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Pesticide Induced Cytotoxicity Analysis in Human Cells

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Abstract—*The aim of our work was to evaluate the effect of pesticides on cell survival, oxidative stress mechanism in hPBMC (human Peripheral Blood Mononucleated Cells) and lung cancer cells (A549 cell line). Cells were exposed to each atrazine, butachlor, chlorpyriphos and Dichlorvos pesticides of varying concentration individually and in combination of pesticides to evaluate pesticide-pesticide interaction. The hPBMNc and A549 cancer cells have been exposed to 1, 10, 50, 100 µg/ml concentration of each atrazine, butachlor, chlorpyriphos and Dichlorvos for 24 h exposure period. The cytotoxicity study revealed that the atrazine, butachlor, chlorpyriphos and Dichlorvos resulted in statistically significant decrease in cell survival rate in dose dependent manner in hPBMC and A549 cells. The cell cycle arrest has also been observed following 10 µg/ml concentration in cancer cells. The oxidative stress analysis has been done to evaluate the toxicity mechanism behind the cytotoxicity of pesticides under investigation. Our results suggest the increased oxidative stress, and cell cytotoxicity in both hPBMC and A549 Cells. The combined treatments of various pesticides suggest their combinatorial and/or synergistic impact on human cells.*

Keywords*: Reactive oxygen species, Cell cycle, cytotoxicity, oxidative stress.*

1. INTRODUCTION

Agriculture sector has always been prime objective that decides the nation's economy. To meets the ever increasing food requirement of the growing population, the extensive use of pesticides and fertilizers resulted in its presence in runoffs that ultimately contaminated our water resources [1,2]. It is perturbing human health as the pesticides contaminates other food items too. Moreover, the population exposed to multiple pesticides at one time via use of pesticides contaminated products that may change the actual toxicokinetic phase (changes with respect to absorption, metabolism, distribution or excretion in the presence of other pesticide) or toxicodynamic phase (changes with respect to interaction with target site) [3]. Further, the pesticides are known to increase ROS level that is responsible for oxidative stress [4-6]. The oxidative stress is a prime cause of most of diseases, Alzheimer, Parkinson, asthma, cataracts, atherosclerosis, diabetes, aging of skin, and cancer [7-8]. The respiration system of sprayer affected most severely while their spraying practice in the field. So, the lung problem in agrochemical sprayer could be more as pesticides enter in respiration system via inhalation route. So, the aim of present study is to evaluate toxicological potential of individual pesticides, pesticides mixture and its impact on cell survival, oxidative status and cell cycle in hPBMC as well as lung cancer cells (A549 cell line).

2. MATERIAL AND METHOD

2.1 Cytotoxicity analysis

The cell toxicity has been evaluated using MTT assay. The hPBMC (peripheral Blood Cells) was seeded in 96-well culture plates and maintained in a RPMI-1640 medium supplemented with 5% fetal calf serum and antibiotics at 37^{o}C with 5% CO2. The hPBMC and A549 cells have been exposed to 1, 10, 50, 100 µg/ml concentration of each atrazine, butachlor, chlorpyriphos and dichlorvos for 24 h exposure period and non-treated cells were used as control. Then, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 3 h in dark at 37oC. Added 100 µL DMSO and incubated for 30 min. The resultant formazan formed by viable cells measured using a multi-mode plate reader at a 570 nm wavelength.

2.2 Intracellular ROS production using DHE staining

In order to evaluate the intracellular reactive oxygen species, the dihydroethidium (DHE) stain (non-fluorescent) was used to evaluate the intracellular ROS levels in A549 cells, seeded in 96-well plates at $1.0–1.2 \times 10^4$ cells per well. The H_2O_2 treatment was used as positive control. The A549 cells have been exposed to 1, 10, 50, 100 µg/ml concentration of each atrazine, butachlor, chlorpyriphos and Dichlorvos for 48 h exposure period and non-treated cells were used as control. Then, the DHE solution was added and incubated for 30 min in absence of light which becomes fluorescent upon superoxide oxidation. The fluorescence with an excitation wavelength of 518 nm and emission wavelength of 605 nm was measured using a microplate reader.

