Applied Ecology and Environmental Sciences, 2020, Vol. 8, No. 4, 166-173
Available online at http://pubs.sciepub.com/aees/8/4/4
Published by Science and Education Publishing
DOI:10.12691/aees-8-4-4

Genotoxic Effect of Certain Pesticide Mixtures in CHO Cell Lines

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Received April 24, 2020; Revised May 26, 2020; Accepted June 02, 2020

Abstract The objective of the present study is to identify Chromosomal Aberrations (CA) and Micronuclei (MN) formation in Chinese hamster ovary (CHO) cell lines when treated with commercially available pesticide mixtures for 24 and 48hrs. CHO cell lines are exposed to pesticide mixtures (Deltamethrin 1% + Triazophos 35% EC (D+T) and Profenofos 40% EC + Cypermethrin 4% EC (P+C)) at a dose of 78.1 µg/ml to 5000 µg/ml with and without metabolic activation (S9). 10 µg/ml of Cyclophosphamide and Mitomycin C at a concentration of 0.4 - 0.8 µg/ml were used as the positive controls. Dose dependant increase of aberrations were recorded for both the pesticide mixtures. Chromosomal Aberrations were mainly observed in the form of chromatid gaps and breaks for both (D+T) and (P+C) treated cell lines of the CHO in comparison to negative control. An increased number of binucleated cells with micronuclei was found at the concentrations >625µg/ml of pesticide mixtures and is statistically significantly different from the control. Our findings suggest that both these pesticide mixtures at higher concentration are found to be a bit genotoxic.

Keywords: *Genotoxicity, Chromosome aberrations, Micronucleus, CHO, Chromatid gaps, Emulsifiable concentrate (EC)*

Cite This Article: B. Reddi Bhargavi, Rajanikanth Akurathi, Ravi Kumar Vellanki, and Jagadeeswara Reddy Kanala, "Genotoxic Effect of Certain Pesticide Mixtures in CHO Cell Lines." *Applied Ecology and Environmental Sciences*, vol. 8, no. 4 (2020): 166-173. doi: 10.12691/aees-8-4-4.

1. Introduction

About 95% of the pesticide toxicity related studies were performed on individual chemicals [1]; however, studies on examining the extent to which these chemical mixtures affect human systems are very few. Potential of pesticides in mixtures was reported by various scientists [2,3]. Nontoxic doses of few pesticide combinations induced significant chromatid breaks and fragments in mouse bone marrow cells while independent exposure of similar doses failed to induce similar effect [4]. Certain pesticides are capable of chemically interacting when combined in mixtures, mainly because the metabolism of one chemical can affect the metabolism of the other. It is possible that mixtures of pesticides can produce additive or synergistic effects or can even produce antagonism [5].

Cytogenetic analysis for chromosomal aberrations such as breaks, satellite associations, and gaps has been used to monitor pesticide induced genotoxic effects on chromosomes under in-vitro and in-vivo conditions [6,7]. To understand the mechanisms of pesticides interactions in human and or animals, usually in vitro assays are carried out. However, it is very difficult to extrapolate the data generated from in vitro studies to the whole organism and also several literatures say that often an in vitro study is a compromise between relevance and convenience [8,9].

Profenofos and cypermethrin separately or in ready-mix, have been found effective in controlling insect-pests. However, information available on the persistence behaviour of these ready-mix products in soil are scare under present environmental conditions [10].

An investigation of a mixture of Deltamethrin and Triazophos on Bt cotton indicated that they increased crop yield (28.45%) and controlled thrips [11]. No data available on the combination of these pesticides in literature. Furthermore, there is no evidence study on genotoxicity and/or cytotoxicity of these pesticide mixtures in vitro. In this backdrop, present investigation was designed to evaluate the genotoxic potential of two commercially available pesticides mixtures, *viz.*, Deltamethrin 1% + Triazophos 35% EC (D+T) and Profenofos 40% EC + Cypermethrin 4% EC (P+C).

2. Materials and Methods

2.1. Pesticide Mixtures and Cell Culture

The two pesticide mixtures (Deltamethrin 1% + Triazophos 35% EC and Profenofos 40% EC + Cypermethrin 4% EC) were procured locally. The

CHO-K1-BH4 cells were purchased from Sigma chemicals and maintained in Ham's F-12 nutrient mix supplemented with 5% foetal bovine serum, penicillin G-100 units/ml; streptomycin sulfate - 0.1 mg/ml; fungizone - 0.25 µg/ml and an additional 2 mM L-glutamine.

