

Dose-Dependent Differential Neuroprotective Effects of Epigallocatechin Gallate Against Aluminum Maltolate-Induced Neurotoxicity Through Modulation of Oxidative Stress, Apoptosis, and NMDAR Modulation

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Abstract

Aluminum (Al) is a ubiquitous environmental neurotoxicant increasingly implicated in the pathogenesis of neurodegenerative disorders. Aluminum maltolate (Al-Malt), has been shown to exert significant cytotoxic effects in neuronal cell models. The present study was designed to investigate the impact of oxidative, apoptotic, and glutamatergic alterations induced by Al-Malt and to evaluate the modulatory effects of epigallocatechin gallate (EGCG) in human neuroblastoma SH-SY5Y cells. For this purpose, cytotoxicity, oxidative stress markers, apoptotic parameters, amyloid precursor protein (APP) expression, and N-methyl-D-aspartate receptor (NMDAR) subunit mRNA expression levels were assessed. Exposure to Al-Malt resulted in significant cytotoxicity, as evidenced by elevated lactate dehydrogenase (LDH) release, increased lipid peroxidation (MDA), depleted glutathione (GSH) levels, and activation of caspase-3 and caspase-9, indicative of mitochondria-mediated apoptosis. In addition, Al-Malt upregulated APP expression, suggesting a potential amyloidogenic mechanism. EGCG treatment significantly attenuated Al-Malt induced oxidative stress by reducing lipid peroxidation and improving intracellular antioxidant status. Moreover, EGCG suppressed LDH release, inhibited caspase-3 and caspase-9 activation, and downregulated APP expression and partially restored Al-Malt-induced alterations in NMDAR1 and NMDAR2B mRNA expression. Notably, the neuroprotective effects of EGCG were concentration-dependent with lower concentrations providing greater protection against GSH depletion, apoptosis, APP overexpression, and glutamatergic dysregulation, whereas higher concentrations retained the ability to reduce lipid peroxidation despite exhibiting weaker effects on other neurotoxicity-related parameters. In conclusion, EGCG protects SH-SY5Y cells against Al-Malt-induced neurotoxicity by reducing oxidative stress, inhibiting apoptotic pathways, and modulating glutamatergic receptor expression. These findings demonstrate that the neuroprotective efficacy of EGCG is critically influenced by concentration, emphasizing the importance of dose optimization for maximizing its therapeutic potential against aluminum-associated neurodegeneration. These findings highlight the therapeutic potential of EGCG against Al-associated neurodegeneration; however, its efficacy appears to be critically dependent on dose optimization.

Highlights

- Al-Malt (200 μ M) induced significant cytotoxicity in SH-SY5Y cells at 24 and 48 h
- Al-Malt triggered mitochondria-mediated apoptosis via activation of caspase-3 and caspase-9
- Al-Malt promoted oxidative stress through increased lipid peroxidation and GSH depletion
- Al-Malt disrupted synaptic plasticity by altering NMDAR subunit expression
- EGCG attenuated Al-Malt-induced oxidative stress, apoptosis, APP overexpression and NMDAR dysregulation

- The neuroprotective efficacy of EGCG was concentration-dependent, with lower concentrations providing greater protection than higher concentrations.

1. Introduction

Aluminum (Al) is an extremely abundant, non-essential element in the Earth's crust and is widely utilized across various industries and in daily human life (Cheng et al., 2021; Wang et al., 2019). Numerous studies have demonstrated that humans are exposed to this potentially toxic element through oral intake at high concentrations via drinking water, food, pharmaceuticals, cosmetics, water purifiers, packaging materials, and aluminum cookware (Alasfar and Isaifan, 2021; Cheng et al., 2021; Wang et al., 2019). Al can accumulate in the human body, leading to damage in various organs and systems, including the liver, kidneys, skeletal system, hematopoietic system, nervous system, immune system, and reproductive system. Among these, the central nervous system is considered the primary target organ for Al toxicity (Cheng et al., 2021). In *in vivo* neurotoxicity studies, Al salts such as Al lactate, citrate, and chloride are commonly used. However, under physiological pH conditions, these salts undergo hydrolysis and form aluminum hydroxide [Al(OH)₃] precipitates, which can significantly reduce the bioavailability of Al (Cheng et al., 2021; Wang et al., 2019). In contrast, Al maltolate (Al-Malt) is a stable Al complex capable of dissolving a large amount of Al³⁺ at physiological pH. Due to its high membrane permeability, Al-Malt enhances both the bioavailability and toxicity of Al, making it a widely used compound in recent *in vitro* neurotoxicity studies. (Cheng et al., 2021; Pan et al., 2020; Wang et al., 2019). Therefore, Al-Malt was selected as the toxic agent in the present study.

Accumulation of Al in neural tissues is considered inevitable, as Al can cross the blood–brain barrier and subsequently cause brain damage (Das et al., 2021; Wang et al., 2019). Although the precise mechanisms underlying Al-induced neurotoxicity remain incompletely understood, several pathways have been proposed as key contributors to Al-associated neurodegenerative processes (Ullah et al., 2021). These include increased production of reactive oxygen species (ROS) and induction of lipid peroxidation (Cheng et al., 2021; Wang et al., 2019), neuronal apoptosis (Cheng et al., 2021; Dhivya Bharathi et al., 2019; Iranpak et al., 2019; Wang et al., 2019), amyloid beta-protein accumulation (Pan et al., 2020), alterations in synaptic plasticity (Pan et al., 2020) and neuroinflammation (Palabiyik-Yuceli et al., 2023). Among these, oxidative stress plays a central role in Al-induced neurotoxicity, as it promotes both apoptosis and neuronal cell death. Therefore, therapeutic strategies involving antioxidant compounds are considered promising approaches to mitigate Al-induced neurotoxicity.

In recent years, both epidemiological and molecular studies have increasingly focused on the detrimental effects of long-term Al exposure on the nervous system, particularly in relation to various neurodegenerative disorders, including Alzheimer's disease (AD) (Exley and Vickers, 2014; Khalifa et al., 2020; Pan et al., 2020). Several studies in the literature have reported elevated Al concentrations in individuals who died from AD and had a history of occupational exposure Al (Exley and Vickers, 2014). Additionally, animal studies have demonstrated a correlation between Al accumulation in brain tissue and pathological alterations associated with AD (Khalifa et al., 2020). In patients with AD, learning ability,

memory, and cognitive functions are notably impaired, and these functions are closely dependent on synaptic plasticity. N-methyl-D-aspartate receptors (NMDARs) play a critical role in regulating this plasticity (Pan et al., 2020).

Various natural plants and plant-derived products have been investigated by researchers as potential therapeutic agents for the treatment of AI-induced neurotoxicity. These compounds have been proposed as promising strategies for managing disorders associated with AI-induced neurodegeneration (Dhivya Bharathi et al., 2019; Rashedinia et al., 2019; Tirumanyam et al., 2019). In our previous *in vivo* study, we evaluated the effects of epigallocatechin-3-gallate (EGCG), the primary polyphenolic compound in green tea, administered either simultaneously or after AI exposure, on AI-induced neurotoxicity in rats through various mechanisms (Palabiyik-Yuceli et al., 2023). Building on these findings, we have now conducted a study to assess the effects of EGCG on AI-induced neurotoxicity in a neuroblastoma cell line. Clinical and laboratory studies have suggested that green tea, and particularly its major catechin EGCG, exerts protective effects against various chronic diseases such as cardiovascular diseases, diabetes, neurodegenerative disorders (including Parkinson's and Alzheimer's diseases), cancer, and obesity. These protective effects are thought to be mediated through multiple mechanisms, including inhibition of cell growth factors, suppression of key cellular enzymes, inhibition of gene transcription, and antioxidant activity (Lambert and Elias, 2010). EGCG is a natural, non-toxic, lipophilic compound capable of crossing the blood–brain barrier, making it a promising candidate as a neuroprotective agent. Future studies should focus on the specific cellular targets affected by this compound and aim to elucidate its underlying protective mechanisms (Mandel et al., 2004).

