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Full Length Research Article

Neuroprotective Effect of Garcinol Against Aluminium Maltolate Al(mal)3 Induced Alzheimer's Disease in SK-N-SH Cells

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Abstract

The process of ageing is the significant feature in the development of Alzheimer's disease (AD), which is one of the most prevalent, claiming the affected individual's personality and sociality beyond the health wise wellbeing. Formations of senile plaques, neurofibrillary tangles (NFT) are the cardinal features which are formed due to environmental and genetic factors. Aluminium (Al) in drinking water has been proposed to be one of the top most environmental factors. In the present study, we examined the effects of aluminum maltolate (Almal) in SK-N-SH cells to elucidate the effect of Al accumulation in the cells leads to Al toxicity and mimic the pathology of AD, Since $Al(mal)_3$ is an electroneutral water-soluble complex that is stable at neutral pH. Determination of Reactive Oxygen Species (ROS), Mitochondrial membrane potential (MMP) to study the integrity of mitochondria and Dual staining (Acridine orange, Ethidium Bromide) for investigating the apoptosis were carried out in the experimental groups of SK-N-SH cells. The study results show that garcinol is promising in suppressing the $Al(mal)_3$ induced AD alike pathological events like ROS formation, mitochondrial membrane damage and apoptosis and there by promotes the neuronal cell survival.

***Key words*:** Alzheimer's disease, Aluminium maldolate, SK-N-SH, Aging, Neurodegenerative disorder

Neurodegenerative diseases are multifactorial disorders commonly under the impact of various reasons such as environmental and genetic factors. Alzheimer's disease (AD) is a severe and progressive neurodegenerative disease of high incidence, in which the disease progression increases with increasing in age. The major pathological features are plaques formed by extracellular Beta-amyloid protein (amyloid-β, Aβ) deposition [1] and tangles of nerve fibres formed by intracellular hyperphosphorylated Tau protein. The imbalance between the production and removal of misfolded proteins (Aβ and Tau) produces a cascade effect, oxidative stress, inflammation and neuronal apoptosis, all these events in turn aggravates the progression of AD. Free radicals are major culprit leads to oxidative stress, which grounds for the brain damage by the imbalance between the production of reactive oxygen species (ROS) and antioxidant defence mechanism of the system. Additionally environmental factors such as smoking, diet, toxins such as pesticides, herbicides, heavy metals and infection have significant roles in the etiology of AD. Al-maltolate as novel compound for inducing AD like pathology in aged rabbits Al-maltolate (Al(mal)3) complex [2] on experimental animals since this compound can deliver a significant amount of free aqueous Al at physiological pH. In contrast, most other Al salts, such as AlCl3, produce insoluble complexes at neutral pH. The uniqueness of Al-maltolate compound is that this Al-complex increases the soluble Al concentration from 4-6 mM compared to other organic Al salts like Allactate or Al-aspartate (soluble Al concentration is 55-330 μM). Al-maltolate is soluble from pH 3.0 to 10.0, possesses hydrolytic stability at pH 7.0, and does not have speciation chemistry problems [3]. Al-maltolate is preferred over other Al compounds because of its following properties: (a) very high metal solubility at pH 7.0, (b) prominent kinetic restrictions to ligand exchange reactions in neutral solution [4-5], hence suitable for toxicological studies and also to understand the neuropathology. Aluminum maltolate Al(mal)3) is one of the metals which have been implicated in the experimental induction of Alzheimer's disease.

The present study was aimed to explore the neuroprotective effect of garcinol against aluminum maltolate ($Al(mal)_3$ induced neurotoxicity by assessing cell viability, mitochondrial membrane potential, levels of reactive oxygen species (ROS), DNA damage and apoptosis (Dual staining). Pre-treatment with garcinol significantly enhanced cell viability, attenuated rotenone-induced ROS, mitochondrial membrane dysfunction and apoptosis.

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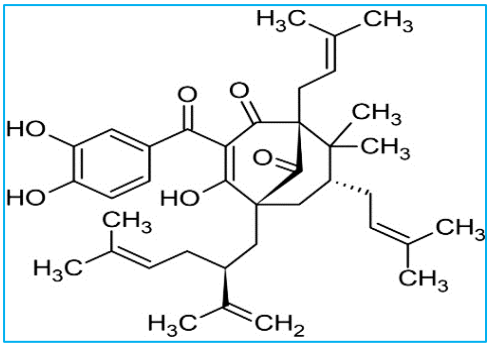

