55±6.89%) (P<0.001). However, in the L-Glu group, the percentage of M1 cells (72.88±15.05%) was higher than M2 cells (27.13±15.05%) (P<0.001), suggesting a higher number of cells with MMP loss in this group. No significant differences were observed in the percentage of M1 (P=0.583) and M2 cells (P=0.583) between the control and ANXA5 group. L-Glu increased the percentage of M1 cells compared to the control group (P<0.001), indicating mitochondrial membrane damage by L-Glu. In the SH-SY5Y cells treated with ANXA5 (2.5 µg/mL) and L-Glu (165 mM), the percentage of M1 cells reduced significantly compared to the cells treated with L-Glu alone (P<0.001), indicating the protective effects of ANXA5 against L-Glu-induced MMP loss.

*Effect of ANXA5 on the Expression of L-glutamate-Induced Genes in SH-SY5Y Cells*

Figure 5 demonstrates the effects of various treatments on the expression of *Bax*, *Bcl-2*, and *Nrf-2* genes. SH-SY5Y cells were treated with L-Glu (165 mM) and ANXA5 (2.5 and 5 µg/mL), alone or in combination, and the gene expression was measured using real-time PCR. As shown in figure 5, L-Glu (165 mM) increased *Bax* expression (P<0.001) and increased the *Bax/Bcl-2* ratio compared to the control cells (P<0.001). ANXA5 alone had no significant effects on the expression of *Bax*, but increased the expression of *Bcl-2* (P<0.05) and reduced the *Bax/Bcl-2* ratio (P<0.01). ANXA5 at 2.5 and 5 µg/mL reversed the effects of L-Glu on the expression of *Bax* (P<0.001) and the *Bax*/*Bcl-2* ratio (P<0.001).

The effect of the treatments on the expression of *Nrf-2* in the SH-SY5Y cells was also investigated (figure 5). L-Glu (165 mM) decreased *Nrf-2* expression compared to the control group. *Nrf-2* expression in cells treated with ANXA5 alone was increased compared to the control group. Moreover, when the SH-SY5Y cells were treated with L-Glu (165 mM) and ANXA5 (2.5 and 5.0 µg/mL), Nrf-*2* expression was significantly increased compared to cells treated with L-Glu alone.

## Discussion

L-Glu excitotoxicity is involved in the pathogenesis of a broad range of diseases, notably spinal cord injury, stroke, and neurodegenerative diseases (NDs).[18] The results of this study revealed that ANXA5 mitigated the cytotoxic effects of L-Glu on the viability of SH-SY5Y cells.