The potential preventive or ameliorative effects of dietary supplements with well-known strong antioxidant properties on AI-induced neurological alterations have become a subject of growing research interest. To the best of our knowledge, no study has evaluated the protective role of EGCG against AI-induced neurotoxicity through multiple mechanisms in SH-SY5Y cells, except for our previously published *in vivo* study. Accordingly, the present study was designed to investigate and identify a potential therapeutic approach for AI-induced neurotoxicity and related neurodegenerative disorders. For this purpose, AI-Malt was used to induce neurotoxicity in SH-SY5Y cells, aiming to model *in vitro* neuronal damage via various mechanisms, including cell death, oxidative stress, alterations in N-methyl-D-aspartate receptor (NMDAR) expression, apoptosis, and amyloid precursor protein (APP) changes. Thus, the present study aims to elucidate the neuroprotective mechanisms of EGCG against AI-Malt-induced neurotoxicity in SH-SY5Y cells, with particular focus on oxidative stress, apoptosis, NMDAR expression, and APP processing.

2. Materials and Methods

2.1. Chemicals and Reagents

Aluminum chloride hexahydrate were purchased from Merck (Darmstadt, Germany), maltol, desferoxamin were purchased from Sigma-Aldrich (St. Louis, MO, USA). EGCG is a gift from DSM

Nutritional Products, Turkey. Dulbecco's modified Eagle medium (DMEM), cell culture medium, and reagents such as fetal bovine serum (FBS), penicillin/streptomycin, and trypsin-EDTA were obtained from GIBCO (Invitrogen Inc., NY, USA).

2.2. Preparation of Al(Malt)₃

Al-Malt were prepared from Al chloride hexahydrate (AlCl₃·6H₂O) and maltol (3-hydroxy-2-methyl-4H-pyran-4-one) according to the method in Bertholf et al. study (Bertholf et al., 1989). Briefly, 15.5 g (122.8 mM) maltol and 9.9 g (40.9) of AlCl₃·6H₂O were dissolved by heating to facilitate dissolution in approximately 160 ml deionized water. The pH was adjusted to 8.3 after the particles dissolved. It was then heated to a temperature of 65°C with stirring. After cooling, the resulting white crystals of Al-Malt were filtered and washed several times with acetone and dried overnight in a vacuum desiccator. The aluminum maltolate salt was freshly dissolved in media before exposure to SH-SY5Y cells.

2.3. Cell Culture

SH-SY5Y cells were purchased from American Type Culture Collection (ATCC, Manassas, VA. USA). SH-SY5Y cells were grown in DMEM supplemented with 100 U of penicillin/ml, 100 µg streptomycin/ml and 10% calf serum, in a humidified 37°C incubator with an atmosphere of 95% air and 5% CO₂. Cells were plated for RNA extraction and biochemical investigations in 6-well plates (Falcon, BD Biosciences, Heidelberg, Germany). Cultures were grown for 5–6 days at 37°C in 5% CO₂ and the medium was changed the day before treatment.

2.4. Real-Time Monitoring of Cell Growth and Proliferation Assay

2.4.1. Cell proliferation assay

Cell proliferation was assessed using the xCELLigence real-time cell analysis system (Roche Diagnostics, Indianapolis, IN, USA), which detects cellular events via electronic impedance measured through gold electrodes located at the bottom of culture wells. The system enables continuous monitoring of cell proliferation, adhesion, and morphology, with impedance data expressed as the Cell Index (CI) value, reflecting cell attachment and spreading over time (Stefanowicz-Hajduk and Ochocka, 2020),(Tan et al., 2009). All substances were dissolved in sterile water. First, 5,000 cells per well were seeded in normal medium; after 24 h neuroblastoma cells were exposed to different concentrations of Al(malt)₃ (50-100-200-400-800 µM), DES (6,25 – 12,5-25-50-100 µM) and EGCG (6,25 – 12,5-25-50-100 µM) to evaluate the cytotoxicity of the substances alone for 24 and 48 h. xCelligence give IC₅₀ level for Al(malt)₃ approximately 200 µM. And the DES and EGCG did not show any significant cytotoxicity. The cells were treated with 10 µM and 25 µM EGCG; and 50 µM DES as a positive control one hour before 200 µM Al(malt)₃ and the cell proliferation was monitored automatically for 48 hours every 15 minutes. Data are presented as a normalized cell index ± standard deviation (SD).

2.4.2. LDH activity assessment

The proportion of cells with compromised membrane integrity or cell death was quantitatively assessed using a colorimetric method with the Cytotoxicity Detection Kit (LDH) (Roche). Briefly, SH-SY5Y neuroblastoma cells were seeded at a density of 5,000 cells per well in a 96-well plate using standard growth medium. Cells were pretreated with 10 μ M or 25 μ M EGCG, or 50 μ M DES for 1 hour, followed by exposure to 200 μ M Al-Malt for 24 hours. LDH release was measured and expressed as a percentage relative to the untreated control group.

2.4.3. Real-Time PCR

SH-SY5Y cells were seeded at a density of 5×10^6 cells per 10 cm Petri dish in standard growth medium, following routine cell culture protocols. Cells were pre-treated with 10 μ M or 25 μ M EGCG; and 50 μ M DES 1 hour prior to exposure 200 μ M Al(malt)₃ for 24 h. After the incubation period, the medium was removed and cells were washed twice with sterile phosphate-buffered saline (PBS). Cells were then harvested by scraping with a sterile scalpel. Total RNA was extracted, followed by complementary DNA (cDNA) synthesis and gene expression analysis using real-time quantitative PCR (qPCR).

2.4.3.1. Total RNA Extraction and cDNA Synthesis

Cells were homogenized for 2 min using the TissueLyser II (Qiagen). Total RNA was isolated with the RNeasy Mini Kit (Qiagen) on the QIAcube system, following the manufacturer's protocol. RNA (10 μ l) was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 20 μ l reaction containing 2 μ l 10 $\times$ RT Buffer, 0.8 μ l 25 $\times$ dNTP Mix, 2 μ l 10 $\times$ RT Random Primers, 1 μ l MultiScribe Reverse Transcriptase, and 4.2 μ l DEPC- H₂O. The reaction was run on a Veriti 96-Well Thermal Cycler (Applied Biosystems) under the following conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. cDNA concentration and purity were assessed using the Epoch Spectrophotometer and Take3 Plate (Biotek).

2.4.3.2. Relative Quantification of Gene Expression

mRNA expression levels of NMDAR1, NMDAR2A, NMDAR2B, APP, NF- κ B, eNOS, caspase-3, and caspase-9 were quantified by real-time PCR using the StepOne Plus Real-Time PCR System (Applied Biosystems) with cDNA derived from SH-SY5Y cell RNA. Results were expressed as relative-fold and compared to control groups. β -actin was used as the endogenous control for normalization. For each cell group, qPCR reactions were performed in triplicate using a 96-well optical plate. Each 20 μ l reaction contained 9 μ l cDNA (100 ng), 1 μ l Primer Perfect Probe Mix, and 10 μ l QuantiTect Probe PCR Master Mix (Qiagen, Hilden, Germany). Thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 60 s. All data were expressed as fold-change in expression compared to cell groups using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

2.5. Biochemical investigations

For biochemical assays, 5×10^6 cells were seeded per 10 cm petri dish in standard culture medium according to routine cell culture protocols. Cells were pretreated with 10 μ M or 25 μ M EGCG, or 50 μ M

DES for 1 hour, followed by exposure to 200 μ M Al-Malt for 24 hours.