2.1.1. Invitro Chromosomal Aberration assay (CA)

Pesticide mixtures were tested for their potential to induce chromosomal aberrations in CHO cell lines using *In Vitro* Mammalian Chromosomal Aberration Test [12]. Atypical Chinese Hamster Ovary (CHO) cells containing 21 chromosomes were maintained in Ham's F-12 nutrient mixture supplemented with 5% foetal bovine serum. On the day before the experiment, approximately 5x 10^5 cells from a culture with approximately 60-80% confluence was seeded into each T-75 cm^2 flasks. The cells were incubated overnight in a humidified incubator at 37°C and 5% CO_2. The pesticide mixtures were prepared as 5 mg/ml stock solution in sterile distilled water just prior to use. 10 µg/ml of cyclophosphamide and Mitomycin C at a concentration of 0.4 to 0.8 µg/ml with and without metabolic activation (S9) were used as the positive controls. In the initial test 5 ml of the cell culture medium was supplemented with 5% foetal bovine serum followed by addition of seven concentrations each of (D+T) and (P+C) i.e, 78.1, 156.3, 312.5, 625, 1250, 2500 and 5000 µg/ml. Cells composed with metabolic activation system (S9) were exposed to pesticides mixture for 3hrs and the cells without metabolic activation system were treated for 18 hrs. In the confirmatory test cells were exposed to pesticides mixtures for 3hrs with and without S9, whereas in continuous treatment without S9 exposed to pesticide mixtures for 18 hrs. Concurrent solvent and positive controls were included in each set. Mitomycin C was used as positive control for cultures treated without S9 and Cyclophosphamide was used for cultures treated with S9. The concentrations chosen for (D+T) EC for 3 hrs exposure was 156.3, 312.5 and 625 µg/ml and for continuous exposure the concentrations chosen were 39.1, 78.1 and 156.3 µg/ml. similarly the concentrations selected for (P+C) EC for 3 hrs exposure was 156.3, 312.5 and 625 µg/ml and for continuous exposure the concentrations selected were 78.1, 156.3 and 312.5 µg/ml. Two hours prior to harvesting, 0.1µg/ml of colchicine was added and cells were trypsinzed. Treated with hypotonic solution and fixed in 3:1 methanol followed by glacial acetic acid. Finally, the cells were dropped onto chilled slides and stained in 3% Giemsa stain. The cytotoxicity of the pesticide mixtures was assessed by determining the Relative population doubling (RPD) of the treated cells.

The RPD was calculated using the following formula:

$$\%\ RPD = \frac{Number\ of\ population\ doublings\ in\ treated\ cultures}{Number\ of\ population\ doublings\ in\ control\ cultures} \times 100$$

$$Population\ doubling = \left[log \frac{post\ treatment\ cell\ number}{initial\ cell\ number} \right] \div log 2$$

From the confirmatory experiment, 200 well spread metaphases were scored per concentration of the pesticide's mixtures, solvent control, equally divided amongst the duplicates. From the positive controls minimum of 100 metaphases were scored.

2.1.2. Invitro Micronucleus (MN) Test

CHO cell cultures were prepared as previously described in the chromosomal aberrations assay. After 22-24 hrs the media was removed and the cells were washed with Phosphate buffered saline (PBS). One set of negative and positive controls were run for each experimental day. Harvesting, slide preparation, scoring and examination of binucleated cells for micronuclei in vitro was determined by the method of Fenech [13]. The induction of Micronuclei was determined in at least 1000 binucleated cells with the cytoplasm well preserved and clearly surrounded with nuclear membrane, having an area of less than one third of that of the main nucleus [14]. The criterion for the identification of Micro nucleus was according to Fenech [15] and the cytokinesis-block proliferation index (CBPI) was calculated according to OECD guideline number 487 [16] using the following formula:

$$CBPI = \frac{MI + 2MII + 3MIII + 4MIV}{N}$$

A minimum of 1000 cells/concentration are analyzed (N).

3. Results and Discussion

3.1. *In Vitro* Chromosomal Aberration Assay

An initial assay was performed to assess the cytotoxicity based on RPD, to fix the high dose for the confirmatory experiment. Various concentrations of the pesticide's mixtures (78.1, 156.3, 312.5, 625, 1250, 2500 and 5000 µg/ml) each of (D+T) EC and (P+C) EC were prepared in sterile distilled water and maintained concurrent solvent controls. In the (D+T) EC treated cultures, with or without S9 mix at the end of 3 hrs exposure, mild detachment of cells was observed at a concentration of 1250 µg/ml and complete detachment of cells was observed in the concentrations at 2500-5000 µg/ml and the % RPD was within the cytotoxic limit from 625 to 78.1 µg/ml, whereas at the end of 18 hrs continuous treatment without S9 mix, mild detachment was observed in 625 µg/ml and complete detachment of cells in the concentrations at 1250-5000 µg/ml and the % RPD was within in the cytotoxic limits from 156.3 to 78.1 µg/ml (Table 1).