Fig 1 Garcinol structure

MATERIALS AND METHODS

Preparation of Al(mal)3 was done according to the method followed by Berthold *et al*. [6], Al(mal)3 was synthesized from maltol (3-hydroxy-2-methyl-4-H-pyran-4-one) and aluminum chloride hexahydrate. For 10 - 15 g of complex, 40.9. mM (9.9. g) of $AlCl_3$.$6H_2$ O and 122.8. mM (15.5. g) of maltol were dissolved in 160 ml of deionized water by mild heating and the pH was adjusted to 8.3. While heating the mixture to 65°C, precipitate was formed by stirring the solution and after cooling off-white crystals were filtered, washed with acetone and dried in a vacuum-desiccator for overnight. Preparation of $Al(mal)_3$ [7]. Crystalline aluminum chloride was prepared into a solution of 20 mM PBS. Maltol was dissolved in the preheated 0.1 M Phosphate Buffered Saline to a final concentration of 60 mM, and then the two solutions were mixed by the same volume to prepare $Al(mal)_3$ solution with concentration of 10 mM, pH was adjusted to 7.2 -7.4 by dropwise addition of 10 N NaOH and a 0.22 mm filter was used for extraction. Al(mal)3 solution of 10 mM was used as the mother liquor and diluted into different concentrations of $Al(mal)_3$ solution by using complete medium of corresponding volume, respectively.

MTT assay

The mitochondrial integrity and proliferation of cells were determined by MTT assay [8]. Cells were seeded in 96 well plate at a density of $3x10^3$ cells per well, incubated for 24 h and introduced to different concentrations of $Al(mal)_3$ (100, 200, 400, 500 and 600 µM) and garcinol (0.01, 0.1, 1, 10 and 100 nM) for 24 hours. To evaluate therapeutic efficacy of garcinol against $Al(mal)_3$ toxicity, cells were pretreated with different concentrations of garcinol (0.01, 0.1, 1, 10 and 100 nM) for 2 hours and incubated with Al(mal)3 (effective dose) for 24 h followed by addition of MTT (5 mg/ml) for 4 hours. After the incubation time the media was removed and purple formazan crystals are dissolved by 100 µl of DMSO. The absorbance of formazan product was read by spectrophotometer at 570 nm using a microplate reader. Based on the results obtained from cell viability assay, the effective dose of Garcinol against Al(mal)3 toxicity was employed to study the effect of garcinol by evaluating various parameters.

Measurement of intracellular ROS levels assay

The levels of ROS found in control and experimental cells were determined by fluorescence dye DCFH-DA [9]. SH-SY 5Y cells (1x 105) were pre-treated with garcinol (10 nM) for 2 h and then added and incubated with $Al(mal)_3$ (400 µM) for 24 h, followed by 25 µM DCFH-DA added for the medium after 30 mins, washed twice with phosphate buffered saline and visualized by using fluorescent microscope. Fluorescence Percentage changes in ROS productions of the treated groups were determined by comparing to the untreated control.

Measurement of mitochondrial membrane potential

Determination of MMP changes was done by using the mitochondrial specific fluorescent dye Rhodamine-123. Cells were cultured in 6-well plate ($1x\ 10^5$) and were treated with garcinol for 2 h and Al(mal)3 for 24 h, followed by incubation of Rhodamine-123 (5 mmol/ml) for 15 minutes [10]. Then washed with Phosphate Buffered Saline and fluorescence was quantified by using blue filter (450-490 nm) and fluorescence intensity was measured by using spectrofluorometer at 535 nm.

Determination of apoptosis

Analysis of apoptosis was done by using the acridine orange/ethidium bromide as a dual staining for this assay. Apoptosis was analyzed by treating the control and experimental cells with fluorescent dyes (AO/EB) and quantified by using fluorescence microscope. Cells were incubated with $Al(mal)_3$ alone, garcinol and $Al(mal)_3$, garcinol (10 nM) alone. After incubation period, cells were washed and followed by the addition of AO/EB reagent for 10 mins. Cells were observed by using fluorescence microscopy, normal green nuclei denote live cells, whereas early apoptotic cells denote bright red nuclei and late apoptotic cells appear in orange colored chromatin [11].

Statistical analysis

Statistical analysis was performed by one-way analysis of variance followed by Duncan's multiple range test (DMRT) using Statistical Package for the Social Science (SPSS) software package version 12.0. Results were expressed as mean ± SD for four experiments in each group. $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Effect of Garcinol on $Al(mal)_3$ induced cytotoxicity in cells exposed to different concentration of $Al(mal)_3$ showed a significant ($p < 0.05$) dependent cyto-toxicity. At a dose of 400 µM, it was caused ~ 50% of cell death as compared with control and considered as inhibitory dose. Different concentrations of Garcinol (0.01, 0.1, 1, 10 and 100 nM) reduced toxicity caused by $Al(mal)_3$ and maximum protection was offered at 10 nM concentration and was taken as protective dose (Fig 2). The doses, 400µM of $Al(mal)_3$ and 10 nM of garcinol were used as effective doses and used for further studies.