2.3 Intracellular ROS production using CellROX®Deep Red staining

The generation of intracellular ROS was detected using the fluorescent probe CellROX®Deep Red (Invitrogen). The A549 cells have been exposed to 1, 10, 50, 100 µg/ml concentration of each atrazine, butachlor, chlorpyriphos and dichlorvos for 24 h exposure period and exposed to CellROX® Deep Red (5 µM, 30 min) at 37 °C under 5% CO2 atmosphere in the dark. Then, the florescence was measured at an excitation wavelength of 633 nm and an emission of 665 nm.

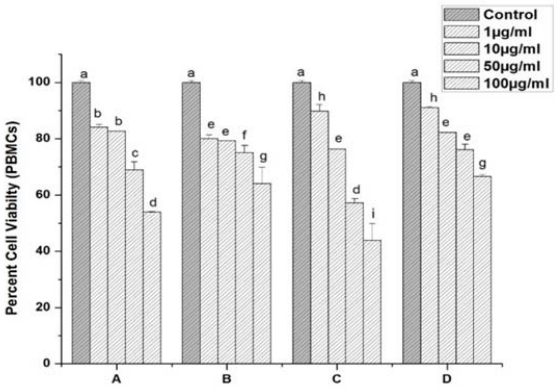
2.4 Cell Cycle analysis

The A549 cells have been exposed to 10µg/ml concentration of each atrazine, butachlor, chlorpyriphos and Dichlorvos for 24 h exposure period for cell cycle analysis and uniformly fixed in ethanol for staining. About 200 µL of ethanol-fixed cells processed according to Muse cell cycle analysis kit. Then, we transfer cell suspension sample to a microcentrifuge tube prior to analysis on Muse™ Cell Analyzer.

3. RESULTS

3.1 Cytotoxicity analysis

The cytotoxicity analysis suggests that the atrazine resulted statistically significant decrease of 15.9%, 17.28%, 31.11%, and 46.08% in cell viability at concentration 1, 10, 50 and 100 µg/ml respectively in hPBMC (Fig. 1). Similarly, there was 5.52%, 24.24%, 26.58% and 33.93% statistically significant decrease at concentration of 1, 10, 50 and 100 µg/ml respectively for A549 cells. The butachlor also significantly decreased 19.98%, 20.63%, 25% and 36.03% of cell viability at 1, 10, 50 and 100 µg/ml concentration respectively in hPBMCs whereas the decrease was statistically significant as 11.51%, 20.67%, 21.94% and 23.56% at 1, 10, 50 and 100 µg/ml concentration respectively for A549 cells. The chlorpyriphos resulted in 10.26%, 23.69%, 42.79% and 56.11% statistically significant decrease in cell viability at 1, 10, 50 and 100 µg/ml concentration respectively in hPBMC whereas the decrease was statistically significant as 4.86%, 22.45%, 27.06% and 34.71% at 1, 10, 50 and 100 µg/ml concentration for A549 cells. The dichlorvos decreased statistically significant as 8.99%, 17.74%, and 23.96% cell viability at 1, 10, 50 and 100 µg/ml concentration respectively in hPBMC whereas the decrease was statistically significant as 3.44%, 19.75%, 33.17% and 78.64% at 1, 10, 50 and 100 µg/ml concentration respectively for A549 cells. The Dichlorvos showed maximum cytotoxicity at 100 µg/ml concentration in A549 cell. So, the cell viability decreased significantly with increase of pesticide dose of atrazine, butachlor, chlorpyriphos and dichlorvos.



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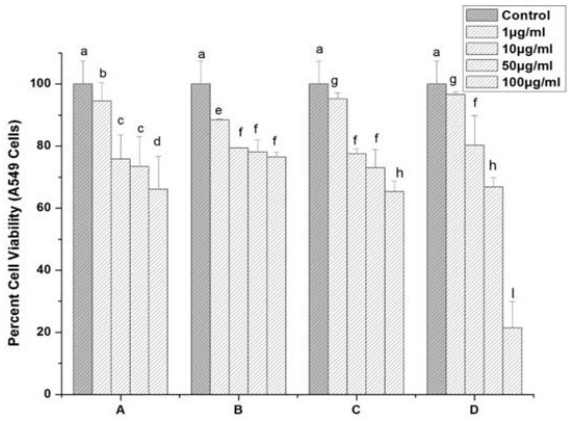