In the (P+C) EC treated cultures, mild detachment of cells was observed at a concentration of 2500 µg/ml and moderate detachment of cells was observed at 5000 µg/ml at the end of 3 hrs with or without S9 mix and the % RPD was within in the acceptable cytotoxic limits between 625 to 78.1 µg/ml concentrations, whereas at the end of 18 hrs continuous treatment without S9 mix, mild detachment was observed at a concentration of 1250 and 2500 µg/ml and complete detachment of cells in the concentrations at 5000 µg/ml and the % RPD was within the acceptable cytotoxic limits between 312.5 to 78.1 µg/ml concentrations (Table 2). The pH of the culture was approximately 7.0, post-treatment. No visible contamination was observed in any of the cultures.

Table 1. Cell Concentration data of D+T EC - Initial Experiment (With and without S9 mix - 3/18 hrs exposure)

Concentration µg/ml	ICN (10^6)			PTCN (10^6)			PTCN ÷ICN(10^6)		
	(+S9/3hrs)	(-S9/ 3hrs)	(-S9/ 18hrs)	(+S9/ 3hrs)	(-S9/ 3hrs)	(-S9/ 18hrs)	(+S9/ 3hrs)	(-S9/ 3hrs)	(-S9/ 18hrs)
SC	1	1	1	3.2	2.8	2.4	3.2	2.8	2.4
78.1	1	1	1	2.9	2.6	2	2.9	2.6	2
156.3	1	1	1	2.4	2.4	1.5	2.4	2.4	1.5
312.5	1	1	1	2	1.9	1.3	2	1.9	1.3
625	1	1	1	1.6	1.5	*	1.6	1.5	-
1250	1	1	1	*	*	#	-	-	-
2500	1	1	1	#	#	#	-	-	-
5000	1	1	1	#	#	#	-	-	-
Concentration µg/ml	**Log (PTCN÷ICN)**			**Log [(PTCN÷ICN)]÷Log2**			**%RPD**		
	(+S9/ 3hrs)	(-S9/ 3hrs)	(-S9/ 18hrs)	(+S9/ 3hrs)	(-S9/ 3hrs)	(-S9/ 18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)
SC	0.5	0.44	0.38	1.7	1.49	1.26	NA	NA	NA
78.1	0.46	0.41	0.3	1.54	1.38	1	90.5	92.6	79.36
156.3	0.38	0.38	0.18	1.26	1.26	0.58	74.11	84.56	46.02
312.5	0.3	0.28	0.11	1	0.92	0.38	58.8	61.74	30.15
625	0.2	0.18	-	0.68	0.58	-	40	38.9	-
1250	-	-	-	-	-	-	-	-	-
2500	-	-	-	-	-	-	-	-	-
5000	-	-	-	-	-	-	-	-	-

* - Mild detachment of the cells; # - Complete detachment of cells; PTCN - Post treatment cell number; ICN - Initial cell number; - No live cells; NA - Not applicable; SC: Solvent control (sterile distilled water, 50 µl/ml culture).

Table 2. Cell Concentration data of P+C EC - Initial Experiment (With and without S9 mix - 3/18 hrs exposure)

Concentration µg/ml	ICN (10 6)			PTCN (10 6)			PTCN ÷ICN(10 6)		
	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)
SC	1	1	1	3.3	3.2	3	3.3	3.2	3
78.1	1	1	1	2.9	2.7	2.7	2.9	2.7	2.7
156.3	1	1	1	3	2.5	2	2.5	2.5	2
312.5	1	1	1	2.3	1.7	1.5	2.3	1.7	1.5
625	1	1	1	1.5	1.5	1.2	1.5	1.5	1.2
1250	1	1	1	1.3	1.2	*	1.3	1.2	-
2500	1	1	1	*	*	*	-	-	-
5000	1	1	1	#	#	#	-	-	-
Concentration µg/ml	**Log (PTCN÷ICN)**			**Log [(PTCN÷ICN)]÷Log2**			**%RPD**		
	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)
SC	0.52	0.5	0.48	1.72	1.68	1.58	NA	NA	NA
78.1	0.46	0.43	0.43	1.54	1.43	1.43	89.53	85.12	90.5
156.3	0.39	0.4	0.3	1.32	1.32	1	76.74	78.57	63,29
312.5	0.36	0.23	0.18	0.58	0.76	0.58	33.72	45.24	36.7
625	0.18	0.18	0.08	0.58	0.58	0.26	33.72	34.52	16.45
1250	0.11	0.08	-	0.38	0.26	-	22.09	15.48	-
2500	-	-	-	-	-	-	-	-	-
5000	-	-	-	-	-	-	-	-	-

* - Mild detachment of the cells; # - Complete detachment of cells; PTCN - Post treatment cell number; ICN - Initial cell number; - No live cells; NA - Not applicable; SC: Solvent control (sterile distilled water, 50 µl/ml culture).