Effect of Garcinol on Al(mal)3 induced intracellular ROS production

Exposure of SK-N-SH cells to $Al(mal)_3$ (400 mM) increased the green fluorescence significantly ($p <0.05$) which is a clear indicator of high levels of ROS, whereas 10 nM Garcinol pretreatment to $Al(mal)_3$(400 mM) exposed cells reduced green fluorescence significantly (Fig 3A-B).

Effect of Garcinol on Al(mal)3 induced reduction in MMP

Rhodamine-123 steadily penetrates the normal cells; which was taken by mitochondria and exhibited high fluorescent intensity. $Al(mal)_3$ exposure decreasing the intracellular green fluorescence significantly ($p <0.05$), which is revealed by increase in fluorescence intensity (Fig 4).

Impact of Garcinol on $Al(mal)_3$ -mediated apoptosis

SK-N-SH cells treated with $Al(mal)_3$ and Garcinol were used to determine apoptosis by using AO/EB in dual staining method. In SK-N-SH, cells exposed to Al(mal)3 induced the formation of orange/red luminescent apoptotic cells significantly ($p <0.05$) whereas pretreatment of Garcinol to

$Al(mal)_3$ exposed cells, increased the cell viability and decreased apoptotic cell death significantly (Fig 5).

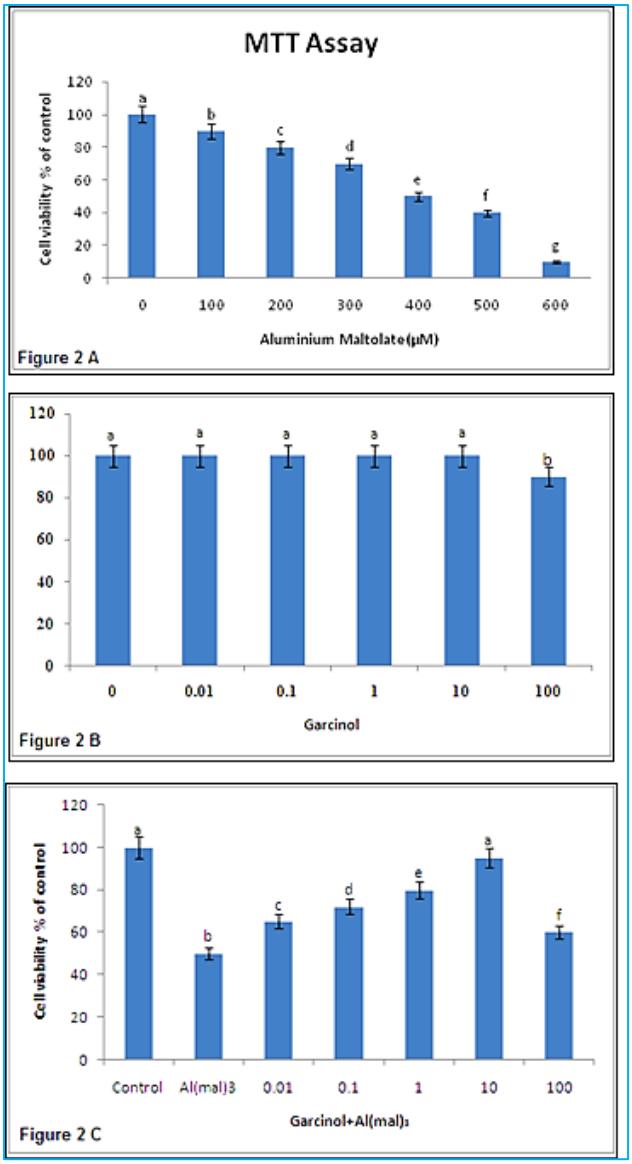

Fig 2 Al(mal)3 (0, 100, 200, 400, 500 and 600 μM) treatment dose dependently diminished the cell viability as compared to control (A). Garcinol (0,0.01, 0.1, 1 and 10 nM) alone treatment did not altered the cell viability, whereas high dose (100 nM) diminished the cell viability slightly (B). Garcinol pretreatment (0.01, 0.1, 1, and 10 nM) dose dependently enhanced the cell viability against Al(mal)3 toxicity, whereas 100 nM of Garcinol reduced cell viability significantly (C). Values are presented as mean ± SD in four experiments each group. Values not sharing a common symbol differ significantly (p <0.05)

In previous studies, aluminum chloride and $Al(mal)_3$ were used [12-14]. $Al(mal)_3$ is a lipophilic complex and easily permeable to the cell membrane. Maltolate is byproducts of the breaking of sucrose and its typical component of the human diet. Maltolate has a strong tendency to combine with aluminum, causing the formation of $Al(mal)_3$ complex in the gastrointestinal tract. Thus, the study of the combined toxicity of $Al(mal)_3$ contributes to human health [15].

