Original Article

Alpha Lipoic Acid Protects Human SH-SY5Y Cells Against Quinolinic Acid-Induced Toxicity: Focusing on ROS Levels and Cell Cycle

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ABSTRACT

Objectives: An abnormal buildup of Quinolinic Acid (QuA) is usually linked to the death of nerve cells and a condition known as neuritis in various forms of neurodegenerative illness. Alpha Lipoic Acid (ALA) has substantial antioxidant properties, according to previous studies. However, the protective effects of ALA against the neurotoxicity induced by QuA are unknown. This work aimed to determine whether ALA could shield the SH-SY5Y neuroblastoma cell line from QuA-induced neurotoxicity.

Methods: Cell viability was assessed using the MTT assay, while cell cycle and apoptotic effects were evaluated using flow cytometry. Cellular levels of reactive oxygen species (ROS) were also examined.

Results: The findings showed that ALA, at non-toxic concentrations, had a protective effect against QuA-induced toxicity. Moreover, pretreatment with ALA reduced the number of cells that underwent apoptosis. Also, it was found that the percentage of apoptotic cells (i.e., those in the sub-G1 phase) was considerably increased following QuA therapy. ALA also dramatically reduced the production of ROS by QuA.

Conclusion: The results suggest that ALA appears to be an effective neuroprotectant and antioxidant against QuA-induced neurotoxicity.

Keywords: Alpha Lipoic Acid; Quinolinic Acid; SH-SY5Y neuroblastoma cell line; Oxidative stress.

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Introduction

Numerous neurodegenerative illnesses manifest and advance as a result of inflammatory processes. Neuroinflammation can cause oxidative stress and excitotoxicity in these conditions and alter several brain energy metabolism parameters (1-3). The pathophysiology of neurodegeneration has been linked to increased inflammatory cytokine production by macrophages and microglia in the central nervous system (CNS), although the precise function of these molecules is still unclear (4). Inflammatory cytokines promote the kynurenine pathway (KP), the most significant route of tryptophan catabolism, resulting in metabolites that can modulate numerous redox systems in diverse physiological activities (5). Kynurenic Acid, kynurenine, and Quinolinic Acid (QuA), among other metabolites produced in this route, are crucial for appropriate brain function (6-9). Many neurodegenerative conditions, including Parkinson's and Alzheimer's diseases, HIV-related cognitive decline, and multiple sclerosis, have been linked to the buildup of QuA (10). In addition, because of rapid oxygen consumption, high content of polyunsaturated fatty acids in the membranes, and deficient antioxidant defenses, the nervous system is particularly vulnerable to damage from reactive oxygen species (ROS) (11). Therefore, limiting the damage caused by oxidation to the nervous system is essential. Many researchers have lately been interested in the ability of diverse natural substances to modify redox in various clinical situations. According to research, natural compounds may improve cognition and memory while slowing or even stopping neurodegeneration (12-14).

The tiny dithiol molecule alpha lipoic Acid (ALA), produced from octanoic acid, may function as a potent micronutrient with various pharmacological and antioxidant characteristics (15). This compound, sometimes known as a vitamin, is a cofactor of several mitochondrial enzymes, including pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and glycine decarboxylase. It is a strong free radical scavenger and therapeutic candidate (16, 17). Moreover, it may be absorbed from food sources like red meat, wheat, vegetables, and fruits to a lesser extent (18, 19). In other words, foods of animal origin (mostly liver and muscles) have significant ALA levels (0.55 to 2.36 ppm), whereas plants have either no detectable ALA or very low ALA (0.09 ppm) (20). In the prevention or treatment of diabetes, neurodegenerative, and hepatic illnesses, it has been utilized as a dietary supplement (17). Additionally, it has been reported that ALA prevents growth and triggers apoptosis in various cancer cell lines, including human lung epithelial cancer NCI-H460 cells (21). To successfully trigger apoptosis in human colon cancer HT-29 cells, ALA has been shown to promote the generation of ROS in the mitochondria (22). ALA has been shown to promote cell death through the activation of caspase-9, -3, and -7 and to increase the cytotoxicity of 5-fluorouracil (5-FU) in HT-29 and Caco-2 cell lines (23). Therefore, the aim in this study was to evaluate the neuroprotective of ALA and its connection with QuA in the SH-SY5Y neuroblastoma cell line.