Based on the cytotoxicity data (RPD) from the initial assay, 156.3 µg/ml for (D+T) EC and 625 µg/ml (P+C) EC were chosen as the highest concentrations for with and without metabolic activation (3 hrs exposure), and 78.1, 156.3 and 312.5 µg/ml for without metabolic activation (continuous 18 hrs exposure) in the confirmatory experiment for (D+T) EC and (P+C) EC, respectively. The pH of the culture was approximately 7.0,

post-treatment. No visible contamination was observed in the culture.

Genotoxic agents have the potential to interact with DNA and may cause DNA damage. CA occurs in proliferating cells and is regarded as a manifestation of damage to the genome. CA assay has been commonly used as a test of mutagenicity in order to evaluate cytogenetic responses to chemical exposure. The results of the present study demonstrated that the frequency of breaks, gaps, fragments, exchanges and endoreduplications were increased significantly in cultures of Chinese hamster cell line treated with D+T and P+C (EC) when compared with the control. The number of total aberrant cells recorded at concentrations of 78.1-625 µg/ml was significantly increased at $p<0.001$ when compared with untreated control. The percentage of structurally damaged cells in the MMC (positive control) treatment group was statistically increased compared to the solvent control indicating the responsiveness of the cells in this test system.

Cell concentration data pertaining to the treatment of (D+T) EC is presented in (Table 3). The % RPDs of (D+T) EC treated cultures with S9 mix for 3 hrs at a concentration of 156.3, 312.5 and 625 µg/ml were 85.71, 79.73 and 41.86. whereas the % RPDs without S9 mix were 80.13, 69.71 and 44.3. For 18 h continuous exposure without S9, % RPDs at a concentration of 156.3, 312.5 and 625µg/ml were 91.3, 69.93 and 42.39.

The % RPDs for (P+C) EC treated cultures for 3 hrs with S9 mix at a concentration of 156.3, 312.5 and 625 µg/ml were 91.11, 81.48 and 56.67 and without S9 mix, the % RPDs were 79.21, 68.32 and 44.55, respectively. For 18 h continuous exposure without S9 at a concentration of 156.3, 312.5 and 625 µg/ml the % RPDs were 91.81, 68.68 and 48.04, respectively (Table 4). The % RPDs calculated for all the pesticides mixtures are within the acceptable cytotoxic limits.

The aberration data of (D+T) EC is presented in Table 5. The (D+T) EC treated cultures did not exhibit any statistically significant change in chromosomal aberration compared to solvent control at any concentration. A marked increase in aberration in the chromatid region, predominantly chromatid breaks, fragments, triradial, quadriradial and deletion was observed in the positive control, thus indicating validation of test system and experimental procedure. A dose-dependent increase in % structural aberrations (exclusive of gaps) with S9 mix and % structural aberrations (exclusive of gaps) without S9 mix at a concentrations of 156.3, 312.5 and 625µg/ml indicates that (D+T) EC may be interpreted as non-mutagenic with a bit of caution (Table 6).

Table 3. Cell Concentration data of D+T EC - Confirmatory Experiment (With and without S9 mix - 3/18 hrs exposure)

Concentration µg/ml	**ICN (10^6)**			**PTCN (10^6)**			**PTCN ÷ICN(10^6)**		
	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)
SC	1	1	1	2.9	3	2.5	2.9	3	2.5
	1	1	1	2.8	2.9	2.6	2.8	2.9	2.6
156.3	1	1	1	2.6	2.5	2.4	2.6	2.5	2.4
	1	1	1	2.5	2.3	2.3	2.5	2.3	2.3
312.5	1	1	1	2.4	2.1	2	2.4	2.1	2
	1	1	1	2.2	2.2	1.8	2.2	2.2	1.8
625	1	1	1	1.7	1.7	1.5	1.7	1.7	1.5
	1	1	1	1.6	1.6	1.4	1.6	1.6	1.4
#CYP-10 µg/ml	1	1	1	2.3	2.2	1.8	2.3	2.2	1.8
	1	1	1	2	2.4	2.3	2	2.4	2.3
Concentration µg/ml	**Log (PTCN÷ICN)**			**Log [(PTCN÷ICN)]÷Log2**			**%RPD**		
	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)
SC	0.46	0.47	0.4	1.58	1.58	1.38	NA	NA	NA
	0.45	0.46	0.41	1.43	1.49	1.38			
156.3	0.41	0.4	0.38	1.26	1.26	1.2	85.71	80.13	91.3
	0.4	0.36	0.36	1.32	1.2	1.32			
312.5	0.38	0.32	0.3	1.26	1	1	79.73	69.71	69.93
	0.34	0.34	0.25	1.14	1.14	0.93			
625	0.23	0.23	0.18	0.58	0.68	0.68	41.86	44.3	42.39
	0.2	0.2	0.15	0.68	0.68	0.49			
#CYP-10 µg/ml	0.36	0.34	0.25	1.14	1.07	0.85	71.1	73.94	76.45
	0.3	0.3	0.36	1	1.2	1.26			