2.5.1. Analysis of glutathione and malondialdehyde levels

Glutathione (GSH) levels (Sedlak and Lindsay, 1968), and malondialdehyde (MDA) levels (Ohkawa et al., 1979) were measured from cell supernatants and standards at room temperature in duplicate using an ELISA reader, based on modified protocols. The average absorbance of each sample and standard were calculated, a standard curve was plotted, and the equation for the absorbance of the standards was obtained. Linear GSH and MDA concentrations were determined from the standard curve equation. Results are expressed as mean $\pm$ standard deviation per mg of protein.

2.5.2. Protein determination. The protein concentrations were determined by the Lowry method using commercial protein standards (Sigma Aldrich, Total protein kit- TP0300-1KT, USA).

2.6. Statistical analysis

The results were expressed as mean $\pm$ standard deviation. The normality of the parameters was assessed using the Kolmogorov–Smirnov test. Subsequently, based on the distribution of the data, a one-way ANOVA was performed, followed by Tukey's post hoc multiple comparison test. A p-value of < 0.05 was considered statistically significant

3. Results

3.1. Cell growth and proliferation

The xCELLigence system was used to determine the appropriate dose of Al-Malt to induce neurotoxicity and to evaluate the protective effects of EGCG and DES against Al-Malt-induced neurotoxicity in SH-SY5Y neuroblastoma cells. Cells were pretreated with Al-Malt, EGCG, and DES. As shown in Fig. 1A, SH-SY5Y cells treated with increasing concentrations of Al-Malt for 24 and 48 hours exhibited a significant decrease in cell viability. A statistically significant reduction was observed at concentrations of 200 μ M and above for 24 hours, and at 100 μ M and above for 48 hours ($p < 0.001$). Figure 1B demonstrates that EGCG treatment up to 100 μ M for 24 hours did not significantly affect cell viability. However, at 48 hours, only 100 μ M EGCG significantly reduced cell viability ($p < 0.001$). The effects of DES on cell viability were also assessed (Fig. 1C). DES treatment significantly increased SH-SY5Y cell proliferation at concentrations of 25 μ M and above ($p < 0.001$). Furthermore, after 48 hours, DES treatment at both 25 μ M and 100 μ M significantly enhanced cell viability ($p < 0.001$). Figure 1D represents the combined effects of Al-Malt exposure with selected doses of EGCG and DES on SH-SY5Y cell viability. As expected, treatment with Al-Malt alone significantly reduced cell viability compared to the control group. Co-treatment with 10 μ M EGCG markedly improved cell viability at both 24 and 48 hours relative to the Al-Malt-only group ($p < 0.001$), indicating a clear cytoprotective effect at this concentration. Notably, this protective effect was not observed at higher EGCG concentrations. Specifically, increasing the dose to 25 μ M abolished the beneficial effect, with cell viability levels comparable to those observed in the Al-Malt-

treated group. This suggests a dose-dependent biphasic response, in which lower concentrations of EGCG exert cytoprotective effects, whereas higher concentrations lose efficacy and may even contribute to cytotoxicity. In contrast, treatment with 50 μ M DES resulted in a modest increase in cell viability at 24 hours, although this effect did not reach statistical significance. However, at 48 hours, the increase became statistically significant ($p < 0.01$), indicating a time-dependent protective effect. Collectively, these findings demonstrate that Al-Malt induces significant cytotoxicity in SH-SY5Y cells, while EGCG exhibits a concentration-dependent dual effect, with optimal protection observed at 10 μ M but diminished or absent effects at higher doses.

Al(Malt)₃: Aluminum maltolate; EGCG: Epigallocatechin gallate; DES: Deferoxamine.

3.2. Al(malt)₃-induced cellular LDH release

Exposure to 200 μ M Al-Malt significantly increased LDH release compared to the control group ($p < 0.001$), confirming enhanced membrane damage and cytotoxicity, consistent with the cell viability findings. Co-treatment with 10 and 25 μ M EGCG, as well as 50 μ M DES, significantly reduced LDH release relative to the Al-Malt treated group ($p < 0.001$ for all), indicating a protective effect. Importantly, this protective effect of EGCG was dose-dependent and diminished at higher concentrations. While 10 μ M EGCG markedly attenuated LDH release, the reduction observed at 25 μ M was less pronounced, indicating a weaker protective effect at the higher dose. This pattern is consistent with the cell viability data, supporting a biphasic response in which lower EGCG concentrations are more effective, whereas higher concentrations exhibit reduced efficacy. Despite these improvements, LDH levels in all co-treatment groups remained significantly higher than in the control group ($p < 0.05$ for 10 μ M EGCG; $p < 0.001$ for 25 μ M EGCG; $p < 0.01$ for 50 μ M DES), indicating that cellular damage was only partially mitigated. Treatment with EGCG or DES alone did not significantly alter LDH release compared to the control group.

Al-Malt: Aluminum maltolate; EGCG: Epigallocatechin gallate; DES: Deferoxamine.

3.3. Al-Malt induced cellular oxidative damage

As shown in Fig. 3A and 3B, intracellular GSH content was markedly decreased, while MDA levels were markedly increased following 24 h exposure to 200 μ M Al-Malt compared to the control group ($p < 0.001$). Co-treatment with 10 μ M EGCG and 50 μ M DES significantly restored GSH levels compared to the Al-Malt group ($p < 0.001$). In contrast, 25 μ M EGCG further reduced GSH levels, showing a significant decrease compared to both the control ($p < 0.001$) and the Al-Malt group ($p < 0.05$), indicating a loss of the protective effect at higher concentrations. This pattern supports a biphasic response of EGCG, with antioxidant effects at lower doses and diminished or adverse effects at higher doses. Similarly, co-treatment with 10 and 25 μ M EGCG in combination with Al-Malt significantly reduced MDA levels compared to the Al-Malt group ($p < 0.01$). However, MDA levels in both EGCG-treated groups remained significantly elevated relative to the control group (both $p < 0.001$), indicating incomplete attenuation of lipid peroxidation. DES treatment also reduced MDA levels relative to the Al-Malt group, but levels

remained significantly higher than those of the control ($p < 0.001$). Treatment with EGCG or DES alone did not significantly alter GSH or MDA levels compared to the control group.

3.4. Effects of EGCG on caspase-3 and caspase 9 mRNA expressions in Al(Malt)₃ treated SH-SY5Y cells

Fold changes in expression were calculated using the $2^{\Delta\Delta C_t}$ method by comparing each group to the control. EGCG: epigallocatechin gallate; Al-Malt: aluminum maltolate. Statistical analysis was performed using One-Way ANOVA followed by Tukey's post hoc multiple comparison test. Pairwise comparisons with the healthy control group are indicated as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; comparisons of the Al-Malt toxicity group with other groups are indicated as ### $p < 0.001$.

SH-SY5Y cells exposed to Al-Malt for 24 hours resulted in a significant upregulation of Caspase-3 and Caspase-9 mRNA expression compared to the control group (both, $p < 0.001$), indicating activation of the apoptotic pathway. Pretreatment with EGCG and DES significantly attenuated the Al-Malt-induced increase in caspase-3 and caspase-9 mRNA levels. Notably, the effect of EGCG was dose-dependent. The lower dose (10 μ M) exerted a more pronounced suppressive effect on caspase expression, whereas the higher dose (25 μ M) showed a weaker attenuation, consistent with a biphasic response. This pattern aligns with the cytotoxicity and oxidative stress findings, supporting reduced efficacy of EGCG at higher concentrations. Collectively, these data indicate that EGCG mitigates Al-Malt-induced apoptosis, at least in part, through suppression of the caspase cascade. Treatment with EGCG or DES alone did not significantly alter caspase-3 and caspase-9 mRNA levels compared to the control group.

