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Original Research

Cytoprotective effect of alpha-2-macroglobulin against pesticide-induced generation of ROS in neuronal SH-SY5Y cells

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ABSTRACT

Introduction: Many clinical studies have demonstrated that continuous exposure to pesticides, especially organophosphates and pyrethroids, causes toxicities such as carcinogenicity and neurotoxicity that lead to disorders such as diabetes, lung cancer, and neurodegenerative diseases. The mechanism underlying pesticide-induced neurotoxicity involves the production of ROS, which causes neuronal injury through oxidative stress. **Methods**: In the present study, the neuronal SH-SY5Y cell line was used to investigate the effect of the pesticides chlorpyrifos (organophosphate), aldicarb (carbamate), and deltamethrin (pyrethroid) on ROS-mediated toxicity and the protective effect of alpha-2-macroglobulin (α2M), a protease inhibitor and beta-amyloid plaque scavenger in the human brain. For cell viability and cytotoxicity, the MTT assay was performed. To monitor ROS production, assays such as DCFHDA, H2O2, and MDA were performed, along with assays of the activity of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase. **Results**: The experimental findings suggest a cytoprotective role for α2M in ROS-mediated toxicity that causes neuronal injury in humans. **Conclusion**: Hence, α2M could be possibly used as a protective agent against oxidative neurotoxicity caused by pesticides.

Key words: Alzheimer's disease, SH-SY5Y, reactive oxygen species, neurotoxicity, deltamethrin, chlorpyrifos, aldicarb, alpha-2-macroglobulin

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INTRODUCTION

The pesticides chlorpyrifos (CPF), aldicarb (ALD), and deltamethrin (DLM; **Figure 1**) are known to cause both acute and chronic neurotoxicity in humans and animals through the inhibition of cholinesterase (ChE) activity in the synaptic junction of neurons, binding to gamma-aminobutyric acid (GABA)-gated channels by preventing the closure of sodium channels[1]. Humans are exposed to these pesticides in a number of ways, such as the consumption of fruits and vegetables contaminated with pesticide sprays and the inhalation of fumigants[2–5]. Apart from causing substantial toxicity, these pesticides interfere with normal neuronal processes *in vivo*, leading to oxidative damage and cellular inflammation in the human brain[6–8]. Some of the modifications caused by these pesticides include oxidative stress[9], disrupted neurotransmission[10] and neuronal differentiation[11], inhibition of replication in neuronal cells[12], cognitive changes, decrease in psychomotor functions[13], and apoptosis in neuronal cells by their metabolites[14–17]. Such changes in neurons may lead to various neurodegenerative diseases such as Alzheimer's disease (AD) and/or Parkinson's disease (PD)[18–20].

Alpha-2-macroglobulin (α_2M) is produced in the human brain and keeps a check on reactive oxygen species (ROS)[21] and ß-amyloid plaque formation, thereby preventing neuronal degeneration and AD progression. α_2M is a serum protease inhibitor that may be involved in AD in mediating the clearance and degradation of Aß, a major constituent of ß-amyloid deposits[22,23]. α_2M, an acute-phase protein and a major component of the innate immune system, is a cerebrospinal fluid (CSF) marker of neuronal injury in preclinical AD [22]. An increased concentration of α_2M in the blood is associated with neuronal injury[24]. Previous studies have shown that α_2M interacts with the above mentioned pesticides[3,21], indicating the probable role of α_2M as an unexplored biomarker in pesticide-induced ROS-mediated neurotoxicity[25]. Hence, in the present study, α_2M was used to study the modulating/impairing effect of pesticide generated ROS on the neuronal SH-SY5Y (NCCS, Pune) cell line.[26].

METHODS

Materials

All pesticides (CPF, ALD, and DLM) and chemicals (MTT dye, DMSO, pyrogallol, DMEM and Ham's F12 medium, fetal bovine serum [FBS], thiobarbituric acid [TBA], 1% penicillin-streptomycin, dichlorodihydrofluorescein diacetate [DCFH-DA], glutathione

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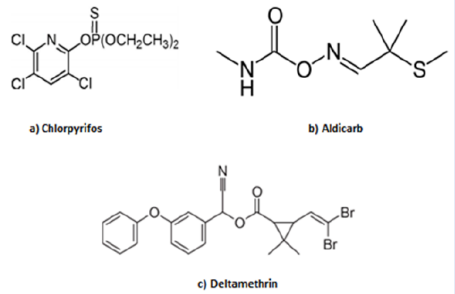

Figure 1: **Structure of pesticides a) chlorpyrifos b) aldicarb c) deltamethrin.**

[GSH], and NADPH) were purchased from Merck-Sigma (St. Louis, MO, USA). All other reagents used were of analytical standard.