Positive control - Cyclophosphamide (CYP); PTCN - Post treatment cell number; ICN - Initial cell number; NA - Not applicable.

Table 4. Cell Concentration data of P+C EC - Confirmatory Experiment (With and without S9 mix - 3/18 hrs exposure)

Concentration µg/ml	ICN (10^6)			PTCN (10^6)			PTCN ÷ICN(10^6)		
	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)
SC	1	1	1	2.5	2.9	2.6	2.5	2.9	2.6
	1	1	1	2.6	2.8	2.7	2.6	2.8	2.7
156.3	1	1	1	2.3	2.2	2.5	2.3	2.2	2.5
	1	1	1	2.4	2.4	2.4	2.4	2.4	2.4
312.5	1	1	1	2.3	2.2	1.9	2.3	2.2	1.9
	1	1	1	2	1.9	2	2	1.9	2
625	1	1	1	1.8	1.7	1.5	1.8	1.7	1.5
	1	1	1	1.6	1.5	1.7	1.6	1.5	1.7
#CYP-10 µg/ml	1	1	1	1.9	1.8	1.9	1.9	1.8	1.9
	1	1	1	1.8	2.3	2.4	1.8	2.3	2.4
Concentration µg/ml	**Log (PTCN÷ICN)**			**Log [(PTCN÷ICN)]÷Log2**			**%RPD**		
	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)	(+S9/3hrs)	(-S9/3hrs)	(-S9/18hrs)
SC	0.4	0.46	0.41	1.32	1.54	1.38	NA	NA	NA
	0.41	0.45	0.43	1.38	1.49	1.43			
156.3	0.36	0.34	0.4	1.2	1.14	1.32	91.11	79.21	91.81
	0.38	0.38	0.38	1.26	1.26	1.26			
312.5	0.36	0.34	0.28	1.2	1.14	0.93	81.48	68.32	68.68
	0.3	0.28	0.3	1	0.93	1			
625	0.26	0.23	0.18	0.85	0.77	0.58	56.67	44.55	48.04
	0.2	0.18	0.23	0.68	0.58	0.77			
#CYP-10 µg/ml	0.28	0.26	0.28	0.93	0.85	0.93	65.93	67.66	77.94
	0.26	0.36	0.38	0.85	1.2	1.26			

Table 5. Chromosomal Aberration Data of D+T EC - Confirmatory Experiment (with and without S9 mix - 3/18 hrs exposure)

	Structural aberrations																						
Concentration (µg/ml)	**Chromatid**																						
	(+S9/3hrs)							**(-S9/3hrs)**							**(-S9/18hrs)**								
	tg	tb	tf	del	ex			tg	tb	tf	del	ex			tg	tb	tf	del	ex				
					tr	qr	cr					tr	qr	cr					tr	qr	cr		
SC	2	5	1	0	2	0	0	2	3	3	1	1	0	0	2	4	2	1	1	0	0		
156.3	3	5	1	4	2	0	0	2	4	1	2	2	2	0	1	6	0	0	2	1	0		
312.5	1	6	2	3	3	1	0	0	6	2	2	1	3	0	2	5	2	3	2	0	0		
625	0	5	0	5	4	4	1	2	4	3	2	4	2	1	1	4	2	5	5	2	0		
CYP - 10 µg/ml	3	10	4	4	16	6	0																
MMC								2	15	4	7	12	7	2	3	14	2	3	15	3	2		
Concentration (µg/ml)	**Chromosome**																						
	(+S9/3hrs)								**(-S9/3hrs)**								**(-S9/18hrs)**							
	ig	sb	af	del	ex				ig	sb	af	del	ex				ig	sb	af	del	ex			
					r/acr	d	tc	plc					r/acr	d	tc	plc					r/acr	d	tc	plc
SC	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
156.3	0	1	1	2	1	1	0	0	2	0	0	1	1	2	0	0	0	0	0	1	2	1	0	0
312.5	1	0	0	1	3	2	0	0	0	1	0	2	1	1	0	0	0	0	0	0	1	0	0	0
625	1	0	0	2	2	1	0	0	1	2	0	1	1	1	0	0	2	1	0	1	1	1	0	0
CYP - 10 µg/ml	2	0	0	4	2	0	0	0																
MMC									2	0	0	2	2	1	0	0	0	1	0	2	2	1	0	0
Concentration (µg/ml)	**Numerical aberrations**																						
	(+S9/3hrs)							**(-S9/3hrs)**							**(-S9/18hrs)**								
SC	p+/pc	Gt	en	po	an	others		p+/pc	gt	en	po	An	others		p+/pc	gt	en	po	an	Others			
	0	0	2	0	0	0		0	0	1	1	0	0		0	0	2	2	0	0			
156.3	0	0	2	0	0	0		0	1	2	0	0	0		0	0	2	1	0	0			
312.5	0	0	1	1	0	0		0	0	1	2	0	0		0	0	1	1	0	0			
625	0	0	2	0	0	0		0	0	0	1	2	0		0	0	2	0	0	0			
CYP - 10 µg/ml	0	0	1	0	0	0																	
MMC								0	0	1	0	0	0		0	0	0	1	0	0			