3.5. Effects of EGCG on NMDAR1, NMDAR2A and NMDAR2B mRNA expressions in Al-Malt treated SH-SY5Y cells

Fold changes in expression were calculated using the $2^{\Delta\Delta C_t}$ method by comparing each group to the control. EGCG: epigallocatechin gallate; Al-Malt: aluminum maltolate. Statistical analysis was performed using One-Way ANOVA followed by Tukey's post hoc multiple comparison test. Pairwise comparisons with the healthy control group are indicated as ** $p < 0.01$; comparisons of the Al-Malt toxicity group with other groups are indicated as # $p < 0.05$; ## $p < 0.01$.

Treatment of SH-SY5Y cells with Al-Malt resulted in a significant downregulation of NMDAR1 and NMDAR2B mRNA expression levels ($p < 0.01$ and $p < 0.05$, respectively), while no significant change was observed in NMDAR2A expression. EGCG treatment at both concentrations increased NMDAR1 and NMDAR2B mRNA expression levels; however, this effect was more pronounced at the lower dose. Specifically, a statistically significant increase was observed only for NMDAR2B in the 10 μ M EGCG group ($p < 0.001$), whereas the higher dose (25 μ M) failed to produce a significant effect, indicating reduced efficacy at elevated concentrations. This pattern is consistent with the biphasic response observed in other results. Treatment with EGCG alone did not induce any significant changes in

NMDAR1, NMDAR2A, or NMDAR2B mRNA expression levels. Similarly, DES treatment did not result in significant alterations, except for a change observed in NMDAR1 expression ($p < 0.05$).

3.6. Effects of EGCG on APP mRNA expressions in Al(Malt)₃ treated SH-SY5Y cells

Fold changes in expression were calculated using the $2^{\Delta\Delta Ct}$ method by comparing each group to the control. EGCG: epigallocatechin gallate; Al-Malt: aluminum maltolate. Statistical analysis was performed using One-Way ANOVA followed by Tukey's post hoc multiple comparison test. Pairwise comparisons with the healthy control group are indicated as *** $p < 0.001$; comparisons of the Al-Malt toxicity group with other groups are indicated as ### $p < 0.001$.

Analysis of APP mRNA expression revealed a significant increase following Al-Malt treatment compared to the control group ($p < 0.001$). Co-treatment with 10 μ M EGCG significantly reduced APP mRNA levels compared to the toxicity group ($p < 0.001$), although expression remained significantly higher than in the control group ($p < 0.001$). In contrast, 25 μ M EGCG failed to produce a significant reduction, and APP expression remained elevated compared to the control ($p < 0.001$), indicating diminished efficacy at the higher dose. This finding further supports a biphasic response pattern, with greater protective effects observed at lower EGCG concentrations. Similarly, co-treatment with Al-Malt and 50 μ M DES significantly reduced APP mRNA levels relative to the toxicity group ($p < 0.001$), but levels remained significantly higher than those of the control group ($p < 0.001$), indicating partial but incomplete recovery. Treatment with DES or EGCG alone did not produce any significant changes in APP mRNA expression compared to the control.

DISCUSSION

In the present study, EGCG and DES did not exert cytotoxic effects on SH-SY5Y cells, whereas Al-Malt induced significant cytotoxicity at concentrations $\geq 200 \mu$ M. Both compounds attenuated Al-Malt-induced cytotoxicity; however, EGCG demonstrated a broader protective profile, including suppression of oxidative stress, downregulation of apoptosis-related caspase expression, and partial restoration of NMDAR and APP mRNA levels. Notably, the protective effects of EGCG exhibited a concentration-dependent biphasic pattern, with greater efficacy observed at lower doses and diminished or absent effects at higher concentrations. This dual response suggests that EGCG may shift from an antioxidant to a less protective or potentially pro-oxidant profile at elevated doses. Collectively, these findings indicate that EGCG exerts multi-target neuroprotective effects against Al-Malt-induced neurotoxicity, although its efficacy is critically dependent on concentration. These results warrant further investigation in *in vivo* and clinical settings.

SH-SY5Y neuroblastoma cells were used in this study, as they are widely regarded as a suitable *in vitro* model for experimental neurotoxicity and neuroprotection research (Dhivya Bharathi et al., 2019). Deferoxamine is used in chelation therapy for aluminum toxicity and was employed as a positive control

in this study (Das et al., 2021). To determine the appropriate Al-Malt dose, SH-SY5Y cells were exposed to concentrations ranging from 50 to 800 μM for 24 and 48 hours, assessed via the xCELLigence real-time cell analysis system. Al-Malt induced a dose- and time-dependent decrease in cell viability, with significant cytotoxicity observed at $\geq 200 \mu\text{M}$; therefore, 200 μM was selected for subsequent experiments. To identify cytoprotective doses, cells were pretreated with EGCG (6.25–100 μM) or DES (6.25–100 μM) for 24 hours prior to Al-Malt exposure, with 10 μM and 25 μM EGCG, and 50 μM DES selected based on dose- and time-dependent analyses. LDH release assay independently corroborated these findings, confirming significant membrane damage at $\geq 200 \mu\text{M}$ Al-Malt and demonstrating that pretreatment with EGCG (10 and 25 μM) and DES (50 μM) significantly attenuated LDH release, with 10 μM EGCG showing the most pronounced cytoprotective effect. These results are consistent with previous studies reporting Al-Malt-induced cytotoxicity across multiple neuronal cell models, including PC-12, SH-SY5Y, N2a, and NT2 cells (Griffioen et al., 2004; Johnson et al., 2005; Wang et al., 2019) and collectively support the neuroprotective potential of EGCG against Al-Malt-induced cytotoxicity.

Oxidative stress represents a central key mechanism underlying metal-induced neurotoxicity and is a well-established contributing factor in the pathogenesis of age related neurodegenerative conditions (Dey and Singh, 2022). The brain is particularly susceptible to oxidative damage due to its exceptionally high oxygen consumption, elevated polyunsaturated fatty acid content, and relatively limited antioxidant capacity. Excessive generation of reactive oxygen species (ROS) under conditions of oxidative stress can induced neuronal cell death through multiple pathways, including apoptotic signaling cascades and excitotoxic calcium dysregulation. A substantial body of evidence indicates that various Al salts are capable of inducing oxidative stress both *in vitro* and *in vivo* (Dey and Singh, 2022; Iranpak et al., 2019). Accumulation of Al in brain tissue exacerbates ROS mediated damage and promotes neuroinflammation, processes that are closely associated with the pathogenesis of AD (Dey and Singh, 2022). In support of this, aluminum chloride has been shown to impair memory and increase oxidative damage in neuronal tissues in mice (Al-Amin et al., 2016). Furthermore, Iranpak et al. (2019) reported that Al exposure stimulated ROS production and induced apoptosis in PC12 cells, further implicating oxidative mechanisms in aluminum-mediated neurotoxicity (Iranpak et al., 2019).