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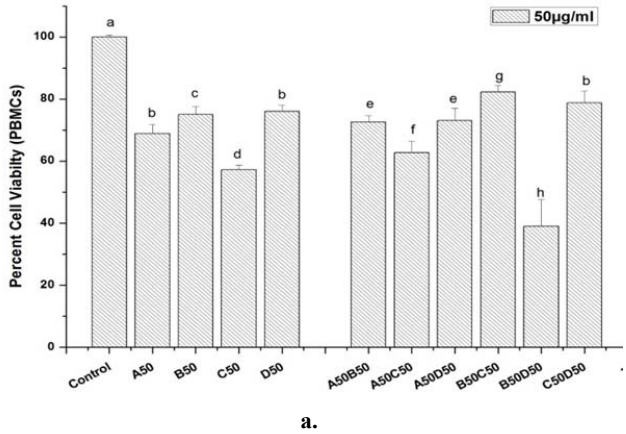
Fig. 1: Percent cell viability of pesticides in response to Atrazine (A), Butachlor (B), Chlorpyriphos (C) and Dichlorvos (D). a) hPBMC b) A549 Cells. Data had been analysed by student's t test between different concentrations of Pesticides and compared with respect to control; expressed as mean values ± S.E. (n=3). Statistically significant results were indicated by different alphabets ($p<0.05$).

3.2 Cytotoxicity of Pesticide-Pesticide Interaction

The result highlights for MTT assay of dose combination of two pesticides in hPBMC and A549 cell are summarized in Fig. 2. The higher dose combination of butachlor and dichlorvos resulted in significant decrease of 60.98% and 69.69% in cell viability in hPBMC and A549 cell respectively as compared to their individual cell toxicity response. Interestingly, there was more significant decrease of 39.39% cell viability with atrazine and dichlorvos higher dose combination in A549 cells as compared to 26.96% in case of hPBMC. Also, there has not been any significant change in cell viability in response to other higher dose combinations such as atrazine with butachlor; atrazine with chlorpyriphos; butachlor with chlorpyriphos and chlorpyriphos with dichlorvos with respect to their individual response in both the cells.



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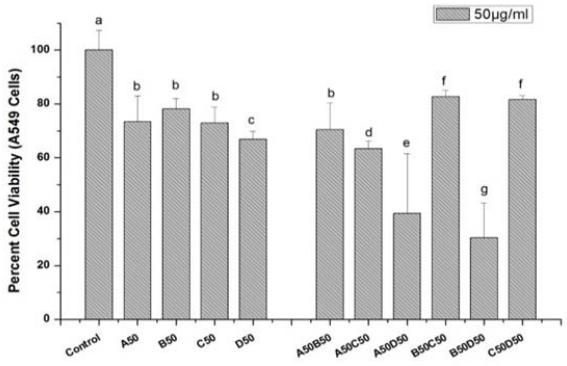

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Fig. 2: Cell viability with pesticide-pesticide interaction Atrazine (A), Butachlor (B), Chlorpyriphos (C) and Dichlorvos (D) with highest concentration combination. **a)** hPBMCs **b)** A549 Cells. Data had been analysed by student's t test between different concentrations of Pesticides and compared with respect to control; expressed as mean values ± S.E. (n=3). Statistically significant results were indicated by different alphabets with ($p<0.05$).

3.3 Intracellular Oxidative Stress Analysis

The intracellular ROS production has been measured using fluorescent probe CellROX®Deep Red. The H_2O_2 is known to increase the ROS production inside the cell. The cells were treated with H_2O_2 and 10µg/ml concentration of Atrazine, Butachlor, Chlorpyriphos and Dichlorvos. Next day, the treated cells were exposed to CellROX®Deep Red reagent in absence of light to evaluate intracellular ROS in cell.

The H_2O_2 treatment significantly increase in the fluorescence intensity indicating higher intracellular ROS production (Fig. 3). Similarly, the atrazine, butachlor, chlorpyriphos and dichlorvos also resulted in increased signal intensity as compared to control. Further, the free radical production was more with atrazine, butachlor, chlorpyriphos and dichlorvos treatment as compared to H_2O_2 exposure too (positive control) (Fig. 3). So, this indicate that the pesticide exposure resulted in oxidative stress indicated for more free radical produced.