Materials and methods

Materials

ALA and QuA (purity > 97%) were provided by Gol ExirPars (Iran). Propidium iodide (PI), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), and RNase A were purchased from Sigma-Aldrich (USA). Trypsin, fetal calf serum (FCS), DMEM/F12, and penicillin/streptomycin were all provided by Gibco (UK). DMSO, ethanol, and TritonTM X-100 were provided by Mojallali Co. (Iran). The Cellular ROS Assay Kit was prepared (Abcam, Cambridge, UK; Cat. No. ab113851).

Cell culture

The SH-SY5Y neuroblastoma cell line was provided by The Pasteur Institute in Iran (Tehran, Iran). These cells have an SK-N-SH origin. The neurotoxicity of various substances in neurodegenerative disorders is assessed using the SH-SY5Y cell line. Undifferentiated cells were chosen in this investigation since they have previously been used in numerous studies to evaluate neurotoxicity (24, 25). Cells were maintained in DMEM/F12 at 37°C with 5% CO_2 and supplemented with FCS (10% v/v), penicillin (100 units/ml), and streptomycin (100 µg/ml).

Cell viability measurement with the MTT assay

The IC_{50} of QuA and the inherent cytotoxicity of the drug were determined using the MTT method. The experiment also tested how effectively this drug shielded the cells from the harm that QuA inflicted. In brief, 1×10^4 SH-SY5Y cells were cultured in 96-well plates and incubated for 24 h at 37 °C and 5% CO_2. The cells were then exposed to various concentrations of QuA (2.5-16 mM) and ALA (200–25000 µM) for the required time. Stock solutions of these compounds (2 M QuA and 10 mM ALA) were created by dissolving them in DMSO. These stock solutions were diluted to working concentrations using DMEM/F12 media. After the treatments, the media was removed, and the cells were then incubated in 100µL of fresh medium containing MTT solution (0.5mg/mL). After 4 h, 200µL of DMSO was added to each well to dissolve the formed formazan crystals. The OD (optical density) of wells was finally

measured at 570nm using a Stat FAX303 plate reader (Awareness Technologies Inc., USA) (26). As a negative control, cells that had not been treated were employed. Each experiment was repeated three times.

The protective effects of ALA against QuA-induced cytotoxicity

1×10^4 SH-SY5Y cells were seeded into each well of the 96-well plates, which were subsequently incubated for 24 h at 37 °C with 5% CO_2. The wells were then divided (in triplicate) into three groups: The control group received nothing except culture media. In the ALA group, the cells were pre-treated with a range of non-toxic doses of ALA (200, 400, 800, and 1560 µM) for 24 h before exposure to QuA for an additional 24 h. Only 6 mM of QuA, or almost the IC_{50} value, were applied to the cells in the QuA-positive control group. Finally, the MTT assay was used to examine the cell viability of each group in triplicate.

Flow cytometry assay

SH-SY5Y cells were seeded onto 6-well plates at a density of 7×10^5, and they were then incubated for 24 h at 37 °C with 5% CO_2 to measure cell apoptosis. After that, the cells were pre-treated with ALA at non-toxic concentrations (200 and 400 µM). After adding a final concentration of 6 mM of QuA, the plates underwent a second 24 h incubation period (at 37°C and 5% CO_2). After trypsinization, collection, and resuspension in cold PBS, the cells were fixed with 70% ethanol (v/v) at 4 °C for two hours. The fixed cells were pelleted after being spun down at 268× g for 5 min and treated for 20 min at 37 °C in PBS containing Triton X-100 (0.1% v/v) and RNase A (100 µg/mL). The cells were stained with 200 µL of PI solution (1 mg/mL) after 15 min of dark incubation. Afterward, the cell cycle was evaluated using flow cytometry. Pre-phase peaks (sub-G1 phase) quantified the number of apoptotic cells (27).