Therapeutic interventions that target oxidative stress pathways represent promising strategies for preventing or treating oxidative stress-related neuronal disorders (Uttara et al., 2009). Oxidative stress represents a well-established mechanism underlying Al-Malt-induced neurotoxicity. In the present study, Al-Malt exposure significantly depleted intracellular GSH levels and elevated MDA concentrations in SH-SY5Y cells, indicative of impaired antioxidant defense and enhanced lipid peroxidation. These findings are consistent with previous reports demonstrating Al-Malt-induced GSH depletion in PC12 cells (Cheng et al., 2021), elevated MDA levels in PC12 and SH-SY5Y cells (Saberzadeh et al., 2016a; Wang et al., 2019) and increased ROS generation in neuronal cell models (Iranpak et al., 2019). EGCG pretreatment attenuated MDA accumulation and partially restored the antioxidant status of the cells. Interestingly, although GSH levels were not fully restored at the higher EGCG concentration, this reduction in intracellular GSH occurred concomitantly with decreased lipid peroxidation. EGCG is known to undergo auto-oxidation under physiological conditions, generating quinone intermediates that readily react with

glutathione to form GSH conjugates (Sang et al., 2007). Therefore, depletion of reduced GSH at higher EGCG concentrations may reflect increased GSH utilization and turnover during antioxidant defense rather than impaired antioxidant activity (Zwolak, 2021). DES, included as a positive control based on its established aluminum-chelating capacity, similarly attenuated oxidative damage markers, in line with *in vivo* evidence documenting its efficacy against Al-induced alterations in GSH, MDA, and ROS levels (Zhu et al., 2022). Collectively, these results indicate that both EGCG and DES confer meaningful protection against Al-Malt-induced oxidative damage in SH-SY5Y cells. Accordingly, the simultaneous reduction in MDA despite lower intracellular GSH levels observed in our study is more likely to reflect enhanced GSH consumption during ROS detoxification than a loss of the antioxidant efficacy of EGCG.

In addition to oxidative stress, **apoptosis** represents a key mechanism of Al-Malt-induced neurotoxicity, and is itself likely triggered downstream of ROS-mediated mitochondrial damage (Das et al., 2021; Dey and Singh, 2022; Wang et al., 2019). Apoptotic neuronal loss has also been implicated in the pathogenesis of neurodegenerative diseases (Zhang et al., 2016), including AD, where active caspase-3 and BAX have been detected in neurons exhibiting neurofibrillary tangle pathology (Mattson, 2004). Converging evidence from multiple neuronal models demonstrates that Al-Malt exposure elevates apoptotic rates, upregulates pro-apoptotic markers including BAX, caspase-3, caspase-9, and cytochrome c, and downregulates BCL-2, as documented across PC12 (Ohyashiki et al., 2002; Wang et al., 2019), (Saberzadeh et al., 2016b). (Saberzadeh et al., 2016a) SH-SY5Y (Dhivya Bharathi et al., 2019; Wang et al., 2019) and *in vivo* rodent models (Zhang et al., 2016). Consistent with these findings, the present study demonstrated that Al-Malt significantly upregulated caspase-3 and caspase-9 mRNA expression in SH-SY5Y cells, indicative of intrinsic apoptotic pathway activation. EGCG pretreatment significantly attenuated both caspase gene expressions, consistent with its reported anti-apoptotic properties (Islam et al., 2025). These results suggest that EGCG confers neuroprotection against Al-Malt-induced apoptosis at least in part through suppression of caspase-dependent signaling, potentially linking aluminum neurotoxicity to the neuronal loss observed in AD pathology.

NMDARs play a fundamental role in synaptic plasticity, learning, and memory, and their dysregulation has been implicated in the pathophysiology of AD and other neurodegenerative conditions (Malik et al., February 2025). In the present study, Al-Malt significantly downregulated NMDAR1 and NMDAR2B mRNA expression in SH-SY5Y cells, consistent with previous *in vivo* reports of dose-dependent NMDAR1 and NMDAR2B downregulation in hippocampal regions following Al exposure (Pan et al., 2020). Al-Malt-induced perturbation of ER stress and intracellular calcium homeostasis, processes tightly coupled to NMDAR stability, may further contribute to this dysregulation, as previously reported (Sahin-Mazlumoglu and Palabiyik-Yucelik, 2026). EGCG pretreatment partially mitigated Al-induced NMDAR downregulation, with statistically significant restoration observed specifically for NMDAR2B at 10 μ M, while NMDAR1 showed a non-significant trend toward recovery. This subunit-specific response may reflect the preferential involvement of NMDAR2B in stress-responsive signaling and is consistent with evidence that polyphenols can modulate glutamatergic neurotransmission and attenuate excitotoxicity (Syarifah-Noratiqah et al., 2018). Notably, EGCG alone did not alter NMDAR expression, suggesting that its modulatory effects are context-dependent and manifest primarily under conditions of cellular stress. In

contrast, DES exhibited minimal impact on NMDAR subunit expression. Collectively, these findings indicate that selective suppression of NMDAR1 and NMDAR2B represents an important mechanism of Al-Malt-induced neurotoxicity, and that EGCG may confer partial neuroprotection by preserving glutamatergic signaling integrity under toxic conditions. Targeting NMDAR subunit regulation may therefore represent a promising therapeutic avenue in aluminum-associated neurodegeneration and related disorders.

Aluminum exposure is known to promote amyloidogenic processing by enhancing β -secretase activity and upregulating APP expression via oxidative stress, thereby facilitating A β peptide generation (Huang et al., 2017; Misonou et al., 2000). In line with this, Al-Malt exposure significantly increased APP mRNA expression in the present study, supporting the contribution of aluminum toxicity to amyloidogenic pathology. Importantly EGCG at 10 μ M significantly attenuated Al-induced APP upregulation, whereas the higher dose (25 μ M) did not produce a significant effect, suggesting a biphasic or dose-dependent response possibly reflecting complex antioxidant-signaling interactions at higher concentrations. However, neither compound fully normalized APP expression to control levels, indicating partial rather than complete reversal of aluminum-induced amyloidogenic signaling. Notably, neither EGCG nor DES alone altered basal APP expression, suggesting that their modulatory effects are specifically associated with counteracting oxidative stress rather than directly regulating APP transcription. These findings are consistent with previous reports of Al-induced upregulation of APP and BACE1 expression (Huang et al., 2017), and with evidence that antioxidant interventions can suppress APP and secretase expression through oxidative stress-related pathways (Justin Thenmozhi et al., 2016).

In conclusion, the present study demonstrates that EGCG exerts significant neuroprotective effects against Al-Malt-induced oxidative stress, apoptosis, and dysregulation of NMDAR subunits and APP mRNA expression in SH-SY5Y cells. Importantly, these neuroprotective effects were concentration-dependent, with lower concentrations providing more consistent protection against GSH depletion, apoptosis, APP overexpression, and glutamatergic dysregulation. These findings suggest a concentration-dependent differential neuroprotective profile of EGCG, highlighting the importance of dose optimization for maximizing its therapeutic efficacy against Al-induced neurodegeneration. Therefore, further preclinical and clinical studies are required to validate its neuroprotective efficacy and to establish safe and effective dosing strategies.

Limitation

Several limitations of the present study should be acknowledged. First, the reliance on mRNA expression data limits the ability to draw definitive conclusions regarding corresponding protein levels, receptor assembly, and functional activity. Second, the use of a single transformed neuronal cell line (SH-SY5Y) limits the generalizability of the findings to primary neuronal systems and *in vivo* conditions. Future studies incorporating protein-level analyses, primary neuron cultures, calcium influx measurements, and electrophysiological assessments are warranted to provide a more comprehensive understanding of the mechanisms underlying EGCG-mediated neuroprotection.