tg = chromatid gap, tb = chromatid break, tf = fragment, del = deletion, ex = exchange, tr = triradial, qr = quadriradial, cr = complex rearrangement, ig = isochromatid gap, sb = chromosome break, af = acentric fragment, dmin = double minutes, r = centric ring, acr = acentric ring, d = dicentric, tc = tricentric, plc = polycentric, p+ = pulverized chromosomes, pc = pulverized cell, gt = cell with greater than 10 aberrations/multiple aberations, po = polyploidy, en = endoreduplication, an = aneuploidy. Chromatid structural aberrations (tb, tf, del and ex combined) of CYP- 10 µg/ml significantly different from Solvent control at 5% probability level (Mann Whitney - U test).

Table 6. Confirmatory experiment summary on *In vitro* chromosomal aberrations in CHO cell lines - 3 hrs exposure to D+TEC (with and without S9 mix) and 18 hrs continuous exposure (without S9 mix)

Concentration (µg/ml)	3hrs exposure				18 hrs Continuous exposure	
	With S9 (10%) mix		Without S9 mix		Without S9 mix	
	% Structural aberrations (exclusive of gaps)	**% Numerical aberrations**	**% Structural aberrations (exclusive of gaps)**	**% Numerical aberrations**	**% Structural aberrations (exclusive of gaps)**	**% Numerical aberrations**
SC	5.0	1.0	4.0	1.0	5.0	2.0
39.1	NA	NA	NA	NA	6.5	1.5
78.1	NA	NA	NA	NA	6.5	1.0
156.3	9.0	1.0	7.5	1.0	11.0	1.0
312.5	10.5	1.0	9.5	1.5	NA	NA
625	12.0	1.0	10.5	1.5	NA	NA
Cyclophosphamide (10 µg/ml)	46.0	1.0	NA	NA	NA	NA
MMC - 0.4 µg/ml	NA	NA	NA	NA	45.0	1.0
MMC - 0.8 µg/ml	NA	NA	52.0	1.0	NA	NA

NA - Not applicable; Total Number of metaphases counted = 200 (for the solvent control and all the pesticides mixtures concentrations); Total Number of metaphases counted = 100 (for the positive controls).

$$\%\ \text{structural aberrations / numerical aberrations} \lim_{x\to\infty} = \frac{\text{Total aberrations}\left(\text{excluding gaps}\right)}{\text{Total no. of metaphases scored}} \times 100$$

The pesticides mixtures (P+C) EC did not exhibit any statistically significant increase in the aberration frequency, except in the concentration 156.3 µg/ml (3 hrs exposure without S9). A marked increase in structural chromatid aberrations was observed in the positive control, thus indicating validation of test system and experimental procedure (Table 7). Summary of percent structural aberrations of chromosomes indicates (D+T) EC did not induce structural chromosomes aberrations in CHO cells (Table 8).