METHODS

Purification and characterization of human α_2M

The α_2M protein was isolated and purified from human blood plasma using ammonium sulfate precipitation followed by gel exclusion chromatography as per the method described previously [3]. A 5% (w/v) native PAGE was performed [21] and the gel was stained with Coomassie brilliant blue R-250 (0.15% in 10% acetic acid). The gel was de-stained for 12 h in the de-staining solution (10% acetic acid), and the purified α_2M formed a single band on the gel.

Cell culture

The SH-SY5Y cell line was cultured in a medium containing 1:1 DMEM and Ham's F12 medium, 10% FBS, and 1% penicillin-streptomycin [26]. The cells were treated with a standard solution of pesticides and α_2M, accordingly, to perform the experiments. Cells were used at 3–7 passages. The cells were divided into five groups based on the treatment with pesticides and proteins to obtain results for various stress markers. Group I was the control group comprising only SH-SY5Y cells under standard conditions (37 °C and 5% CO_2). Group II consisted of SH-SY5Y cells incubated with α_2M. The concentration of protein was 2 μM and the incubation time was 3 h (37 °C). Group III comprised pesticide (CPF, ALD, and DLM)-treated SH-SY5Y cells. Cells were treated with 5 μM of each pesticide (CPF, ALD, and DLM) separately for 3 h under standard conditions (37 °C and 5% CO_2). Group IV was the pesticides (CPF, ALD, and DLM) and α_2M group, in which the SH-SY5Y cells were treated with 5 μM of the pesticides for 3 h and then treated with α_2M for 3 h.

Cell viability and proliferation assay

To determine the cytotoxicity and cell viability of the SH-SY5Y cells, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide MTT assay was performed in 96-well plates [27]. The cells were used at 70–80% confluence. Later, the medium was removed and the cells were treated with pesticides (CPF, ALD, and DLM; 5 μM) for 6 h and later incubated with α_2M for 3 h. Afterward, the cells were treated with MTT solution (5 mg/mL stock solution) at a final concentration of 0.5 mg/mL MTT. The cells were incubated for 4 h at 37 °C in 5% CO_2. Finally, the cells were treated with 100 μL DMSO for 10 min to dissolve the formazan crystals. The absorbance was measured using an ELISA plate reader at 570 nm with a reference wavelength of 630 nm and was directly proportional to the number of viable cells. The experiments were performed in triplicate.

Measurement of ROS

ROS production was measured using DCFH-DA [28]. The cells were treated with α_2M and the pesticides, following which 10 μM DCFH-DA was added to the medium for 1 h at 37 °C for diffusion into the cells. A multi-detection microplate reader was used for fluorescence measurement. The excitation and emission wavelengths for DCFH-DA were 485 and 535 nm, respectively [28–30].

Measurement of malonaldehyde (MDA) levels

To detect the generation of ROS by pesticides in the neuronal cell line, the MDA levels were measured. The MDA levels in SH-SY5Y cells were quantified with the TBA reaction. Thiobarbituric acid reactive substances (TBARS) were measured by comparing the absorption to the standard curve of MDA equivalents generated by the acid-catalyzed hydrolysis of tetramethoxypropane [31]. The absorbance was recorded at 532 nm.

Measurement of superoxide dismutase (SOD) activity

The antioxidant enzyme SOD converts superoxide ($O_2^{-\cdot}$) into H_2O_2 and O_2, which is converted to water by other enzymes. The measurement was carried out as described previously [32]. To 80 μL of the cell suspension, 2.82 ml of 0.05 mM tris-succinate buffer was added, and the sample was treated for 30 min in a CO_2 incubator. The reaction was initiated by adding 100 μL of 8 mM pyrogallol solution to each well [32]. The absorbance was read at 420 nm.

Measurement of glutathione peroxidase (GPx) levels

GPx are cytosolic enzymes that catalyze the conversion of H_2O_2 into H_2O and O_2 and the reduction of peroxide radicals ($ROO^{\cdot}$) into alcohol and oxygen. GPx levels were measured by determining the decrease in absorbance at 340 nm upon the oxidation of NADPH to $NADP^+$ [33].

Statistically analysis

All experiments were repeated thrice and the data shown is the mean +- SD. P-value < 0.05 is considered as significant difference.