MTT assay is widely used methods to analyze the cell proliferation and viability. Previous experiments indicated that $Al(mal)_3$ treatment (400 μM and 150 μM) caused ~ 50% of cell death in SH-SY 5Y [16] and PC 12 cells [17], which is consistent with our current results. However, Garcinol pretreatment significantly protected the $Al(mal)_3$-treated cells in a dose-dependent manner.

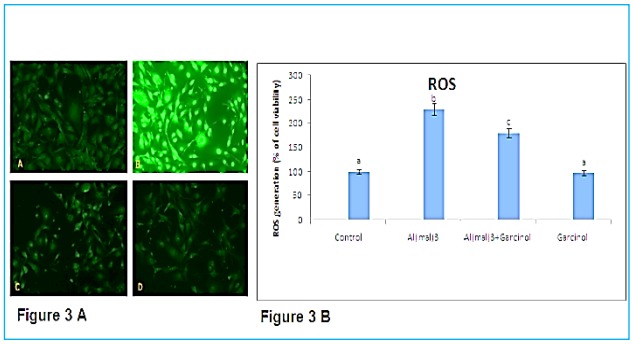

Fig 3A-B Al(mal)3 treatment enhanced the ROS levels as compared to control, whereas Garcinol pretreatment attenuated the levels of ROS in SK-N-SH neuroblastoma cells. Values are given as mean ± SD of four experiments in each group. *p <0.05 compared to control; p <0.05 compared to Al(mal)3 group (Duncan's multiple range Test-DMRT)

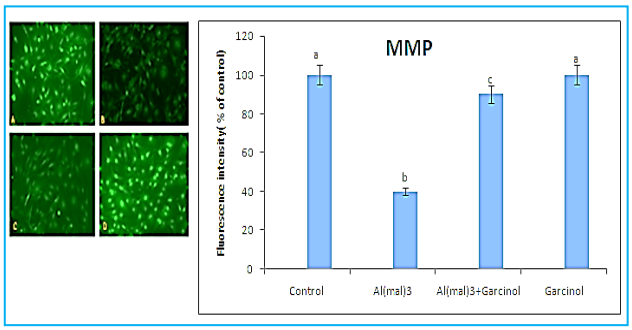

Fig 4 Al(mal)3 treatment reduced mitochondrial membrane potential as compared to control, whereas Garcinol pretreatment enhanced mitochondrial membrane potential in SK-N-SH neuroblastoma cells. Values are given as mean ± SD of four experiments in each group. *p <0.05 compared to Al(mal)3 group (Duncan's multiple range Test-DMRT)

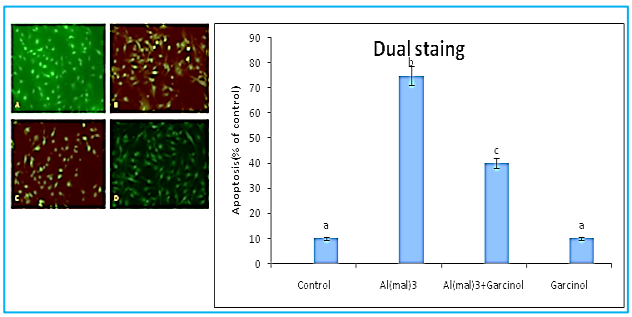

Fig 5 Al(mal)3 treatment enhanced apoptosis as compared to control, whereas Garcinol pretreatment showed antiapoptotic effect in SK-N-SH neuroblastoma cells. Values are given as mean ± SD of four experiments in each group are given as mean ± SD of four experiments in each group. * p <0.05 compared to control; p <0.05 compared to Al(mal)3 group (Duncan's multiple range Test-DMRT)

The mitochondrial dehydrogenases catalyze the formation of blue formazan product by the reduction of the MTT tetrazolium salt and this assay is widely used for evaluating neuronal cell survival [18]. Neuronal apoptosis is a significant characteristic of neurodegeneration and well-known form of cell death in many NDDs including AD [19]. The observation of dual staining techniques confirmed these apoptotic changes in $Al(mal)_3$ treated cells. Previous studies indicated that the treatment with Al(mal)3 induced cell death in brain [20-21] and as well in in vitro studies [22-24], which is corroborated with our study. The neuroprotective effect of Garcinol in MTT assay paralleled the morphological analyses

obtained with dual staining. Previous reports on garcinol exposure increased the survival of PC12 cells against oxygen-glucose deprivation/reoxygenation injury, [25] SH SY 5Y cells against glutamate toxicity [26] and cultured rat hepatocytes against D-galactosamine or carbon tetrachloride injury [27], which strengthen our finding.