Table 7. Chromosomal Aberration Data of P+C EC - Confirmatory Experiment (with and without S9 mix - 3/18 hrs exposure)

	Structural aberrations																						
Concentration (µg/ml)	**Chromatid**																						
	(+S9/3hrs)							**(-S9/3hrs)**							**(-S9/18hrs)**								
	tg	tb	tf	del	ex			tg	tb	tf	del	ex			tg	tb	tf	del	ex				
					tr	qr	cr					tr	qr	cr					tr	tr	qr		
SC	0	1	1	0	0	0	0	1	0	0	1	1	0	0	1	0	1	1	0	0	0		
156.3	1	1	2	2	0	1	0	0	1	0	1	1	0	0	2	1	1	2	2	0	1		
312.5	0	2	0	1	1	0	0	1	0	1	0	0	0	1	0	0	2	0	1	1	0		
625	2	1	2	2	2	1	0	1	1	1	0	1	1	0	0	2	1	2	2	2	1		
CYP - 10 µg/ml	1	8	2	1	12	0	1																
MMC								0	5	0	2	10	8	2	2	6	2	0	15	6	0		
Concentration (µg/ml)	**Chromosome**																						
	(+S9/3hrs)								**(-S9/3hrs)**								**(-S9/18hrs)**							
	ig	sb	af	del	ex				ig	sb	af	del	ex				ig	sb	af	del	ex			
					r/acr	d	tc	plc					r/acr	d	tc	plc					r/acr	d	tc	plc
SC	0	0	2	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
156.3	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
312.5	1	0	1	1	0	1	0	0	0	2	0	1	0	0	0	0	0	1	0	1	0	0	0	0
625	0	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	2	0	1	0	0	0
CYP - 10 µg/ml	0	0	1	2	0	1	0	0																
MMC									1	0	0	3	2	0	0	0	0	1	0	0	1	0	0	0
Concentration (µg/ml)	**Numerical aberrations**																						
	(+S9/3hrs)							**(-S9/3hrs)**							**(-S9/18hrs)**								
SC	p+/pc	Gt	en	po	an	others		p+/pc	gt	en	po	An	others		p+/pc	gt	en	po	an	Others			
	0	0	1	0	0	0		0	0	1	0	0	0		0	0	0	0	0	0			
156.3	0	0	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0			
312.5	0	0	0	0	0	0		0	0	1	0	0	0		0	0	2	0	0	0			
625	0	0	2	1	0	0		0	0	0	0	0	0		0	0	0	0	0	0			
CYP - 10 µg/ml	0	0	0	3	0	0																	
MMC								0	0	0	1	0	0		0	0	0	2	0	0			

tg = chromatid gap, tb = chromatid break, tf = fragment, del = deletion, ex = exchange, tr = triradial, qr = quadriradial, cr = complex rearrangement, ig = isochromatid gap, sb = chromosome break, af = acentric fragment, dmin = double minutes, r = centric ring, acr = acentric ring, d = dicentric, tc = tricentric, plc = polycentric, p+ = pulverized chromosomes, pc = pulverized cell, gt = cell with greater than 10 aberrations/multiple aberations, po = polyploidy, en = endoreduplication, an = aneuploidy. Chromatid structural aberrations (tb, tf, del and ex combined) of MMC -0.4 µg/ml significantly different from Solvent control at 5% probability level (Mann Whitney - U test).

Table 8. Confirmatory experiment summary on *In vitro* chromosomal aberrations in CHO cell lines - 3 hrs exposure to P+CEC(with and without S9 mix) and 18 hrs continuous exposure (without S9 mix)

Concentration (µg/ml)	3hrs exposure				18 hrs continuous exposure	
	With S9 (10%) mix		Without S9 mix		Without S9 mix	
	% Structural aberrations (exclusive of gaps)	% Numerical aberrations	% Structural aberrations (exclusive of gaps)	% Numerical aberrations	% Structural aberrations (exclusive of gaps)	% Numerical aberrations
SC	2.5	0.5	2.0	0.5	2.0	0.0
78.1	NA	NA	NA	NA	2.0	0.0
156.3	4.0	0.0	2.0	0.0	3.0	1.0
312.5	3.5	0.0	2.5	0.5	3.5	0.0
625	4.5	1.5	3.5	0.0	NA	NA
Cyclophosphamide (10 µg/ml)	28	3.0	NA	NA	NA	NA
MMC - 0.4 µg/ml	NA	NA	NA	NA	31	2.0
MMC - 0.8 µg/ml	NA	NA	32	1.0	NA	NA

NA - Not applicable; Total Number of metaphases counted = 200 (for the solvent control and all the pesticides mixtures concentrations); Total Number of metaphases counted = 100 (for the positive controls); Mitomycin C (MMC).

$$\%\ structural\ aberrations\ /\ numerical\ aberrations = \frac{Total\ aberrations\ \left(excluding\ gaps\right)}{Total\ no.\ of\ metaphase\ scored} \times 100.$$

3.2. Micronucleus Induction

Treatment of CHO cells with D+T(EC) at a concentration of 39.1, 78.1, 156.3, 312.5 and 625µg/ml caused dose-dependent MN induction. An increased number of binucleated cells with micronuclei was found at the higher concentrations (>39.1 µg/ml of D+T(EC) and was statistically significantly different from the control. In the cell cycle kinetics analysis or CBPI, treatment with D+T(EC) in concentrations more than 156.3µg/ml was found to exert an inhibition of cell proliferation in CHO cells (Table 9).