Intracellular Reactive Oxygen Species (ROS) analysis

For this experiment, 2.5×10^4 SH-SY5Y cells were grown in 96-well plates and incubated for 5 h at 37 °C with 5% CO_2. After that, cells were pre-treated with ALA at non-toxic doses (200 and 400 µM) and incubated for 24 h. Cells were pre-treated for one hour at 37°C and 5% CO_2 in the dark with 100 µL of diluted 2',7'-dichloro dichlorofluorescein diacetate (H2DCFDA). The ROS test kit's 1X buffer was then used to clean the cells. The cells were treated for 4 h with 6 mM QuA and the previously prescribed doses of ALA in the growth medium after a second wash with 1X buffer. Untreated cells were the negative control, The positive control was tert-butyl hydroperoxide (TBHP; 10 mM), which can lessen ROS generation. The fluorescence was then measured using a FACScan fluorescent plate reader (excitation/emission: 485/535 nm; Becton Dickinson, San Jose, CA, USA). Each experiment was repeated three times.

Statistical evaluation

The statistical analysis was performed using GraphPad Prism 8. (San Diego, CA, USA). The normal distribution of samples was analyzed using the Shapiro-Wilk normality test. Differences between the groups were assessed using one-way analysis of variance (ANOVA). The data was presented as mean±SD (standard deviation), and a difference between groups of $P<0.05$ was deemed significant.

Results

QuA reduced the viability of SH-SY5Y cells

QuA is toxic to the SH-SY5Y cell line and decreased cell viability dose-dependently. 50% fewer cells were viable after 48h of exposure to QuA at a dosage of 6 mM. The IC_{50} value of 6 mM was chosen for further research (Fig. 1).

Non-toxic concentrations of ALA

The MTT assay was performed to evaluate the effects of various ALA concentrations (200-25000 µM) on the SH-SY5Y cells. According to the findings and after 24 h, four concentrations of ALA between 3125 and 25000 µM could decrease the viability of SH-SY5Y cells (Fig. 2). Therefore, safe doses (200,400,800 and 1560 µM) were selected for subsequent experiments.

ALA protect SH-SY5Y cells against QuA-induced cytotoxicity

Two different concentrations of ALA were used in this experiment. As shown in Fig. 3, ALA significantly decreased QuA-induced cytotoxicity at 200 µM and 400 µM ($P < 0.001$).

ALA can lower the number of apoptotic cells in the sub-G1 phase

The results revealed that compared to the untreated control group, the number of apoptotic cells was significantly increased after QuA treatment of SH-SY5Y cells at a dose of 6 mM (i.e., cells in the sub-G1 phase) (Fig. 4). Pretreatment with ALA could reduce this effect (from 57.1% of apoptotic cells in the QuA-treated group to 45.1% in the 400 µM ALA pre-treated groups). As a result, QuA treatment dramatically increased the percentage of apoptotic cells in the sub-G1 phase (from 7.98% in the untreated control to 57.1% in the 6 mM QuA-treated cells) (Fig. 4).