RESULTS

Cell viability and cell cytotoxicity assay

The MTT assay was performed to assess cell viability and cell proliferation activity, in which the quantity of formazan is directly proportional to the number of viable cells. The SH-SY5Y cells treated with all three pesticides (CPF, ALD, and DLM) in group III showed reduced cell viability ($p < 0.001$; **Figure 1**). Almost no effect on viability was seen for cells treated with α_2M (group II), while the effect of the pesticides appeared to be reversed in cells treated with pesticides after incubation with α_2M (group IV).

Measurement of ROS levels

Figure 3 shows the ROS levels induced by the pesticides as monitored by DCFHDA in the control group of SH-SY5Y cells and all three pesticides in group III ($p < 0.001$). The increased fluorescence in group III as compared to the control group and α_2M group indicates the production of ROS. On the contrary, group IV showed a reduction in the fluorescence intensity as compared to group III, indicating the defensive function of α_2M against ROS production.

Measurement of MDA

Figure 4 shows the TBARS activity in the SH-SY5Y cells in various groups. The highest MDA production was recorded in group III, in which the cells were treated with pesticides. However, group II showed no TBARS production. Group IV (pesticides + cells + α_2M), however, showed a reduction in the TBARS level as compared to group III. The MDA levels were normal in the control group and slightly reduced in the α_2M group ($p < 0.001$). However, MDA levels were significantly decreased in the α_2M + pesticides + cells group ($p < 0.001$) compared to the pesticides group. Hence, the protective effects of α_2M were observed with respect to MDA levels due to pesticide-triggered ROS in SH-SY5Y cells.

Measurement of SOD levels and GPx activity

Figure 5 shows the effect of various treatments on SOD levels in the control and other groups. Group III (pesticides group) showed reduced SOD activity as compared to group I (control) and group II (α_2M; $p < 0.001$), demonstrating the toxicity triggered by the pesticides. On the other hand, group IV (α_2M + pesticides + SH-SY5Y cells) showed a remarkable increase in SOD and GPx activity levels, indicating the protective effect of α_2M against the pesticides. **Table 1** shows the effect of pesticides on the activity of antioxidant enzymes in the cells. The pesticides group showed reduced GPx activity as compared to the control group I and α_2M group II, while group IV (α_2M and pesticides) showed increased GPx activity levels.

DISCUSSION

CPF, ALD, and DLM are potent neurotoxic pesticides that affect various neuronal processes involved in the synaptic transmission and growth of neurons [34–37]. α_2M is an acute-phase protein produced in the brain to keep ROS levels under control [3,21] and prevent amyloid plaque formation by dissolving them through receptor-mediated endocytosis. It also protects the brain from undergoing neuronal degeneration [25]. α_2M is also a major component of the innate immune system and acts as a hallmark of neuronal injury [21]. The human brain synthesizes and secretes α_2M upon stimulation with interleukin-6, which indicates that α_2M is an acute-phase protein in the human CNS. In this study, we characterized ROS formed by CPF, ALD, and DLM in the SH-SY5Y cell line. Furthermore, we investigated the ability of α_2M to modulate the neurotoxic effect of these pesticides. The cellular toxicity of these pesticides on SH-SY5Y cells was first measured by the MTT assay, which indicated the toxic effects of the pesticides on the cell line.

DCFH-DA is a fluorimetric dye that is applied to quantify ROS [38]. This non-fluorescent compound diffuses into the cells, where it is sequentially hydrolyzed to release DCFH as the substrate by intracellular enzymes. ROS oxidize DCFH and convert it into DCF, a fluorescence product that is measured by spectrofluorometry using a microplate reader. A significant increase in the production of DCF was seen in pesticide-treated cells. This increased production of ROS results in oxidative damage to cellular components. However, when α_2M was incubated with pesticide-treated cells, a contrary effect was observed. Similarly, regarding MDA levels, pesticide-treated groups showed a significant increase in absorbance, but in group IV, a