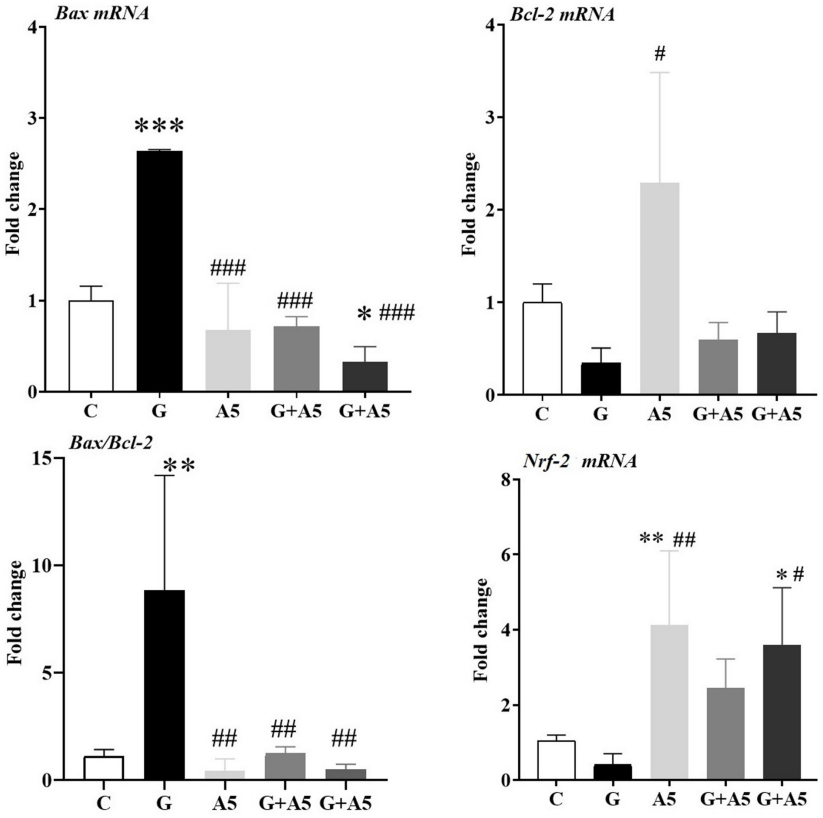


**Figure 5:** Annexin A5 reversed the effects of L-glutamate on the expression of *Bax*, *Bcl-2*, and *Nrf-2*. The cells were treated with L-glutamate (165 mM) and Annexin A5 (2.5 and 5.0 µg/mL) for 24 hours, and gene expression was measured using real-time PCR (n=3). (C: control, A5: Annexin A5, G: L-glutamate, G+A5: L-glutamate+Annexin A5 groups). The significant difference between groups was tested by one-way ANOVA followed by a Tukey *post-hoc* test. The data are as mean±SD. *P<0.05; **P<0.01; and ***P<0.001 *vs.* control group; #P<0.05, ##P<0.01; and ###P<0.001 *vs.* L-glutamate group

ANXA5 also ameliorated MMP dissipation induced by L-Glu. Moreover, it reversed the impact of L-Glu on the expression of apoptotic genes, as well as *Nrf-2*, a key regulator of the cell antioxidant defense system. These data suggest a role of ANXA5 in preventing L-Glu cytotoxicity and treating its related diseases. L-Glu excitotoxicity is mostly caused by calcium-induced mitochondrial damage.[19] ANXA5 plays an important role in intracellular $Ca^{2+}$ homeostasis. Therefore, $Ca^{2+}$ homeostasis may play a role in the protective effects of ANXA5. More research is needed to determine the exact mechanisms involved.

ANXA5 is an intracellular protein that is also found extracellularly in cerebrospinal fluid (CSF) and blood circulation.[20] Extracellular ANXA5 can enter cells via pinocytosis and impact signaling pathways that regulate gene expression. It also regulates calcium signaling, which is involved in many cellular functions. Alternatively, ANXA5 attaches to phospholipid membranes on the cell surface, acting as a shield to prevent molecules from interacting and potentially causing damage.[20] Circulating ANXA5 was linked to AD. Plasma levels of ANXA5 in patients with AD were found than in healthy individuals, and it has been suggested that ANXA5 may play a protective role in AD by interacting with islet amyloid polypeptides and reducing their toxicity.[21] The results of our study revealed that ANXA5 protects SH-SY5Y cells against the toxic effects of L-Glu, consistent with the protective effects of ANXA5 in other studies. In choroid plexus cell cultures, ANXA5 safeguards against the toxicity induced by amyloid-β (Aβ) by reestablishing the dysregulated calcium homeostasis.[21] Moreover, a recent study revealed the safeguarding properties of ANXA5 against necrosis and apoptosis of chondrocytes by binding to phosphatidylserine on the cell membrane and forming a lattice network that maintains membrane integrity.[22] Oflaz and colleagues showed that ANXA5 protects against cisplatin and selenite-induced apoptosis.[23] In a recent study, Hu and colleagues demonstrated that ANXA5 recombinant protein could penetrate the blood-brain barrier, reduce infarct volumes, and improve neurological function after ischemic stroke (IS) in mice, whereas anti-ANXA5 administration increased infarct sizes and aggravated neurological function, implying that ANXA5 may potentially serve as a therapeutic candidate for defending against IS-induced brain injury.[24] However, some studies showed cytotoxic effects of ANXA5 against tumor cells.[25-27] In the present study, ANXA5 alone showed no significant impact on the viability of SH-SY5Y cells, which is consistent with the findings of Kang and colleagues in keratinocytes, who reported that ANXA5 had no significant effects on cell proliferation,[25] implying a cell-specific activity of ANXA5.