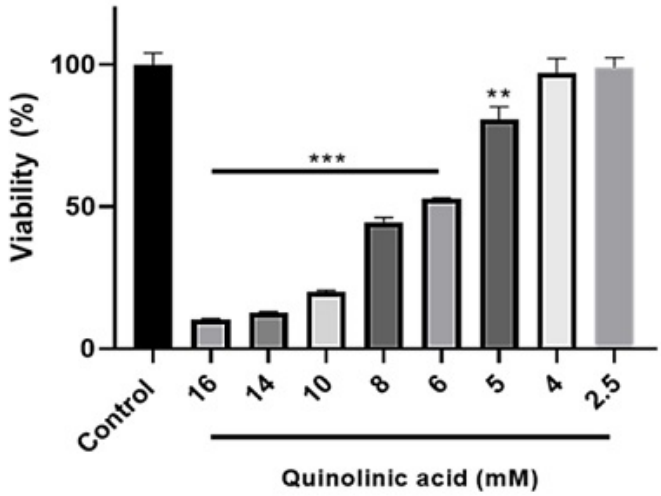


Figure 1: The effects of a 48 h exposure to QuA on the growth of SH-SY5Y cells. Cell viability was evaluated using the MTT test, and results were presented as a percentage of the untreated control (100%). The results are expressed as mean±SD of three separate tests; the control cells had no treatment.

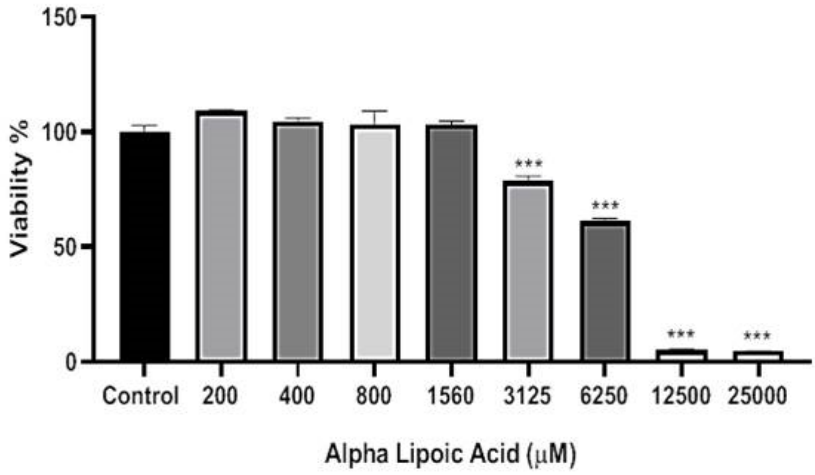


Figure 2: The impact of ALA on the growth of SH-SY5Y cells after a 24 h exposure. The MTT was used to determine the cell viability. The results expressed as mean±SD of three separate tests; the control cells had no treatment. ***P < 0.001 indicates significant differences from the untreated control group.

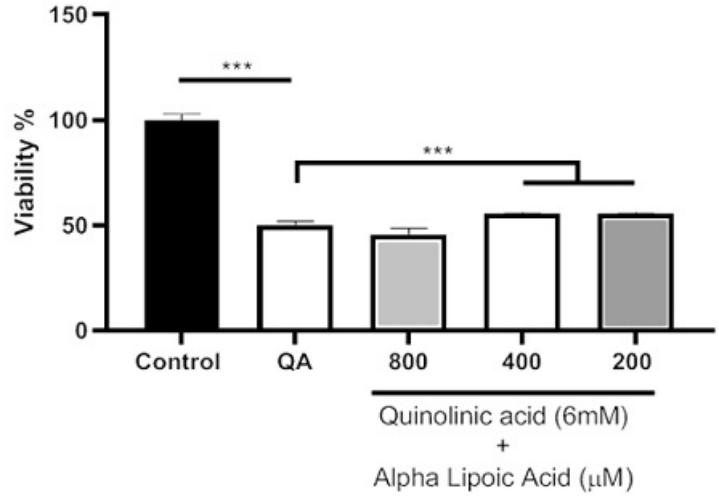


Figure 3: The investigation Cell viability in presence of ALA in SH-SY5Y cell- induced toxicity by QuA. Cell viability was evaluated using the MTT test, and results were expressed as a percentage of the untreated control (100%). Untreated cells are the control. The results expressed as mean ± SD of three independent studies. ***P < 0.001 indicates significant differences from the untreated control group.