Declarations

Acknowledgment

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Figures

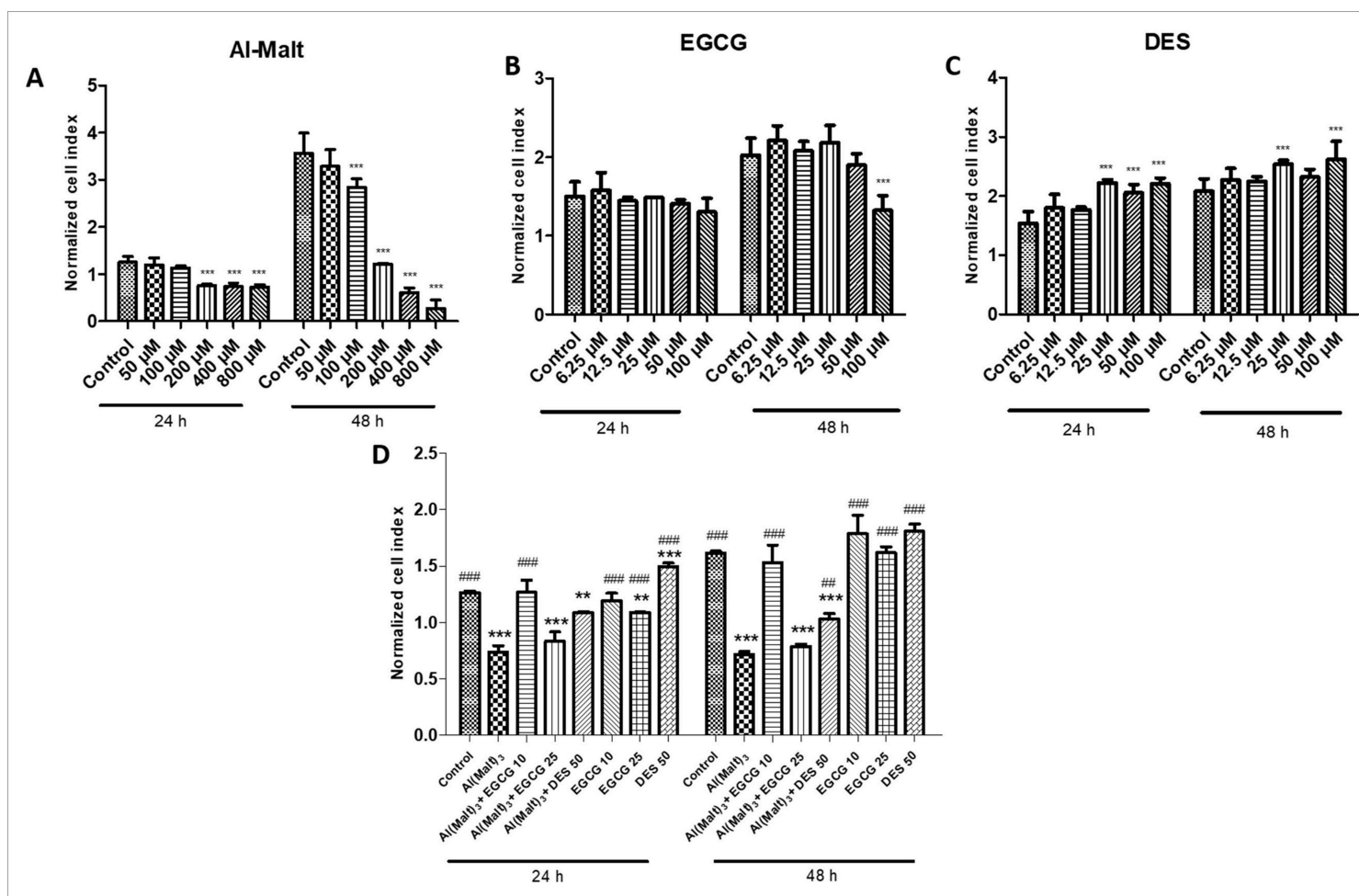


Figure 1

Cell viability following treatment with Al-Malt, EGCG, and deferoxamine (DES). Data were analyzed using one-way ANOVA followed by Tukey's post hoc multiple comparison test. ** $p < 0.01$; *** $p < 0.001$ control vs treatment groups. ## $p < 0.01$ ### $p < 0.001$ Al-Malt vs treatment groups

Al(Malt)₃: Aluminum maltolate; EGCG: Epigallocatechin gallate; DES: Deferoxamine.

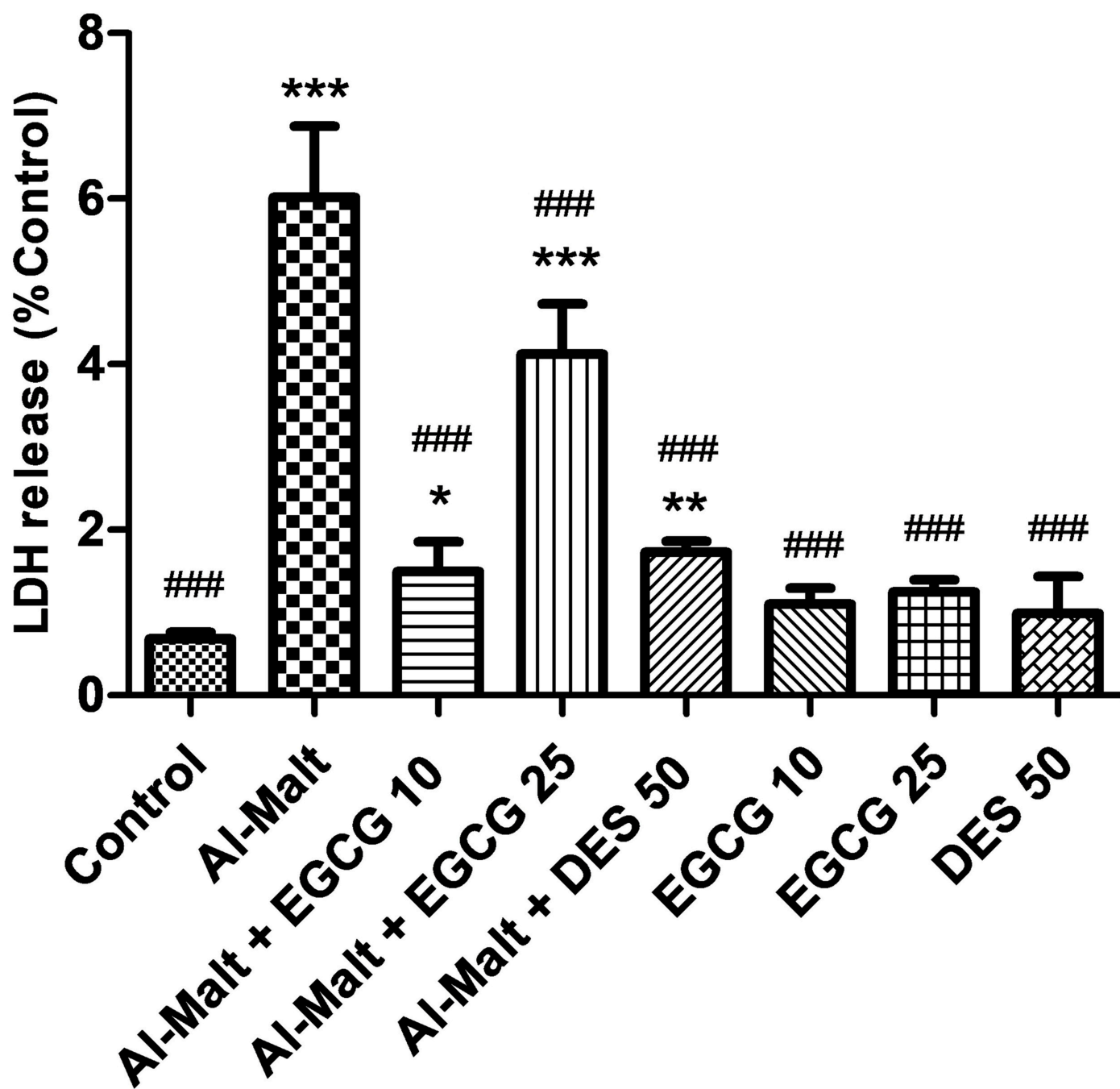


Figure 2

LDH release from SH-SY5Y cells treated with Al-Malt, EGCG, and deferoxamine (DES) for 24 hours. Data represent the mean $\pm$ SD of three independent experiments with similar results. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ control vs treatment groups; ### $p < 0.001$ Al-Malt vs treatment groups

Al-Malt: Aluminum maltolate; EGCG: Epigallocatechin gallate; DES: Deferoxamine.