Similarly in CHO cells treated with P+C (EC) at a concentrationsof 78.1, 156.3, 312.5, 625 and 1250µg/ml induced dose-dependent MN induction. An increased number of binucleated cells with micronuclei was found at the higher concentrations (>156.3 µg/ml) of P+C(EC) and was statistically significantly different from the control. In the cell cycle kinetics analysis or CBPI, treatment with P+C (EC) in concentrations more than 312.3 µg/ml was found to exert an inhibition of cell proliferation in CHO cells (Table 10).

MN assay is a widely used cytogenetic method to assess in vitro chromosomal damage. Analysis of the frequency of occurrence of micronuclei in treated cells provides a comparatively rapid and sensitive indication of both chromosomal aberrations and chromosome loss that lead to numerical chromosomal anomalies [17,18]. Micronuclei are chromatin masses in the cytoplasm with the appearance of small nuclei that arise from chromosome fragments at anaphase or from acentric chromosomal fragments. They provide a quantifiable measure of recent DNA injury that result from when acentric fragments or whole chromosome are left behind the main nucleus at telophase [19]. An increase in the percentage of Micronucleus in a population of cells indicates chromosomal damage occurred as a result of an exposure to either clastogenic or an aneuploidogenic effect [20]. Present study showed that exposure of CHO cells to D+T (EC) and P+C (EC) significantly and dose-dependently increased the frequency of Micronucleus. These results of micronucleus formation support our observations that D+T (EC) and P+C (EC) causes chromosomal damage in Chinese hamster cell lines.

This unexplained genotoxicity is much more common in the in vitro cytogenetics. Some of these positive responses may be clue to true covalent adducts formation whereas, others are most likely cytotoxicity artefacts and still others may be due to non-covalent drug/DNA interactions, i.e. DNA intercalation or groove-binding [21].

Table 9. Frequencies of micronucleus (MN) formation and cell cycle kinetics on CHO cultures treated with different concentrations of D+T (EC) together with Mitomycin C treated and untreated cultures.

Treatment	Conc.	BN	MN	MN (%)	Cell cycle kinetics			BN (%)	CBPI
					M1	M2	M3		
D+T (EC)	0	2000	38	1.9	118	694	157	69.4	1.977
	39.1	2500	51	2.04	98	711	138	71.1	1.934
	78.1	3000	74*	2.46	102	773	102	77.3	1.954
	156.3	2200	96**	4.36	151	701	121	70.1	1.916
	312.5	2000	114**	5.7	386	416	118	41.6	1.572*
	625	High toxicity							
SC		1000	18	1.8	81	772	139	77.2	2.042
MMC	1	1000	62**	6.2	682	241	61	24.1	1.347**

BN:Binucleate, MN:Micronucleus, a The no of mononucleated (M1), binucleated (M2), and polynucleated (M3) cell per 1000 cells were quantitated for cell cycle kinetic analysis, *significantly different from control p<0.05. ** significantly different from control p<0.001.

Table 10. Frequencies of micronucleus (MN) formation and cell cycle kinetics on CHO cultures treated with different concentrations of P+C (EC) together with Mitomycin C treated and untreated cultures

Treatment	Conc.	BN	MN	MN (%)	Cell cycle kinetics			BN (%)	CBPI
					M1	**M2**	**M3**		
P+C (EC)	0	2100	42	2	121	657	146	65.7	1.873
	78.1	2000	59	2.95	94	723	132	72.3	1.936
	156.3	2800	81*	2.89	118	762	109	76.2	1.969
	312.3	2200	118*	5.36	164	697	126	69.7	1.936
	625	2000	134**	6.7	402	431	111	43.1	1.597*
	1250	High toxicity							
SC		1000	16	1.6	76	781	142	78.1	2.064
MMC	1	1000	74*	7.4	681	233	59	23.3	1.324**

4. Conclusion

The present results provide evidence that pesticide mixtures D+T (EC) and P+C (EC) in higher concentrations have a genotoxic and cytotoxic effects on cultured Chinese hamster cell line, bringing into highlight the need for further studies to better understand the molecular mechanisms of action of D+T (EC) and P+C (EC) compound for a better comprehension. Thus, results found, at least under the experimental conditions used in the present study, showed the ability of these pesticide mixtures to induce genotoxicity and cytotoxicity in vitro in CHO cells while it failed to induce mutagenic effects on Ames *Salmonella typhimurium* strains in our previous study [22].

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