ALA reduced the levels of intracellular ROS

Compared to the untreated control cells, QuA treatment of SH-SY5Y cells markedly increased the generation of ROS. Treatment with TBHP alone as a positive control considerably elevated intracellular levels of ROS compared to the control group. Furthermore, it became clear that pretreatment with ALA remarkably reduced the amount of ROS caused by QuA compared to the group that received only QuA (Fig. 5).

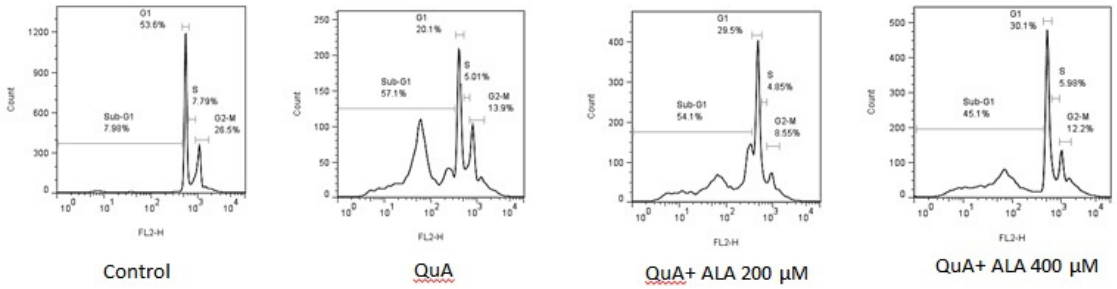
Discussion

QuA has been identified as a pro-oxidant and endogenous excitotoxin of the brain that greatly increases neuroinflammation (28-30). Therefore, detoxification or neutralization of QuA might aid in preventing inflammatory responses in the brain. In the current study, the capacity of ALA, a natural antioxidant, to prevent neurons from being harmed by QuA in vitro was examined. The findings demonstrated that ALA might lessen QuA-induced neurotoxicity in neuroblastoma cells through decrease in ROS generation and apoptosis.

The vitality of SH-SY5Y cells was considerably and concentration-dependently lowered by QuA. Subsequent analysis showed that these cells produced ROS in response to QuA. It is widely accepted that oxidative stress contributes to the etiology of neuroinflammatory diseases (31, 32). Increased oxidative stress, elevated free radical generation, elevated lipid peroxidation, mitochondrial metabolic abnormalities, and severe DNA damage are among the symptoms of the neurotoxicity brought on by QuA. This neurotoxicity is brought on by excessive N-methyl-D-aspartate (NMDA) receptor activation and elevated intracellular Ca2+ concentrations (33, 34). Numerous studies have demonstrated that QuA is toxic for neurons (34). For instance, after seven days of intrastriatal injection of QuA, a Huntington's disease excitotoxic model has been developed (35). Moreover, brain tissue of Alzheimer's patients produces more QuA than normal. QuA was also associated with impairments in NMDA-mediated neuronal function in primary cultures of human neurons (36). By the overactivation of NMDA receptors in rat striatal slices, QuA has also been linked to ROS generation and neuronal injury.

Numerous studies have linked the generation of ROS and the ensuing oxidative stress to the death of neurons (37, 38). It was shown that QuA treatment could lead to accumulation of the neuroblastoma cells in the sub-G1 phase and significantly boost ROS production. It has been consistently demonstrated that QuA is essential for causing cell death due to oxidative damage to the CNS. For instance, it has been shown that QuA induces the death of PC12 cells and increases the generation of ROS (39). Therefore, from these results, it can be concluded that oxidative stress plays an important and key role in the damage of neurons, and therefore any potential antioxidant compound can be effective in preventing the damage of neuron cells caused by oxidative stress. In this regard, Nakai et al. reported that free radical scavengers decreased the neuronal damage induced by QuA through decreasing oxidative stress and apoptosis in the rat striatum (40). Furthermore, Behan et al. discovered that melatonin and deprenyl, two potent free radical scavengers and antioxidant defense boosters, greatly slowed down the oxidative death of hippocampus cells produced by QuA (41).