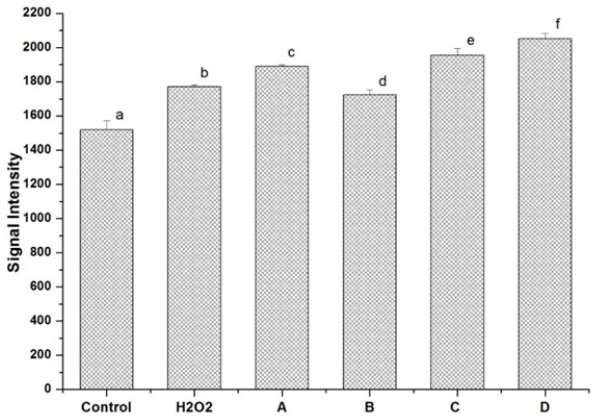


Fig. 3: Intracellular oxidative stress analysis in response to Atrazine (A), Butachlor (B), Chlorpyriphos (C) and Dichlorvos (D) in A549 cells using RedROX cell assay Assay. Data is expressed as mean values ± S.E. (n=3) and had been analyzed student's t test between different concentrations of Pesticides and compared with respect to control. Statistically significant results were indicated by different alphabets with ($p<0.05$).

3.4 Intracellular Superoxide Analysis

The intracellular ROS production has been measured using DHE staining. The intracellular ROS generation revealed similar results as CellROX®Deep Red assay. The cells were treated with H_2O_2 and different concentration of Atrazine, Butachlor, Chlorpyriphos and Dichlorvos. Next day, the treated cells were exposed to DHE in absence of light to evaluate intracellular ROS in cell. The H_2O_2 treatment significantly increase in the fluorescence intensity indicating higher intracellular ROS production (Fig. 4).

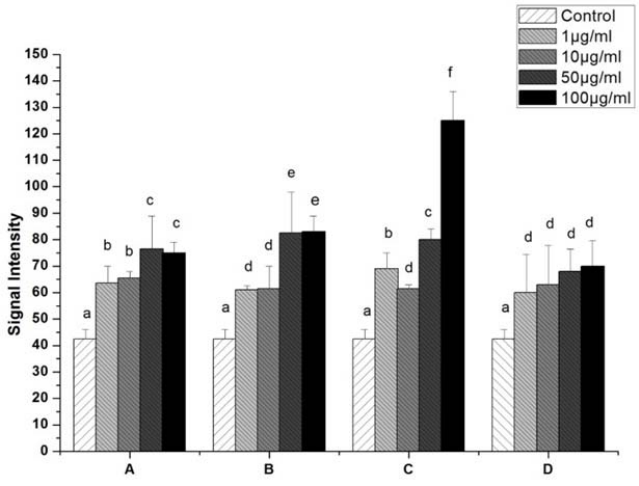


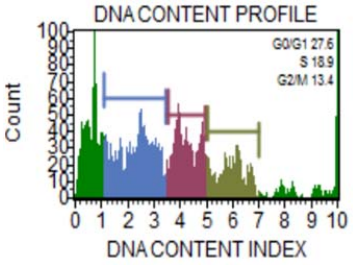
Figure: Intracellular Superoxide Analysis of A549 cells in response to Alachlor (A), Butachlor (B), Chlorpyriphos (C) and Dichlorvos (D) using DHE Assay. Data is expressed as

mean values ± S.E. (n=3) and had been analyzed by one way Anova followed by Dunnett's test between different concentrations of Pesticides and compared with respect to control. Statistically significant results were indicated by "a" and "b" with ($p<0.05$) for A549 and H1299 cells respectively.