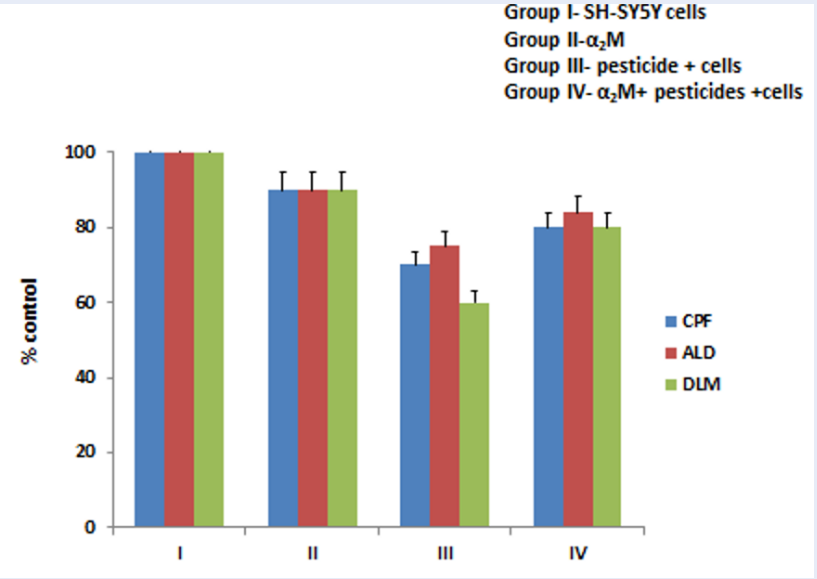

Figure 2: **MTT assay of various groups of SH-SY5Y cells and all three pesticides (CPF, ALD, and DLM)**. Cells were pretreated with pesticides at 5 μM for 3 h and then incubated with α2M for 3 h. After incubation, cell viability was evaluated by the MTT assay. The results are shown as the mean $\pm$ SEM of three experiments, with each experiment performed in triplicate ($p < 0.001$ versus control). Group II is not statistically significant to Groups I, III and IV.

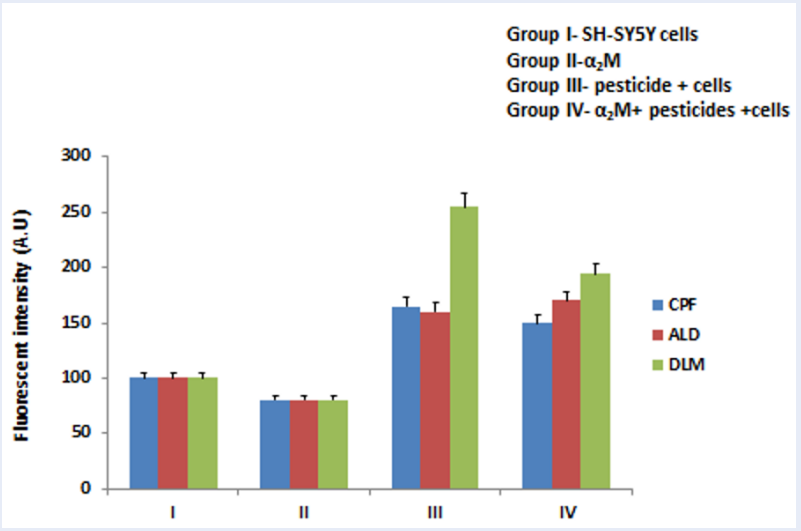

Figure 3: **DCFHDA ROS assay of SH-SY5Y cells. ROS were determined by the DCFH-DA method**. DCFH-DA measurements are reported as fluorescence intensities (AU, arbitrary fluorescence units). Results are mean $\pm$ standard error of triplicates. The error bar depicts the standard error among the six different samples of that group. Cells were incubated with 5 μM of each pesticide for 45 min, and the α2M concentration was 2 μM. Excitation and emission wavelengths for DCFH-DA were 485 and 535 nm, respectively. Group II is not statistically significant to Groups I, III and IV.

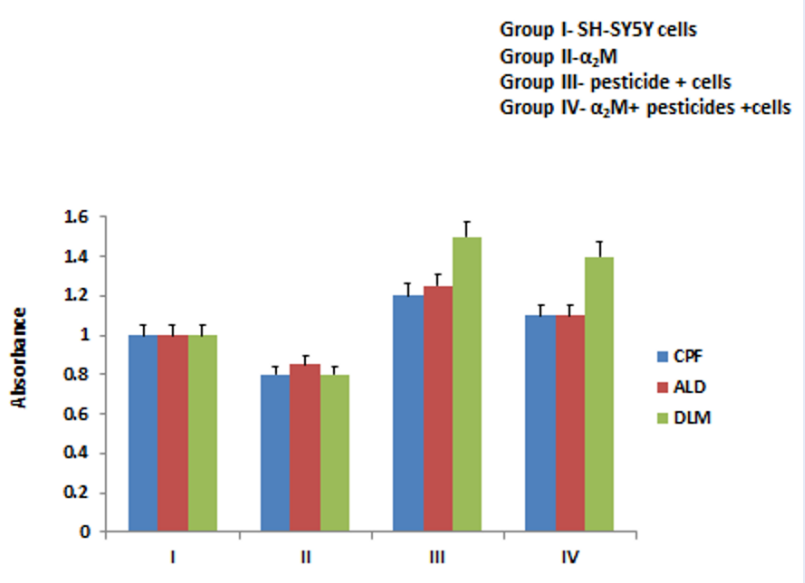

Figure 4: **MDA level assessment in cell lines after pesticide exposure and treatment with α2M**. Absorption spectra were measured at 532 nm. The presented experiments were performed in triplicate ($p < 0.005$). Group II is not statistically significant to Groups I, III and IV.