Evaluating the mitochondrial membrane depolarization using rhodamine-123, which is a mitochondrial sensitive dye that is taken up only by mitochondria with intact membrane polarity, which emits green fluorescence. The observed low green fluorescence in mitochondria from Al(mal)3- treated cells indicates its depolarized state, and more green fluorescence found in garcinol pre-treated cells represents the improved mitochondrial membrane integrity [28]. Yamamoto *et al*. [28] demonstrated that the treatment of garcinol enhances mitochondrial function by enhancing mitochondrial energy production and antioxidant systems in a murine cell lines. Mitochondrial damage able to triggered the ROS production, and its leads to oxidative stress in the cells. Organisms appear to modulate several antioxidant enzymes and stress-related gene expression in response to oxidative stress. In the present study, garcinol treatment significantly suppressed the $Al(mal)_3$-induced ROS levels. Previous study from our lab also demonstrated that garcinol administration significantly attenuated AlCl3-induced lipid peroxidation process by improving the antioxidant content [29], which is line with our present results. Garcinol improving the antioxidant status by removing free radicals, which is three times higher than vitamin C. $Al(mal)_3$ initiates apoptosis involving both the ER stress and the mitochondrial dysfunction [30], consistent with increasing evidence that suggests signaling between the ER and mitochondria may be participated in the regulation of apoptosis [31].

Garcinol enhance neuronal cell survival by modulating ERK pathway and promote neurite growth in epidermal growth factor (EGF)-responsive neural precursor cell [32]. Antioxidant nature of garcinol has been experimentally demonstrated by many groups. Garcinol readily scavenge free radicals and can remove oxidative stress in the cell; garcinol having the neuroprotection against damaging effects of free radicals. Garcinol also reduce inducible nitric oxide synthase (iNOS) level (induced by lipopolysaccharide) in primary astrocytes and could enhance survival of neurons in neuron–astrocyte co-culture system [33]. This finding increasing the possibility of garcinol considered in the development of therapeutic drug for neurodegenerative diseases such as Alzheimers's, Parkinson's to attenuate oxidative stress-induced neurotoxicity.

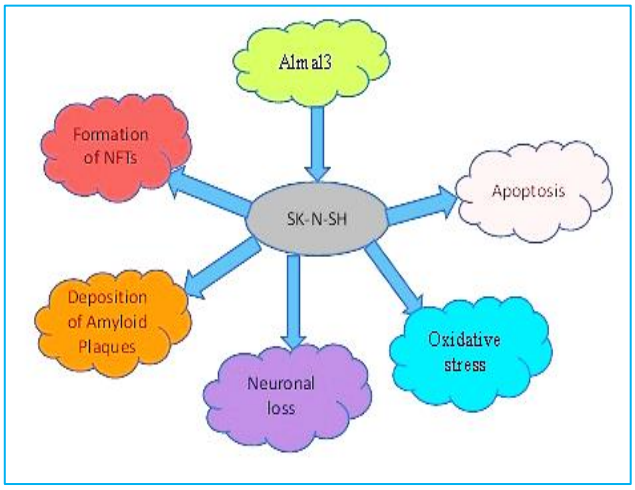

Fig 6 Schematic representation of Al-maltolate-treated SK-N-SH Neuroblastoma cells

Garcinol, a phytochemical nature of the plants belongs to the genera Garcinia has also been reported to have anti-oxidant and anti-inflammatory properties, additionally having neuroprotection against oxidative stress occurring in AD patients. Thus, the present finding is huge importance in the treatment concept of Alzheimer's disease and demands for further in vivo and in vitro investigations [34].

CONCLUSION

Garcinol have significant protective effects of neuroprotection by circumventing all the major pathological events that are for AD. Thus, the current requirement is to understand the extended role of garcinol in varieties of Alzhiemer's models through effective research techniques. For the early recruitment of garcinol in AD therapeutics, its toxicological profiles, safe dosage as well as its bioavailability improving strategies stand in the need of being elucidated. These studies will put forth the implementation of garcinol as a promising compound in the clinical research and treatment of AD.

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