Several possible mechanisms have been proposed for the protective effects of ANXA5. Mitochondrial dysfunction plays a pivotal role in L-Glu-induced excitotoxicity. L-Glu increases $Ca^{2+}$ influx, resulting in $Ca^{2+}$ overload in the mitochondria. $Ca^{2+}$ overload leads to the inhibition of the respiratory chain and inactivation of antioxidant enzymes, which increases ROS production. ROS react with mitochondrial DNA, leading to DNA fragmentation and peroxidation of mitochondrial membrane lipids. The result of these changes is cell apoptosis and necrosis.[28-30] Recently, Mehdieh and colleagues reported the protective effects of ANXA5 against $H_2O_2$-induced oxidative damage by reducing DNA fragmentation and preventing MMP loss.[9] Similarly, the results of the present study revealed that ANXA5 effectively reversed the effects of L-Glu on the loss of MMP. ANXA5 includes four $Ca^{2+}$ binding domains. Following $Ca^{2+}$ binding, ANXA5 is assembled at membrane injury sites and stabilizes the membrane.[31] Therefore, it is postulated that under OS and increased intracellular $Ca^{2+}$, ANXA5 is activated, protecting the cells against L-Glu-induced apoptosis by regulating $Ca^{2+}$ concentration. Further studies are needed to explore the effects of ANXA5 on regulating $Ca^{2+}$ concentration and signaling pathways. Oflaz and colleagues showed that ANXA5 is closely linked to the voltage-dependent anion channel (VDAC1) located in the outer mitochondrial membrane, and by regulating the activity of these channels, protects against cisplatin and selenite-induced apoptotic cell death.[23]

It is postulated that the protective effects of ANXA5 are mediated through its antiapoptotic and antioxidant effects. In our recent study, ANXA5 reduced the cytotoxic effects of H□O□ by decreasing the accumulation of reactive oxygen species and increasing the expression of *Nrf-2*. Moreover, ANXA5 reversed the effects of H□O□ on increasing the Bax/Bcl-2 ratio.[9] Zhang and colleagues have demonstrated the ameliorating effects of ANXA5 against Di-N-butylphthalate-induced OS through activating the ERK/Nrf2 pathway.[32] Moreover, an *in vivo* study revealed the protective effects of ANXA5 in reducing intestinal injury by reducing apoptosis and enhancing the activity of the Nrf2 antioxidant pathway.[33] This investigation, consistent with the previous research, found that ANXA5 effectively reversed the effects of L-Glu on the expression of *Bax*, *Bcl-2*, and *Nrf-2* at the mRNA level. This suggests that the benefits of ANXA5 in reducing L-Glu cytotoxicity may be mediated through anti-apoptotic and antioxidant pathways. Conversely, Zhao and colleagues demonstrated that ANXA5 knockdown can inhibit rat cardiomyocyte apoptosis by decreasing the Bax/Bcl-2 ratio.[27] Cell-specific effects and the differences between the intracellular and extracellular effects of ANXA5 may explain the discrepancies observed in the studies.[34, 35]

This research faces several limitations. First, the effects of the treatments on the expression of *Bax*, *Bcl-2*, and *Nrf-2* were evaluated at the mRNA level in this study. Estimating the levels of these markers at the protein level using Western Blot analysis is essential. Second, regulating $Ca^{2+}$ concentration is implicated in the protective effects of ANXA5. Therefore, further studies are needed to determine the role of ANXA5 in regulating cellular $Ca^{2+}$ concentration.

## Conclusion

Our study showed that ANXA5 can mitigate the toxic effects of L-Glu in SH-SY5Y cells. This mitigation of toxicity occurs through the prevention of mitochondrial dysfunction, oxidative stress, and apoptosis, indicating a potential beneficial role for ANXA5 in the treatment of conditions associated with L-Glu-induced excitotoxicity.

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## Authors' Contribution

Z.A: Study design, data analysis and interpretation, drafting, M.M: Study design, data analysis and interpretation, drafting, MA.T: Study design, data analysis and interpretation, supervision of the research, critical reviewing, 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.

**Conflict of Interest:** None declared.

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