The atrazine, butachlor, chlorpyriphos and dichlorvos also resulted in increased signal intensity as compared to control. The chlorpyriphos at 100 µg/ml concentration showed maximum ROS production in A549 cells as compared to other concentrations of atrazine, butachlor, dichlorvos and chlorpyriphos as well as control. Similarly, the free radical production was more with atrazine, butachlor, chlorpyriphos and dichlorvos treatment as compared to H_2O_2 exposure (positive control) (Fig. 4). The dichlorvos pesticide showed increased signal intensity in almost equal proportion at all the concentration although ROS production was more as compared to control. The free radical production trend was observed to be same in both the cells i.e. hPBMCs and A549 cells. Non-florescent DHE easily permeates cell membranes that get oxidized by O2– and converted to ethidium bromide (fluorescent) intercalates into nuclear DNA. So, this indicate that the pesticide exposure resulted in oxidative stress indicated for more free radical produced.

3.5 Cell Cycle Analysis

The cell cycle analysis has been to evaluate the effect of agrochemicals on cell cycle progression in the A549 cells. The pesticides treatment effect the cell cycle progression in the A549 cells as demonstrated in Fig. 6.



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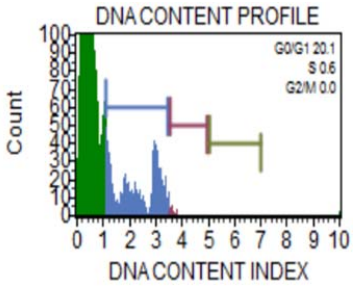

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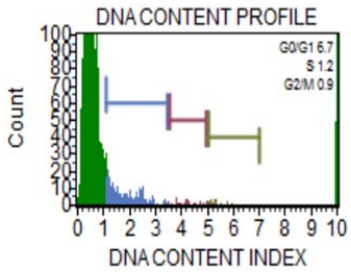

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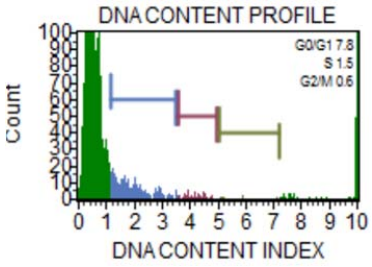

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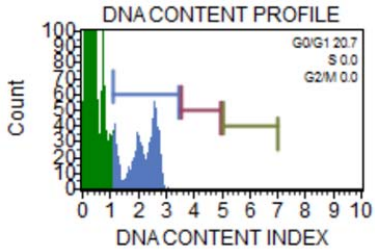

e.

Fig. 6: Cell Cycle Analysis on pesticide treatment in A549 Cells. a. Control b. Atrazine. c. Butachlor c. Chlorpyriphos d. Dichlorvos

The Muse histogram displayed peaks at G0/G1 (27.6), G2/M (13.4), S (18.9) phases for control. In contrast, the atrazine treatment decreased in G0/G1, G2/M peak and S peak values (Fig. 4) to 20.1, 0.0 and 0.6 respectively (Fig. 7). Butachlor pesticide also resulted in decrease of G0/G1, G2/M peak and S peak values to 6.7, 0.9 and 1.2 respectively. The chlorpyriphos treatment showed 7.8, 0.6 and 1.5 peak for G0/G1, G2/M peak and S phase respectively. The Dichlorvos treatment resulted in arrest of cell cycle after Go/G1 phase (20.7), as the S and G2/M phase showed 0 peak value. The arrest at G2/M checkpoint decides survival with DNA damage that may activate either repair or apoptosis-like program. So the pesticide treatment resulted in a change in cell cycle dynamics with respect to untreated cells.

4. DISCUSSION

The pesticides amalgamate with food commodities while agricultural practice that are used to enhance food production from last decade. Although the organophosphate pesticides

(Op), among pesticides, have been choice nowadays to eradicate pest from farms, but their associated poisoning is still a public health challenge. Our results suggest that there is significant toxicity posed by herbicides and organophosphate pesticides. The results showed combinatorial and synergistic effect of combinations of various pesticides. The organophosphate chlorpyriphos and dichlorvos are more toxic as compared to herbicides atrazine and butachlor. The pesticides posed toxicity via free radical mechanism as results indicates. The cell cycle arrest has also been observed at G2/M and S phase in response to treatment of both classes i.e. herbicides as well as organophosphates. The organophosphate pesticides have toxic effect on both human as well as animals. Despite its tremendous side effects, these agrochemicals are continued to use to protect the farms from pest infestation. So, there is need to implement new polices to protect the lives form mixed exposure of pesticides.

5. ACKNOWLEDGEMENTS

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