In the current study, the antioxidant effect of ALA in the SH-SY5Y cells treated with QuA was examined. ALA decreased ROS generation and increased the survival of the neuroblastoma cells treated with QuA. In agreement with these findings, ALA has been demonstrated to cross



Groups	Proportion of cells in sub-G1 phase (% of total cell count)
Control (untreated)	7.98
6 mM QuA only	57.1
200 µM lipoic acid + 6 mM QuA	54.1
400 µM lipoic acid + 6 mM QuA	45.1

Figure 4: The percent of apoptosis caused by QuA in presence of different concentration of ALA. The amount of the total number of cells in subG1 showed as percent. The precent of subG1decreased in ALA group in compared to control.

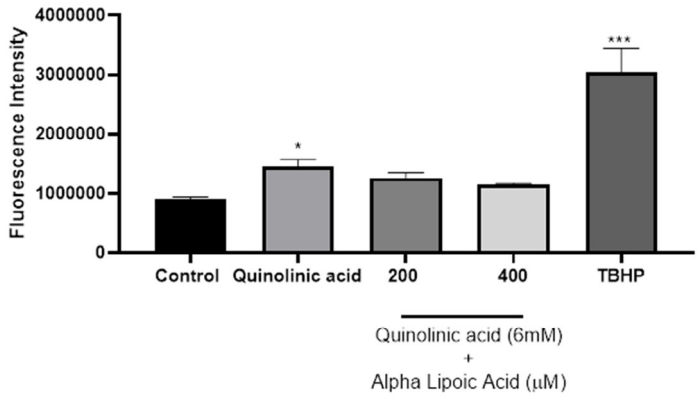


Figure 5: The effect of ALA on ROS levels in the cells treated by QuA. Intracellular levels of ROS was measured using the fluorescent ROS marker. The results are displayed as the mean±SD of triplicate tests. Compared to the control group, a significant difference was considered with *P<0.05 and ***P<0.001 the QuA-treated group.

the blood brain barrier successfully, protect neurons from oxidative damage, and act as an anti-inflammatory drug (43). The neuroprotective effect of ALA has also been demonstrated (44). ALA has also been shown to impact several molecular targets, including transcription factors, cellular kinases, cell cycle mediators, drug transporters, and inflammatory cytokines (45). After ischemic stroke, ALA triggers neurological recovery to terminate oxidative damage through activation of the Nrf2/Ho-1 pathway (46). It was found that ALA decreased the number of sub-G1 cells in QuA-treated SH-SY5Y cells. As already mentioned, the neurotoxic effects of QuA may be at least largely due to oxidative stress and ROS production. ALA can scavenge free radicals and shield neuroblastoma cells from QuA-induced toxicity because it lowers the quantity of ROS that QuA brings. Another study revealed that ALA can induce cell cycle arrest in a p27Kip-dependent manner in non-transformed cell lines, while it induces apoptosis in tumor cells (47). In summary, the findings suggest that ALA dramatically reduced the oxidative damage and cell death induced by QuA in neuroblastoma cells. Therefore, this compound can be suggested as a protective agent against brain disorder, although more investigations are needed in this field.

Declarations

Contributions of authors

Mehdi Rostami and Seyed Amir Vaezzade contributed to the manuscripts' design, conception, acquisition, and drafting. Arezoo Rajabian, Hanieh Nadi Yazdi and Elaheh Gheybi contributed to the interpretation and critically revised the manuscript. Fatemeh Forouzanfar and Mohammad Soukhtanloo each contributed to the idea and drew on their respective expertise to contribute. All writers have read and given their consent to the final manuscript.

Conflict of Interest

Authors have nothing to declare.

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