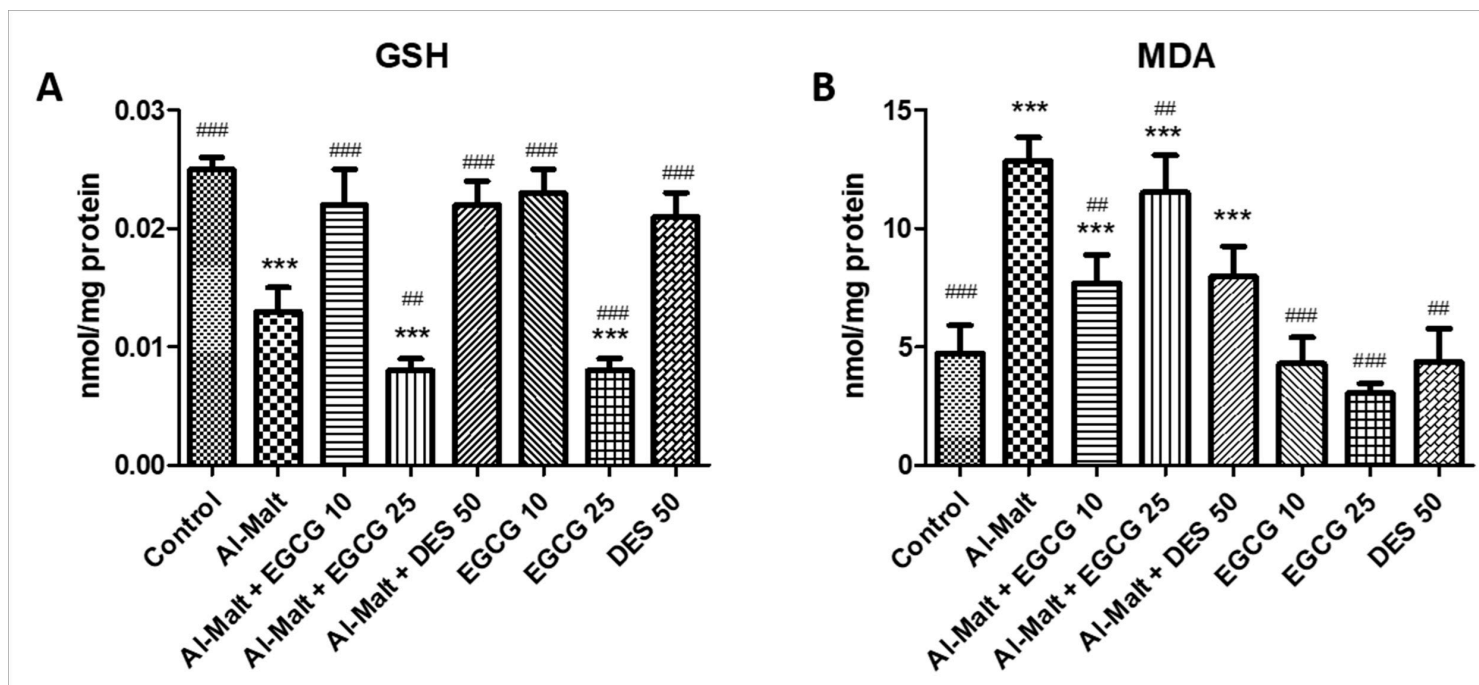


Figure 3

GSH (A) and MDA (B) levels in SH-SY5Y cells treated with AI-Malt, EGCG, and deferoxamine (DES). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc multiple comparison test. *** $p < 0.001$ vs. control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. AI-Malt group.

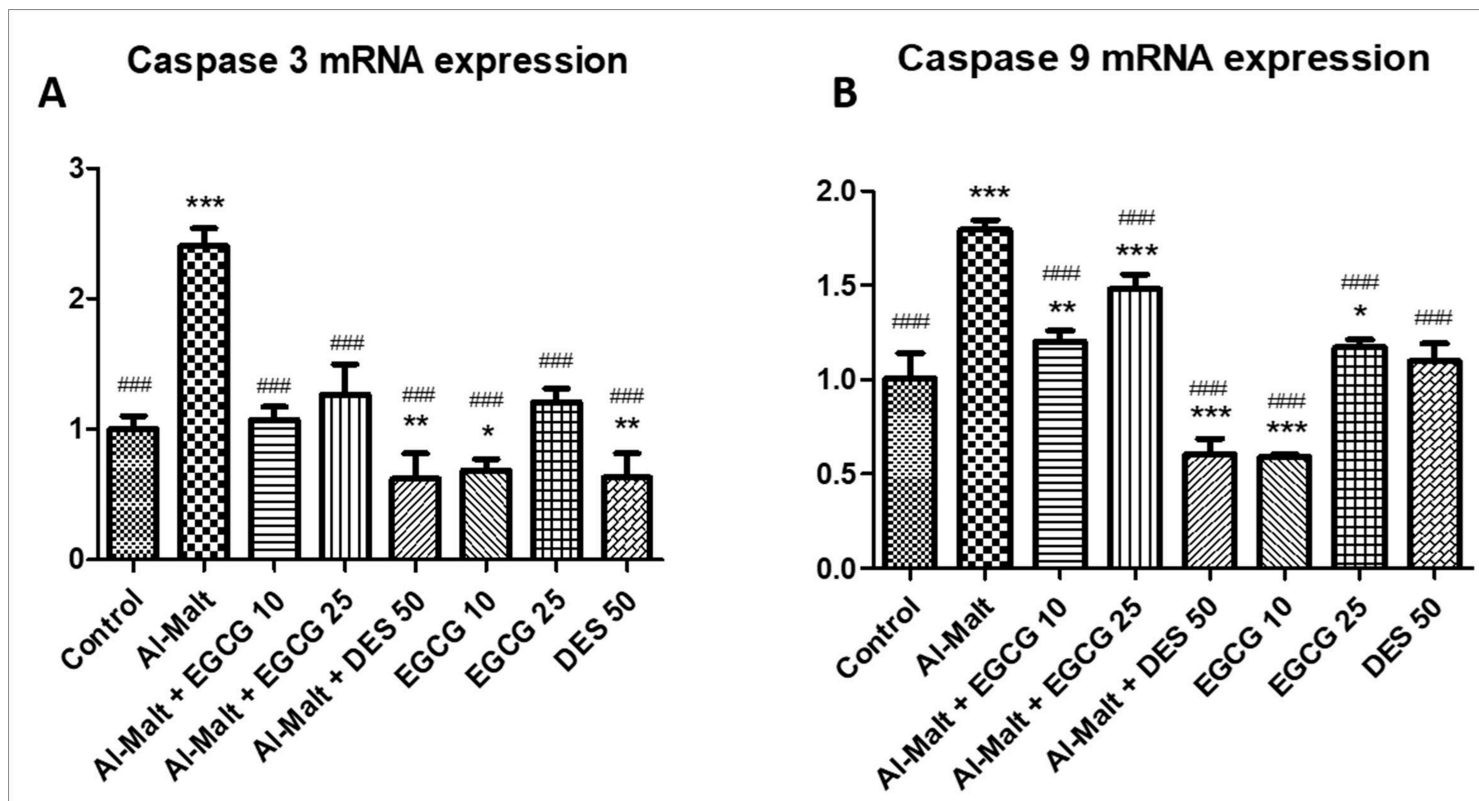


Figure 4

Caspase-3 (A) and Caspase-9 (B) mRNA expressions in SH-SY5Y cells treated with Al-Malt, EGCG, and DES

Fold changes in expression were calculated using the $2^{\Delta\Delta Ct}$ method by comparing each group to the control. EGCG: epigallocatechin gallate; Al-Malt: aluminum maltolate. Statistical analysis was performed using One-Way ANOVA followed by Tukey’s post hoc multiple comparison test. Pairwise comparisons with the healthy control group are indicated as *p < 0.05; **p<0.01; ***p<0.001; comparisons of the Al-Malt toxicity group with other groups are indicated as ###p < 0.001.