Table 1: Effect of pesticides on the activity of antioxidant enzymes of the groups

Enzyme	Control	CPF	ALD	DLM
SOD	1.6 ± 0.140	0.90 ± 0.086	1.4 ± 0.131	0.88 ± 0.041
GPx	24.4 ± 2.25	18.3 ± 1.31	20 ± 0.100	16.3 ± 0.07

SOD and GPx measurements are reported in μmol/min/mg cells for SOD and nmol/min/mg cells for GPx (SOD, superoxide dismutase; GPx, glutathione peroxidase).

decrease in absorbance was observed, suggesting the potential effect of α_2M on pesticide-treated cells, in contradiction with results obtained for group IV. Regarding antioxidant enzymes, SOD and GPx activities were quantified at 420 nm and 340 nm, respectively, and reported as units per milligram of cells for SOD and nanomoles per minute per milligram of cells for GPx[39]. The results obtained for groups III, IV, and V suggest a cytoprotective role for α_2M against ROS-mediated toxicity induced by the pesticides. Because proteases and ROS work through a common mechanism of inflammation, like in diseases such as atherosclerosis[40], these findings also indicate the protective role of α_2M in preventing damage to various cellular species by ROS apart from trapping proteases.

CONCLUSIONS

Our data showed that the selected pesticides were potent neurotoxic agents, while α_2M decreased the cytotoxic effect of these pesticides. Hence, this study showed that α_2M modulates pesticide-induced ROS-based neurotoxicity based on different parameters. Thus, α_2M could possibly be used as a protective agent against neurotoxicity caused by ROS and pesticides, warranting further experimental studies.

ABBREVIATIONS

a2M: alpha-2-macroglobulin, **ALD**: aldicarb, **CPF**: chlorpyrifos, **DCFHDA**: dichlorodihydrofluorescein diacetate, **DLM**: deltamethrin, **GPx**: glutathione peroxidase, **H_2O_2**: hydrogen peroxide, **MDA**: malondialdehyde, melonaldehyde, **ROS**: reactive oxyhen species, **SOD**: superoxide dismutase, **TBA**: thiobarbituric acid, **TBARS**: thiobarbituric acid reactive substances

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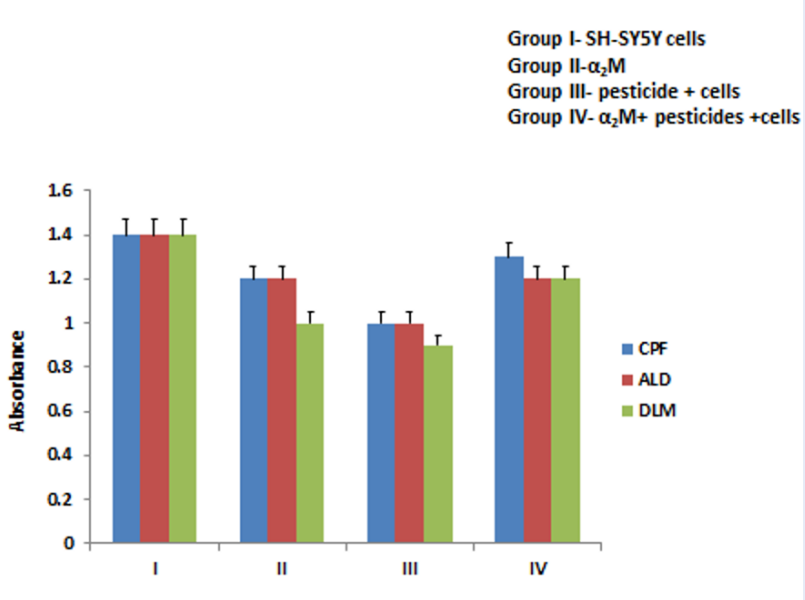

Figure 5: **Measurement of SOD activity at 420 nm**. The activity was reported in mmol/cells for SOD. Group II is not statistically significant to Groups I, III and IV.

acknowledged.

AUTHOR'S CONTRIBUTIONS

SD: data collection, analysis and interpretation of results; drafted the manuscript.
HA: drafted the manuscript; critically revised and proofread the manuscript; approved the final version of manuscript. FHK: conception and design of work; approved the final version of manuscript. All authors read and approved the final manuscript.

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AVAILABILITY OF DATA AND MATERIALS

Not applicable.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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