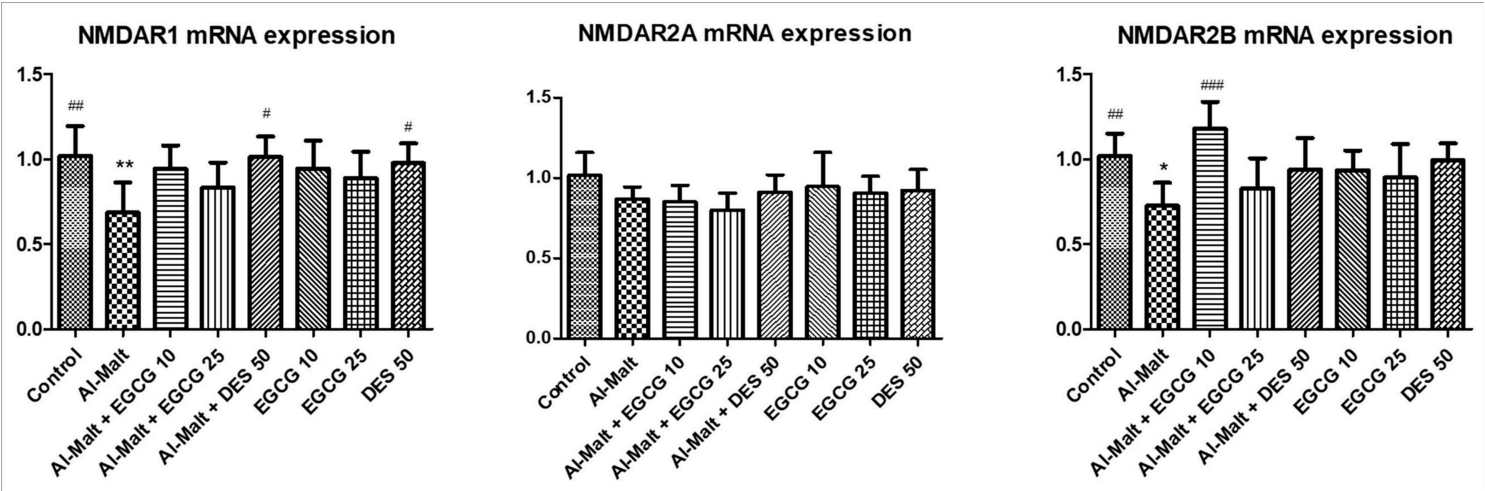


Figure 5

NMDAR1 (A), NMDAR2A (B) and NMDAR2B (C) mRNA expressions in SH-SY5Y cells treated with Al-Malt, EGCG, and DES

Fold changes in expression were calculated using the $2^{\Delta\Delta Ct}$ method by comparing each group to the control. EGCG: epigallocatechin gallate; Al-Malt: aluminum maltolate. Statistical analysis was performed using One-Way ANOVA followed by Tukey’s post hoc multiple comparison test. Pairwise comparisons with the healthy control group are indicated as **p<0.01; comparisons of the Al-Malt toxicity group with other groups are indicated as #p < 0.05; ##p < 0.01.

APP mRNA expression

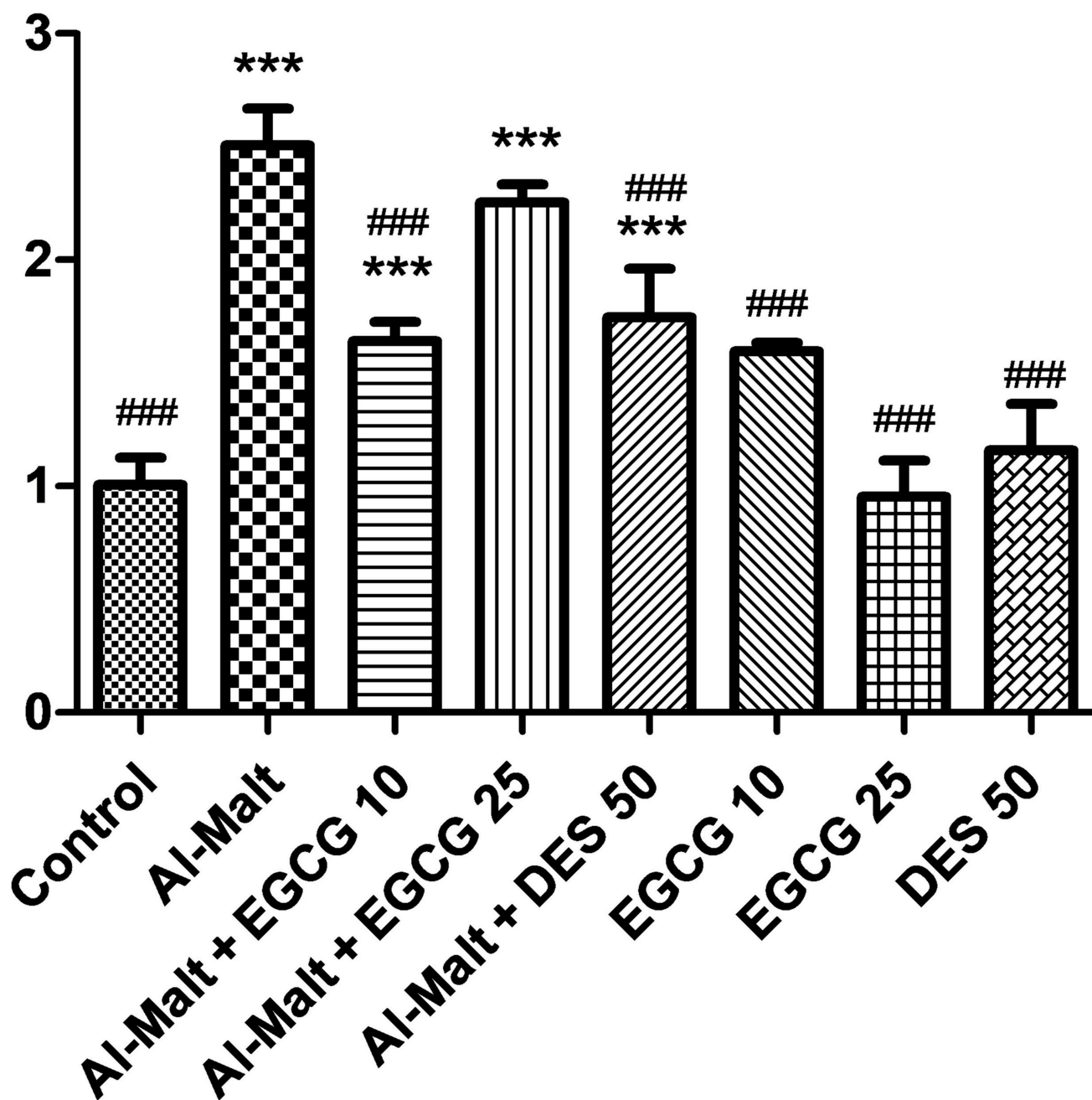


Figure 6

APP mRNA expressions in SH-SY5Y cells treated with Al-Malt, EGCG, and DES

Fold changes in expression were calculated using the $2^{\Delta\Delta Ct}$ method by comparing each group to the control. EGCG: epigallocatechin gallate; Al-Malt: aluminum maltolate. Statistical analysis was performed using One-Way ANOVA followed by Tukey's post hoc multiple comparison test. Pairwise comparisons

with the healthy control group are indicated as *** $p < 0.001$; comparisons of the Al-Malt toxicity group with other groups are indicated as ### $p < 0.001$.

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